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Ecotoxicology and environmental safety toxicity of pyrethroid cypermethrin on the freshwater snail *Chilina parchappii*: Lethal and sublethal effects

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ABSTRACT

The aim of the present work was to study the effect of the pyrethroid cypermethrin (CYP) on the non-target freshwater snail *Chilina parchappi*. Initially, the sensitivity of adult snails to CYP was evaluated via the 96-h LC_{50} test. Then, snails were exposed to subtethal CYP concentrations (0.1 and 10 mg/l) for 1, 4 and 10 days and the digestive glands were dissected for biomarkers analyses. Enzymatic activity of catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), as well as total glutathione reduced (GSH) levels, were determined. Histological analyses of morphology, intracellular accumulation of lipofucsins and neutral lipids accumulation in the digestive gland were also evaluated. As compared to other molluscs, *C. parchappi* showed high resistance to CYP exposure evidenced by the 96-h LC_{50} value (44.59 mg/l). Snails exposed to sublethal CYP concentrations showed a statistically significant increase ($p < 0.01$) in GST (79–116%) and GPx (45–190%) activities with respect to controls. However, CAT activity showed a tendency to decrease with CYP treatment but was not statistically significantly different compared to control. Only high CYP concentration caused a statistically significant increase ($p < 0.01$) in GSH content (95–196%). There was evidence of structural changes in the digestive gland of snails exposed to CYP, showing a dose-dependent response. In exposed snails, some of the main symptoms included a reduction in the thickness of the epithelium, vacuolisation of the digestive cells and an increase in the number of excretory cells. Accumulation of lipofuscins (933–1006%) and neutral lipids (403%) were statistically significantly higher ($p < 0.05$) in snails exposed to CYP compared to control. This study showed that *C. parchappii* is quite tolerant to CYP exposure and that at sublethal concentrations, GSH metabolism could play a protective role against the pesticide harm in snails. Therefore, it would be interesting to study the response of this organism to other environmental stressors to assess its potential use in monitoring programs.

1. Introduction

Cypermethrin (α-cyano-3- phenoxybenzyl 1-cis, trans-3- (2, 2- dichlorovinyl) −2, 2 dimethylcyclopropane carboxylate, CYP) belongs to the class of synthetic organic insecticides derived from pyrethrins. This pesticide is widely used throughout the world due to its high effectiveness and low toxicity compared to organophosphates and carbamates (Palmquist et al., 2012). In South America, the expansion of soybean cultivation in recent decades has also promoted the use of this insecticide (CASAFE, 2013). However, despite its lower toxicity compared to other compounds, recent research reveals that pyrethroids like CYP are also dangerous for aquatic biota and humans (Tang et al., 2018). This pesticide usually reaches the freshwater systems that surround the application areas through different routes such as runoff, accidental spillages and/or excessive direct spraying, exposing aquatic organisms to their sublethal or lethal concentrations (Manjunatha et al., 2015; Wei and Yang, 2014).

Due to the lipophilic nature of CYP, this compound penetrates rapidly through the membranes of the gills, skin, and digestive tract of aquatic organisms. The main toxic targets are the voltage-gated sodium channels (Soderlund and Bloomquist, 1989) and the integral protein ATPase in the neuronal membrane (Kakko et al., 2003), which affects

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nerve impulse transmission. Besides, in the organs responsible for biotransformation reactions, the oxidative CYP metabolism may produce reactive oxygen species (ROS) that, if are not neutralised by the antioxidant defence system, induce oxidative stress (Khazri et al., 2016). These biological responses at the biochemical and physiological level are usually the first ones to appear and most sensitive ones. In addition, if at the molecular level the defence mechanisms are not able to mitigate the effects of such substances, this can be reflected in alterations of the tissue morphology (Hinton et al., 1992). In this sense, histological or histochemical analyses represent a very sensitive tool for detecting sublethal effects of pesticides in organisms and to interpret their toxic mechanisms on target organs.

Some parameters determined at the biochemical, physiological and histological level, could work as biomarkers since they are considered particularly valuable as early warning tools to monitor exposures to pollutants (Howcroft et al., 2009). Molluscs arouse great interest as model organisms when studying biomarkers of contamination due to their wide distribution, adaptability to different environmental conditions (Khazri et al., 2016) and high resistance to pesticides like CYP (Maund et al., 2012). Within molluscs, the gastropod *Chilina parchappii* is an abundant and representative species of the benthic assemblages of streams in the Pampas region in Argentina, an area that concentrates the largest agricultural activities of the country (Estebenet et al., 2002; Tietze and De Francesco, 2010). Thus, the aim of this study was to evaluate the sensitivity of *C. parchappii* to CYP using biomarkers commonly applied in environmental monitoring studies. For this, lethal concentrations (LC_{50}), parameters related to the antioxidant status and histopathological alterations, as well as lipofuscins and neutral lipids levels were evaluated. It is expected that this work would contribute to the knowledge of pyrethroids impact on non-target aquatic organisms.

2. Materials and methods

2.1. Sample collection

Adults of *Chilina parchappii* were collected in Tandileofú stream in Tandil, Argentina (37°21′30.3″S/59°01′15.9″W), during the pre-reproductive season (end of Southern winter). This place was selected because it has a low anthropogenic impact and pesticides concentration in sediments could be lower than the detection limit (CYP < 0.01 μg/kg; Atrazine < 0.1 μg/kg; Chlorpyrifos < 0.2 μg/Kg; Metsulfuron methyl, 2,4-D and Acetochlor < 0.5 μg/kg, manuscript in progress). The snails were taken to the laboratory and kept in dechlorinated tap water (pH 6.8–7.5 and dissolved oxygen 7.3–8 mg/l) with continuous aeration. During the acclimation time, which was performed for one week before the experiments, the temperature was maintained at 20 ± 2 °C, the photoperiod was 12:12-h L: D and snails were fed with commercial formulated fish food with high vegetable content.

The snails were selected according to their weight (1.15 ± 0.15 g) and size (1.85 ± 0.005 cm total shell length). All experiments were performed according to guidelines of the Institutional Animal Care and Use Committee of National University of the Centre of the Province of Buenos Aires (UNCPBA).

2.2. Determination of LC_{50}

The sensitivity of *C. parchappii* to CYP was evaluated using lethality as the endpoint. After the acclimation period, several bioassays were performed exposing snails for 96 h to different concentrations of CYP in the range of 10–70 mg/l with a dilution factor of 1.05 between doses. Additionally, two control treatments were included (Control 1: no pesticides or solvents; Control 2: without pesticides but with solvents). For each treatment, 10 snails were randomly placed into glasses aquarium (3 l) containing 1.5 l of the test solution. A stock solution of 150 g/l CYP (Glextrin 25 formulated solution containing 25% of active principle purchased from GLEBA S.A. La Plata, Argentina) was prepared in acetone (grade p.a.) and maintained in the dark at 4 °C. The subsequent working stock solutions were prepared by diluting the main stock with acetone. The final solvent concentration remained below 0.033% for all treatments. Mortality was recorded every 24 h, and the test solution was completely replaced in each aquarium.

The bioassay was performed at 20–22 °C and an L:D photoperiod of 12:12 h with continuous aeration. The snails were not fed the two days before the assay nor during the exposure period. Each treatment was performed in triplicate.

To determine the real pesticide water concentration in the test solutions, CYP was quantified by GC-ECD following the method of Hladik et al. (2009) as described in Lavarías et al. (2017). Method recoveries were 85% for the water samples, so the nominal concentrations were corrected reporting only these values.

In order to compare the sensitivity of *C. parchappii* with other freshwater invertebrates, the species sensitivity distributions (SSDs) for CYP were calculated using the USEPA SSD Generator software (http://www.epa.gov/caddis/da_software_ssdmacro.html). Comparisons were based on the acute 24-h toxicity data (LC_{50}) for freshwater invertebrates in similar experimental conditions. Data for CYP were obtained from the USEPA ECOTOX database (http://cfpub.epa.gov/ecotox/).

2.3. Sublethal toxicity assays

To evaluate the effect of CYP trough a selection of biomarkers, the snails were exposed to sublethal CYP concentrations (0.1 and 10 mg/l CYP) for 1, 4 and 10 days. These concentrations were chosen according to CYP concentrations reported in contaminated waters of the Pampean region (Mugni et al., 2010). The CYP exposure conditions were the same as those described for the LC_{50} assay. No mortality was observed in any treatment.

At the end of each exposure time, the snails were anaesthetized on ice for 10 min for organs dissection. Then, the digestive glands were weighed and stored at −80 °C for biochemical determinations. For histological and histochemical studies, only snails exposed for 4 days were analysed. For these histopathological analyses, small sections of the digestive gland were immediately fixed in Bouin solution for 4 h, then washed and stored in 70% ethanol. Other sections were cryopreserved in liquid nitrogen for histochemical determinations.

2.3.1. Biochemical measurements

2.3.1.1. Preparation of tissue homogenate. The digestive glands were weighed and homogenized (1:15 w/v) in 20 mM Tris-base cold buffer solution (pH 7.5), containing 0.15 M KCl and 1 mM EDTA, using a Teflon homogenizer. Homogenates were centrifuged at 2000 × *g* at 4 °C for 15 min and the supernatants were used for biochemical determinations. Total protein concentration was determined as described by Lowry et al. (1951) using bovine serum albumin as standard.

2.3.1.2. Antioxidant enzyme activities. The CAT activity was determined following the decomposition of hydrogen peroxide (H_2O_2) spectrophotometrically at 240 nm, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7) and 10 mM H_2O_2 (Aebi, 1984). Results were expressed as units of CAT per mg of proteins. One CAT unit was the amount of enzyme required to catalyse 1 nmol of H_2O_2/min.

The GPx activity was analysed according to Nebbia et al. (1993) in 0.1 M potassium phosphate buffer (pH 7) and 2 nM EDTA using 1.5 nM cumene hydroperoxide as substrate. The absorbance was measured at 340 nm. One GPx unit represented the amount of enzyme required to consume 1 nmol of NADPH/min. Results were expressed as units of GPx per mg of proteins.

The antitoxic defence enzyme GST was analysed spectrometrically at 340 nm according to Habig et al. (1974), using 1-chloro-2, 4-

dinitrobenzene (CDNB) as substrate. The final reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM CDNB and 1 mM GSH. One GST unit represented the amount of enzyme required to conjugate GSH with 1 μmol CDNB/min, and GST activity was expressed as units of GST per mg of protein.

2.3.1.3. Reduced glutathione content. The determination of GSH was performed according to the method of Ellman (1959) with minor modifications. After deproteinization with trichloroacetic acid (TCA 50%), the samples were centrifuged at 3000 × *g* for 30 min at 4 °C. Then, free endogenous GSH was determined using 1 mM of Ellman reactive (5, 5-dithio-bis-2-nitrobenzoic acid DTNB in methanol 1 mM). The absorbance was measured at 412 nm. GSH was used as standard and the results were expressed as nmol per mg of protein.

2.3.2. Histological studies

For the histological analysis, the digestive glands stored in 70% ethanol, were dehydrated at increasing ethanol concentrations and then embedded in glycol-methacrylate resin (Leica Historesin®). Sections were cut at 5 μm with an electronic microtome (Leica® RM 2155), stained with hematoxylin-eosin and observed under the light microscope (AXIOPLAN 2 Zeiss®). All images were recorded in an image analysis system (Axiovision Rel 4.4).

2.3.3. Histochemical analyses

Lipofuscins (LF) analysis was carried out in frozen digestive glands. Tissue sections of 9 μm thick were cut in a Leica CM1850 cryostat (Leica Instruments GmbH, Nusslock, Germany) in a thermostatic cabinet at less than −25 °C. LF content was studied histochemically using the Schmorl reaction (Lowe, 1988). Cryostat sections were fixed in formal calcium for 15 min at 4 °C, rinsed in distilled water and then incubated in the reaction medium containing 1% ferric chloride and 1% potassium ferricyanide (3:1) for 5 min. Sections were washed in 1% acetic acid for 1 min, rinsed in distilled water and mounted with glycerin. Cryosections were examined under a light microscope (AXIOPLAN 2 Zeiss®). The percentage area of tissue section covered by LF was assessed in 8 randomly selected fields per specimen using an objective lens of 65x magnification, analysing 4 snails per group. The total area scanned in each measurement was 300,000 μm^2.

For neutral lipid (NL) determinations, thick (9 μm) cryostat sections were fixed in formal calcium for 15 min at 4 °C, dried at room temperature and washed in isopropanol (60%). Subsequently, sections were stained with 1% oil red in 60% isopropanol for 20 min at room temperature. Sections were washed in 60% isopropanol and then in distilled water, counterstained with 1% Fast Green FCF (Sigma, F-7252) for 20 min and mounted in glycerine. Cryosections were examined under a light microscope (AXIOPLAN 2 Zeiss®). The percentage area of tissue section covered by neutral lipids was assessed in 8 randomly selected fields per specimen using an objective lens of 65x magnification, analysing 4 snails per group. The total area scanned in each measurement was 300,000 μm^2.

2.4. 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). The results of the biomarker analysis are shown as mean ± standard deviation (SD). Statistical comparisons of different treatments according to concentration and exposure time was done by two-way ANOVA. Normality and homogeneity of variances were verified with the Shapiro-Wilk and Levene tests, respectively. The subsequent Tukey test was used in those cases for which statistically significant differences between treatments were found ($p < 0.05$).

Table 1
LC_{50} values for adult snails of *C. parchappii* exposed to CYP. Equation of the Probit analysis $y = 57.47e^{-0.003x}$ $R^2 = 0.99$.

LC_{50}	CYP mg/l	Confidence intervals
24-h	53.62	51.47–59.44
48-h	51.22	48.83–58.4
72-h	47.63	46.26–50.2
96-h	44.59	43.63–45.61

3. Results

3.1. Determination of LC_{50}

No mortality was observed in the two control groups (with and without solvent). As expected, the sensitivity of *C. parchappii* to CYP increased with the exposure time (Table 1). No snail mortality was observed at concentrations below 34 mg/l CYP. Mortality started at 24-h to 42 mg/l CYP exposure and there were not surviving snails at 96-h to 50 mg/l CYP exposure. In surviving snails exposed to concentrations higher than 42 mg/L CYP, neurotoxic effects related to the loss of ability to substrate adhesion were observed. The variation between LC_{50} values for CYP at 24-h and 96-h was of 16.8% ($y = 57.47e^{-0.003x}$ $R^2 = 0.99$). The CYP sensitivity comparisons between *C. parchappii* and other freshwater invertebrate species are shown in Fig. 1. It is noticeable that this snail is the most tolerant of the species reported so far to CYP.

3.2. Sublethal effects of CYP on antioxidant defence

There were no statistically significant differences between control groups with and without acetone, so only these results of these first ones are shown.

Fig. 2 shows the toxic effect of sublethal CYP concentrations (0.22% and 22.4% of 96-h LC_{50}) on oxidative stress parameters in the digestive gland of adult snails. In general, a clear dose-dependent response for these biochemical measures was observed. The pyrethroid caused a decrease in CAT activity with respect to the controls and this behaviour was observed in all tested conditions (Fig. 2A). However, such decrease was not statistically significant in any case ($F_{2,87} = 6.57$; $p > 0.05$, Table 2), so this enzyme could have low sensitivity to CYP exposure. On the other hand, GPx activity was notably higher in snails exposed to CYP with values ranging between 45% and 190% compared to the controls ($F_{2,87} = 33.41$; $p < 0.01$, Table 2) at almost all treatments essayed (Fig. 2B). It was also observed that CYP caused a marked acute effect on the activity of this enzyme, represented by the large increases at day 1 of exposure. Besides, in control snails, CAT and GPx activities remained at very similar values from 1-day to 10-day evidencing that fasting would not have affected these enzymes (Fig. 2A–B).

The GST activity showed a clear concentration-dependent response to CYP exposure in treated snails when compared to controls, and it was statistically significantly different ($F_{2,\ 87} = 28.52$; $p < 0.01$, Table 2) at the highest concentration in the three evaluated times (Fig. 2C). This enzyme showed the highest sensitivity at 4 days of exposure, increasing by 79% and 116% compared to the control at 0.1 and 10 mg/l, respectively.

The antioxidant GSH, which functions as a substrate for GST and GPx, showed a similar tendency to these enzymes (Fig. 2D). Although GSH levels increased with pesticide concentration respect to controls, this parameter was only statistically significantly different ($F_{2,\ 87} = 22.06$; $p < 0.01$, Table 2) on days 4 and 10 at 10 mg/l of CYP by 196 and 957%, respectively. To analyse the effect of time at the two tested concentrations, the percentage variation of treatments as compared with 100% of their respective controls was calculated. It was observed that time affected the antioxidant parameters in a very

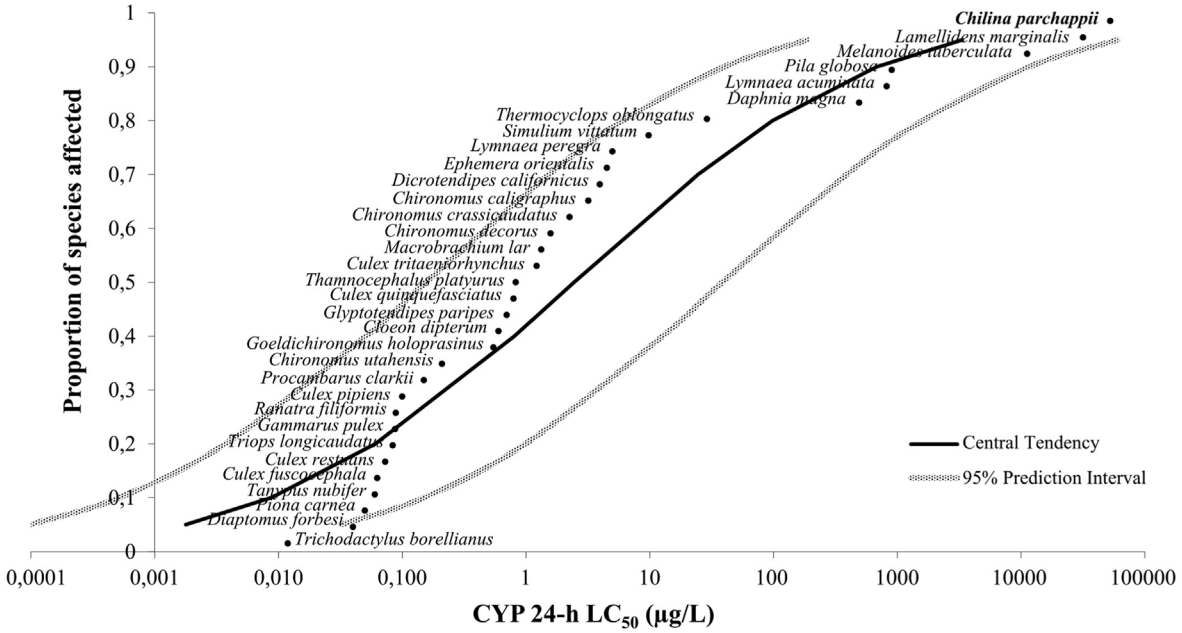

Fig. 1. Sensitivity of *C. parchappii* to CYP compared to other freshwater invertebrates calculated by species sensitivity distributions (SSDs) using acute 24-h toxicity data.

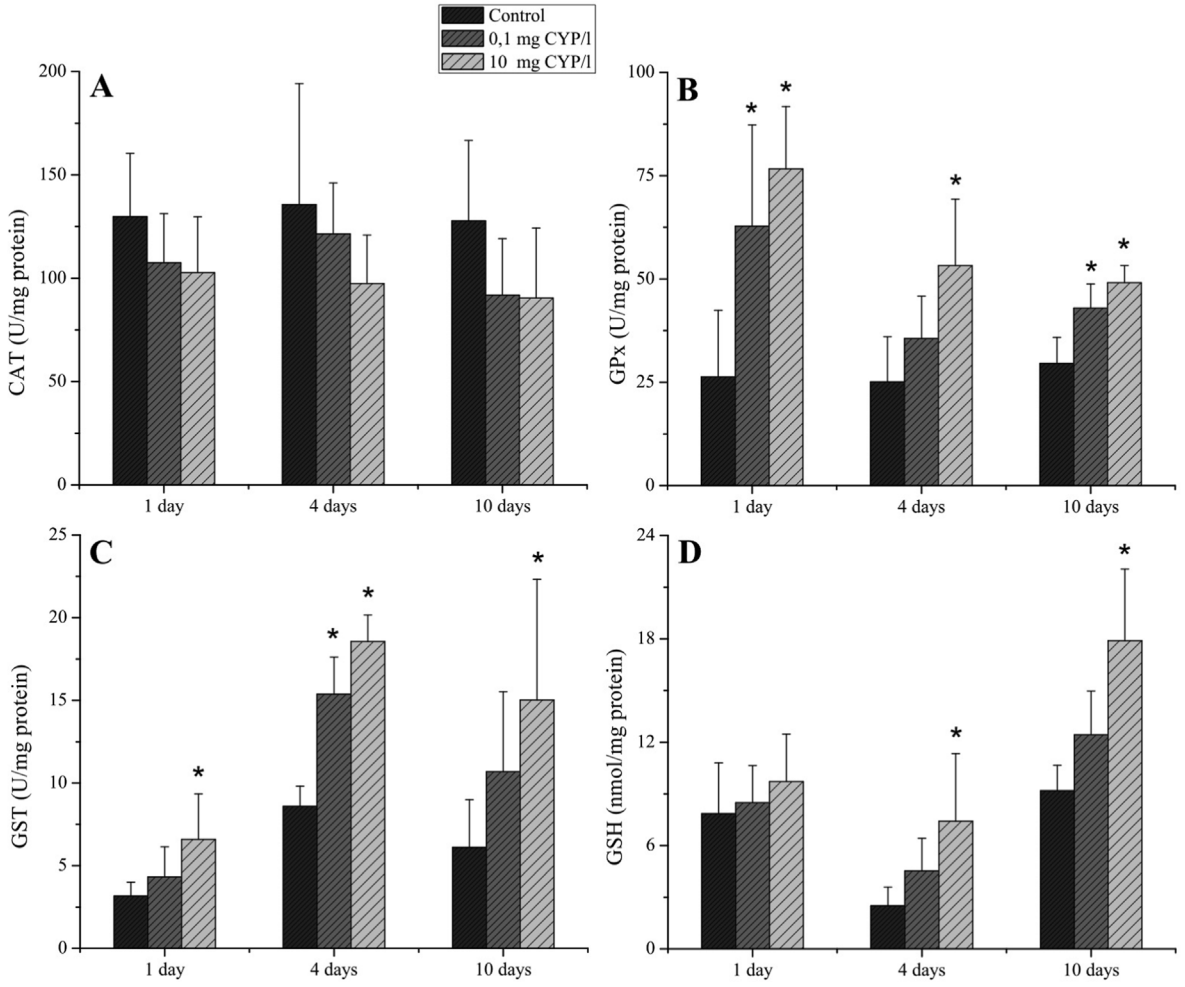

Fig. 2. Effect of sublethal CYP concentrations on CAT, GPx and GST activities and GSH content in the digestive gland of *C. parchappii*. CAT (A), GPx (B), GST (C), GSH (D). Data are shown as mean ± SD (n = 10). Statistical differences from the corresponding control are indicated as *($p < 0.01$) Control 0.1 mg/l 10 mg/l.

Table 2
Results of two-way ANOVA testing the effect of exposure time and CYP concentration on CAT, GPx and GST activities and GSH content in the digestive gland of *C. parchappii*.

Effect	CAT	GPx	GST	GSH
Concentration	$F_{2, 87} = 6.57$	$F_{2, 87} = 33.41^{**}$	$F_{2, 87} = 28.52^{**}$	$F_{2, 87} = 22.06^{**}$
Time	$F_{2, 87} = 1.20$	$F_{2, 87} = 11.80^{**}$	$F_{2, 87} = 47.15$	$F_{2, 87} = 57.40^{**}$
Concentration x time	$F_{4, 87} = 0.35$	$F_{4, 87} = 3.77^{*}$	$F_{4, 87} = 2.45$	$F_{4, 87} = 3.38^{*}$

Statistical differences are indicated as $^{*}(p < 0.05)$, $^{**}(p < 0.01)$.

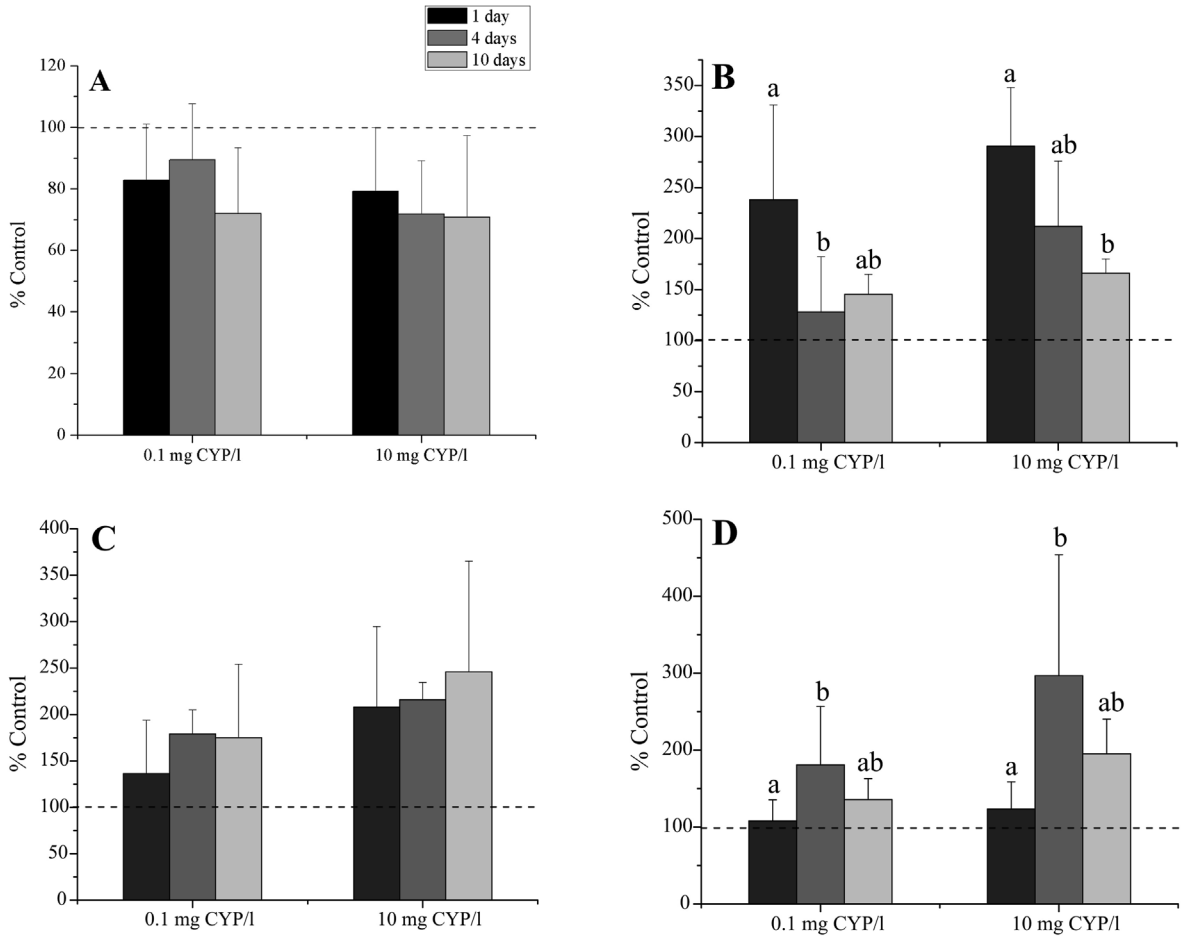

Fig. 3. Effect of time at any sublethal CYP concentrations on CAT, GPx and GST activities and GSH content in the digestive gland of *C. parchappii*. CAT (A), GPx (B) GST (C), GSH (D). Data are shown as mean ± SD (n = 10). **Statistically significant** differences between the effects observed at different times of study ($p < 0.01$) are indicated by different letters. 1 day 4 days 10 days.

different way (Fig. 3). There was no time-dependent response in CAT activity in the digestive gland of *C. parchappii* (Fig. 3A). However, GPx showed a tendency to decrease its activity over time and this effect was very noticeable at 10 mg/l of CYP concentration ($F_{2, 87} = 11.80$; $p < 0.01$, Table 2) (Fig. 3B). Opposite to that, an increasing time-dependent trend in GST activity was observed but there were no statistically significant differences ($F_{2, 87} = 47.15$; $p > 0.05$, Table 2; Fig. 3C). GSH levels showed the same behaviour at 0.1 and 10 mg/l of CYP, resulting in a biphasic pattern with the highest GSH concentrations at day 4 (Fig. 3D).

3.3. Histopathological effects of CYP

The digestive gland of the control *C. parchappii* showed a well-organized structure with numerous blind ended tubules (Fig. 4A). The simple epithelium was formed by three different cellular types: digestive cells, basophilic cells and excretory cells (Fig. 4B). The tubules showed a narrow lumen and were surrounded by connective tissue (Fig. 4A–B).

Pyrethroid exposure caused statistically significant histopathological alterations in the digestive gland of *C. parchappii* as compared to control. Epithelial atrophy was observed in snails exposed to 0.1 mg/l CYP (Fig. 4C). This atrophy is characterised by a lumen enlargement and a reduction of the epithelial thickness. Also, regressing tubules were observed in snails exposed to 0.1 mg/l CYP, characterised by vacuolisation and shedding of digestive cells into the lumen (Fig. 4D). At 10 mg/l CYP exposure, the atrophy was more intense (Fig. 4E) and an increasing number of excretory cells was observed (Fig. 4F).

3.4. Histochemical effects of CYP

Fig. 5A–C shows that LF accumulation is statistically significant ($p < 0.05$) in the digestive gland cells of snails exposed at both CYP concentrations as compared to control, with values ranging between

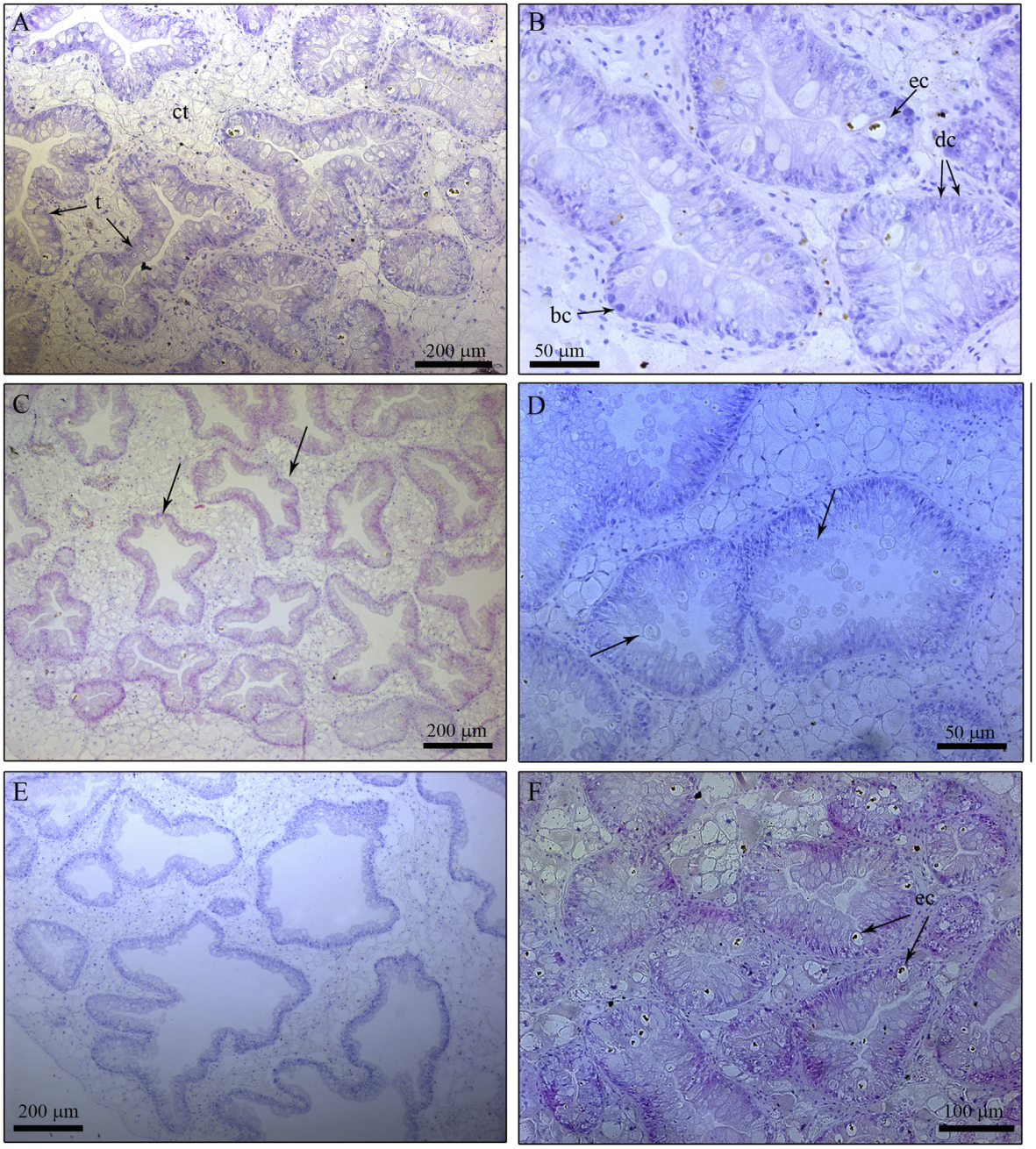

Fig. 4. Histopathological alterations in the digestive gland of *C. parchappii.* **A.** General aspect of the digestive gland of a control snail showing digestive tubules (dt) surrounded by connective tissue (ct). **B.** Detail of the digestive gland epithelia of a control snail formed by digestive cells (dc), basophilic cells (bc) and excretory cells (ec). **C.** Atrophied digestive tubules in a snail exposed to 0.1 mg/l of CYP showing a reduction of the epithelial thickness (arrows). **D.** Detachment of digestive cells (arrows) at the same treatment. **E.** Snail exposed to 10 mg/l CYP with evidence of severe atrophy. **F.** Increased number of excretory cells (ec) in a snail exposed to 10 mg/l CYP.

933% and 1006% (Fig. 6). Besides, it was observed that the insecticide caused a clear tendency to increase LN accumulation (Fig. 5D–F) although it was only statistically significantly different ($p < 0.05$) between control snails and those treated with 10 mg/l of CYP by 403% (Fig. 6).

4. Discussion

As mentioned before, *C. parchappii* is endemic to the central region of Argentina and shows a wide geographical distribution and low dispersion (Nuñez et al., 2010). Besides, it is a key component of benthic fauna and the diet of fishes. It belongs to one of the oldest families of pulmonary gastropods with hermaphrodite reproduction to adapt to the aquatic environment (Tietze and De Francesco, 2010). Also, this snail is easy to identify, collect and maintain under controlled laboratory conditions throughout the year, makes it a good model for ecotoxicological studies.

Recent evidence shows that, at a global scale, CYP is the most frequently detected pyrethroid not only in different environmental compartments but also in organisms (Tang et al., 2018). This kind of insecticides have garnered great interest due to the balance between the high level of effectiveness and low toxicity compared to other insecticides, as many authors have mentioned. However, in aquatic environments, pyrethroids are usually toxic to organisms and may cause

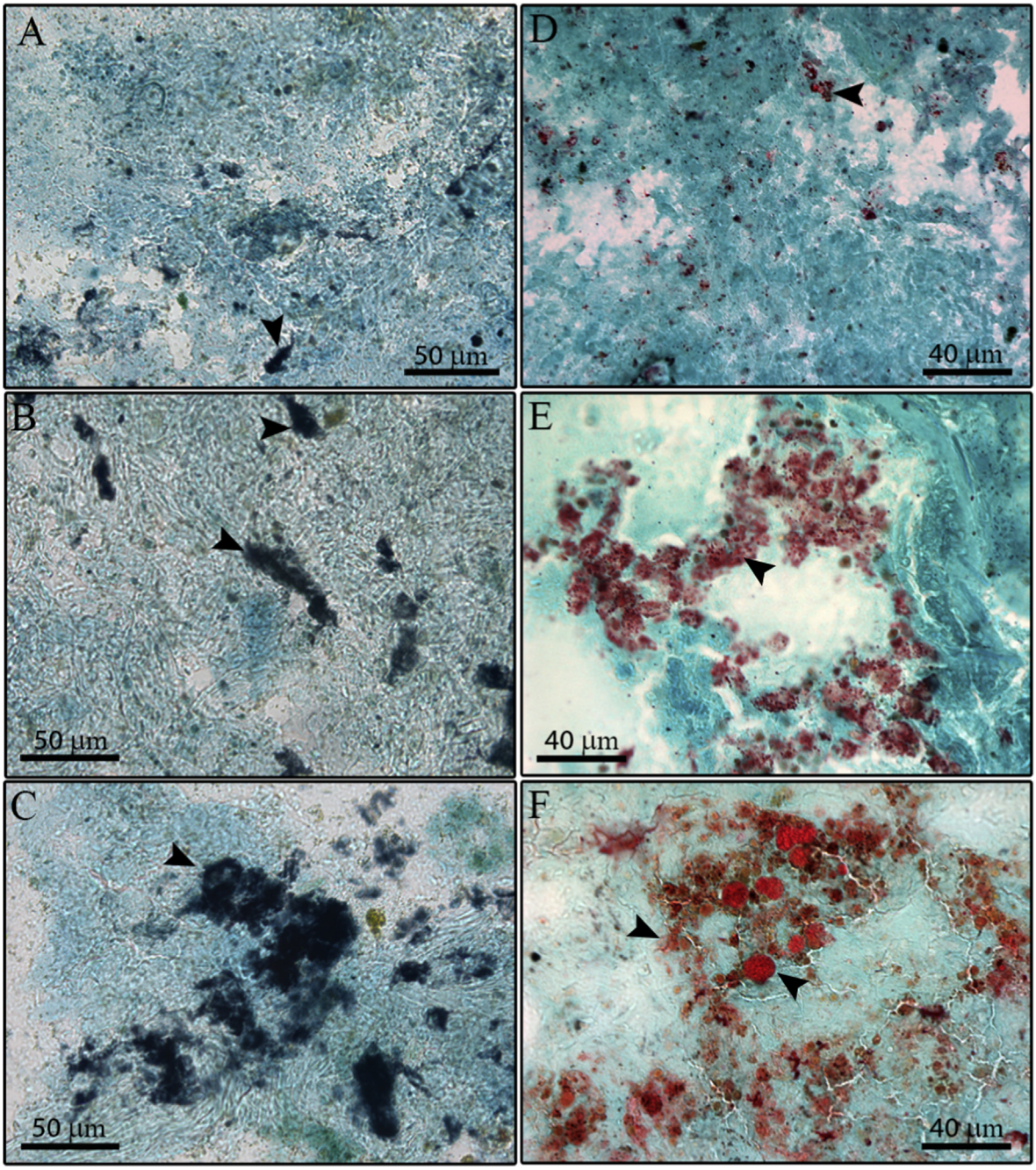

Fig. 5. A-C. Lipofuscin (LF) histochemistry in cryosections of the digestive gland of *C. parchappii* exposed to sublethal CYP concentrations for 4 day. Transverse sections of digestive tubules of control snails (**A**), snails exposed to 0.1 mg/l (**B**) and 10 mg/l (**C**) of CYP. **D-F.** Neutral lipid (NL) histochemistry in cryosections of the digestive gland of *C. parchappii* exposed to sublethal CYP concentrations for 4 day. Transverse sections of digestive tubules of control snails (**D**), snails exposed to 0.1 mg/l (**E**) and 10 mg/l (**F**) of CYP. Arrowhead indicate positive reaction.

long-term adverse effects due to their lipophilic nature. Like other pyrethroids, CYP has a short half-life in the water column (generally less than 2 days), but in the aquatic environment, it adheres to the suspended particulate matter and sediments, affecting, mainly, the benthic community (Ayad et al., 2011). Among freshwater invertebrates, molluscs are usually quite tolerant to pollutants like CYP (Ahirrao and Phand, 2015; Maund et al., 2012; Palmquist et al., 2012). The snail *C. parchappii* showed to be the most resistant species of those reported up to date (USEPA ECOTOX database, http://cfpub.epa.gov/ecotox/). This snail has a value of LC_{50} higher than other species such as *Melanoides tuberculata* (Mule and Lomte, 1992) and *Lamellidens marginalis* (Kumar et al., 2012) with 24-h LC_{50} values of 11.2 and 31.65 mg/l of CYP respectively. On the other hand, it is important to take into account that the differences in the LC_{50} reported values depend on the type of CYP formulation tested. For instance, the snail *Pila globosa* showed to be 2.6 times more sensitive to the commercial formulation than to the technical grade of CYP (Majumder and Kaviraj, 2015). Since commercial formulations are those that really have an environmental impact, it was preferred to use this kind of presentation for this study, as in most recent reports. Additionally, it is important to remark that the recovery time of CYP exposure in the aqueous solution was similar to that prepared using the pure active ingredient (Xu and Huang, 2017) and also to that prepared using a commercial formulation (Poletta et al., 2013).

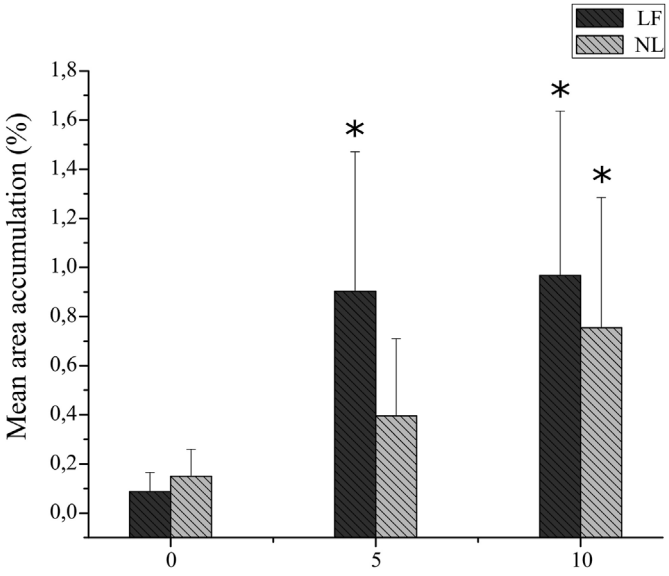

Fig. 6. Lipofuscin (LF) and neutral lipid (NL) accumulation in the digestive gland of *C. parchappii* exposed to sublethal CYP concentrations for 4 day. Data are expressed a mean percentage area filled with lipofuscin or neutral lipids ± SD. Statistical differences from the corresponding control are indicated as * ($p < 0.05$) LF NL.

In Argentina, in some cases, CYP concentrations exceed aquatic ecological guidelines (Carriquiriborde et al., 2007; Jergentz et al., 2005; Mugni et al., 2010), so non-target fauna could be affected at different biological levels. This presents the challenge of searching sensitive markers that help to monitor these pollutants in vulnerable environments. In this context, molluscs arise a great interest as potential sentinel organisms (Ayad et al., 2011; Hartnik et al., 2008; Sacchi et al., 2013; Solé et al., 2018; Usheva et al., 2006). As mentioned before, this taxon is little tolerant to pesticides possibly due to their limited capacity to metabolise them, so they tend to accumulate and transfer these toxicants through the food chain (Bhagat et al., 2016; Faggio et al., 2018). However, different parameters at molecular, biochemical, physiological and cellular levels have responded sensitively in molluscs to the exposure and/or the effects of anthropogenic compounds, providing accurate and reliable biological endpoints (biomarkers) for environmental assessment (Cuevas et al., 2015; El-Gendy et al., 2019; Shawa et al., 2011; Solé et al., 2018; Viarengo et al., 2007).

In molluscs, the digestive gland is usually the main target organ to evaluate the effects of many xenobiotics. Such organ is not only responsible for digestion and storage of nutrients but also for accumulation, detoxification and excretion of toxins, so it is considered suitable for biomarker estimations (Faggio et al., 2018; Mahmoud et al., 2012; Sellami et al., 2015; Usheva et al., 2006; Yao et al., 2017). CYP, like other toxicants, can interfere with optimal antioxidant status promoting oxidative damage, which may lead to biochemical (proteins, lipids and nucleic acids), physiological (cell functions) and morphological alterations (histopathologies) (Arrighetti et al., 2018; Shashikumar and Rajini, 2010). For that reason, the antioxidant system is usually the most studied biomarker of effects in target organisms (Bhagat et al., 2016; Manduzio et al., 2005; Monserrat et al., 2007). Aerobic organisms hold enzymatic and non-enzymatic antioxidant defensive systems to neutralise ROS. Among these oxidizing species, H_2O_2 is detoxified by CAT into O_2 and H_2O in the peroxisomes, and also by GPx using reduced glutathione mainly in the cytosol (Lushchak, 2011). Between these two enzymes, CAT has been the most studied in invertebrates and one of the first enzymes proposed as an effective biomarker for oxidative stress (Sellami et al., 2015). It has been observed that CAT activity increases in the digestive gland of freshwater molluscs under CYP exposition (Arrighetti et al., 2018; Khazri et al. 2015, 2016). However, CAT activity in *C. parchappii* was not altered in this study, probably because of the regulatory mechanism of ROS which may cause an increase, decrease or biphasic antioxidant enzymatic behaviour depending on toxic exposure conditions (Regoli and Giuliani, 2014).

On the other hand, both GPx and its cofactor GSH responded very sensitively to CYP exposition showing a dose-dependent increase in snails. This fact could be indicating that the main route for H_2O_2 metabolisation in *C. parchappii* is through GPx. Some pesticides have caused the increase of this enzyme in snails (Damásio et al., 2010; Khalil, 2015; Leomanni et al., 2015). The main function of GPx is to protect the cytosol and cellular membranes from hydroperoxides released by lipid peroxidation (Lushchak, 2011). Although GPx and GST activities could be affected when the GSH supply is reduced, this would not be the case of *C. parchappii* when exposed to CYP.

GST is the most studied phase II metabolism enzyme in aquatic organisms. Its main role is to conjugate electrophilic metabolites, such as pesticides, with GSH to facilitate their excretion and also protect cells from oxygen toxicity (Livingstone, 2001). It is known that many aquatic organisms could detoxify CYP through GST activity (Shashikumar and Rajini, 2010; Xu and Huang, 2017), and the same can be deduced from *C. parchappii* in this study. Sellami et al. (2015) explained that the increase of GST activity in the digestive gland of the bivalve *Venerupis decussata* exposed to permethrin could be a result of the protective effect of this enzyme when the phase I metabolism is not sufficient. This could explain the high tolerance of *C. parchapii* to the pesticide.

The tripeptide GSH also works as a non-enzymatic antioxidant defence quenching oxyradicals through its –SH group and as a reducing agent (Lushchak, 2011). Even though cells usually tend to maintain GSH at a constant level (Viarengo et al., 2007), CYP caused a clear tendency to increase GSH levels although they turned out statistically significantly different only at the highest concentration tested, presenting a low sensitivity. This result also shows that, despite the stressful conditions of fasting and toxic exposure, the snail *C. parchappii* was able to continue synthesising this essential tripeptide for maintaining redox homeostasis.

It must be pointed out that ROS are able to induce different cellular responses (Klotz and Steinbrenner, 2017). The variation in ROS levels can act as signalling molecules for proliferation, differentiation and stress-responsive survival pathways. In various model animals, it is known that some transcription nuclear factors like p53, FOXO and NRF2 support GSH synthesis and its utilisation by induction expression of the ROS-detoxifying enzymes like GPx and GST. Possibly, this is also taking place in snails exposed to CYP which would explain the up-regulation of these antioxidant defences. Therefore, as mentioned before, the modulation of ROS levels is fundamental for cellular homeostasis and *C. parchappii* would have the ability to cope with the harmful effects of exposure to high pesticide concentrations. However, CYP caused oxidative damage in the organ responsible for its detoxification (the digestive gland) evidenced by the increase in LF content. These metabolites are the end oxidation products of unsaturated NL and other endogenous or exogenous compounds (Moore et al., 2006). They are usually accumulated as insoluble granules within the lysosomes and cytoplasm altering intracellular digestion, as it was observed in the digestive gland of molluscs exposed to xenobiotics (Brooks et al., 2015; Guerlet et al. 2006; Leomanni et al., 2015; Marigómez et al., 2013; Raftopoulou and Dimitriadis, 2012). It has been proposed that the use of LF as a lipoperoxidation biomarker, seems to be more appropriate than the use of thiobarbituric acid reactive compounds (TBARs) (Viarengo et al., 2007). In the case of oxidative damage induced by CYP, it was observed that such biomarker responded very sensitively in *C. parchappii*, whereas the lipoperoxidation measured by TBARs in *P. canaliculata* did not show a direct response with the intensity of stressful conditions (Arrighetti et al., 2018). The differential sensitivity between both biomarkers could be attributed to the fact that these intermediate hydrophilic products (TBARs) are usually rapidly degraded and excreted, while the autofluorescent pigments (LF) are accumulated into

the cells persisting for longer periods in stressed organisms (Viarengo et al., 2007).

Unlike as it was observed in snails, exposure to CYP caused a decrease on GPx activity and GSH levels in the worm *Caenorhabditis elegans* (Shashikumar and Rajini, 2010) and in the freshwater mussel *Unio elongatulus eucirrus* (Köprücü et al., 2010), explaining clearly the deleterious effects of this pesticide on lipids of these invertebrates. In this way, the complementary LF measures are useful to understand the changes of antioxidative enzymatic activities induced by toxicants, evidencing the stress situation that the organism is going through (Viarengo et al., 2007).

In addition to oxidative damage, toxicants can affect NL metabolism producing, in general, its intracellular accumulation (Moore, 1988). In fact, the morphometrical alteration of NL in the digestive gland of molluscs has been proposed as a suitable biomarker of organic pollution (Leomanni et al., 2015; Marigómez et al., 2013; Perrat et al., 2013; Raftopoulou et al., 2006). Such a symptom could be due to the increased synthesis or decreased utilisation in the digestive cells (Marigómez and Baybay-Villacorta, 2003). It was observed that LF accumulation in lysosomes induces its dysfunction altering digestive enzymes in molluscs (Moore et al., 2006; Raftopoulou and Dimitriadis, 2012). Among these lysosomal enzymes, lipases under stress can decrease lipid catabolism, which is an essential pathway for energy supply (Regoli, 1992), especially in cases of fasting. The histopathological alterations observed in *C. parchappii* evidence lysosomal damage. Epithelial atrophy and vacuolisation of digestive cells, as well as excretory cell proliferation, were evident and intensity was dose-dependent. The symptoms of alteration, cell proliferation and epithelial renewal may be suitable biomarkers as it was proposed for other gastropods exposed to pesticides (Arrighetti et al., 2018; Cengiz et al., 2005; Hamed et al., 2007; Klobučar et al., 1997; Otludil et al., 2004). The increment of excretory activity in the snails could be associated to “old” or “exhausted” digestive cells under a stress condition as previously suggested (Marigómez et al., 1996; Zaldibar et al., 2007). On the other hand, the intensity of oxidative damage could be directly associated with lipophilia of xenobiotics (Marigómez and Baybay-Villacorta, 2003). Therefore, autophagocytic processes induced by LF trigger NL accumulation that would function as pesticide reservoirs in the digestive gland of *C. parchappii*, decreasing its bioavailability towards other susceptible organs. The parameter that defines such characteristic of the compounds is the n-Octanol/Water Partition Coefficient (*Kow*) and, as CYP has a high *Kow* value (*Kow* 6.3), a direct correlation between the accumulation of the pesticide and the lipid content would be expected in stressed snails (study in progress). This could explain the high tolerance showed by the snails to the pyrethroid, as **it was** observed in other molluscs (Werner et al., 2002). Likewise, other defence mechanisms such as the p-glycoprotein, which mediates multixenobiotic resistance, could be acting as observed in the freshwater snail *Physa acuta* (Assef et al., 2014). Therefore, this could be another interesting parameter to evaluate in future research.

5. Conclusion

Due to the worldwide continuous and increasing use of pesticides, it is necessary to detect pollutants in ecosystems and evaluate their effects on natural populations. The study of biomarkers in key species is essential for the diagnosis of environmental problems associated with modern agriculture. In this context, the present work shows that the freshwater snail *C. parchappii* is very tolerant to CYP exposure. The GSH metabolism could play a protective role in pesticide harm in snails. The antioxidant status and histopathological alterations in the digestive gland are sensitive biological parameters that could be used as potential biomarkers of aquatic pollution by CYP. In addition, it could be interesting to analyse the resistance mechanisms of this species to other pollutants in order to evaluate its potential use in monitoring programs.

CRediT authorship contribution statement

M.R. Fernández San Juan: Investigation, Validation, Data curation, Formal analysis, Writing - original draft. **A. Cortelezzi:** Conceptualization, Formal analysis, Writing - review & editing. **C.B. Albornoz:** Resources, Methodology. **S.M. Landro:** Data curation, Writing - review & editing. **F. Arrighetti:** Resources, Methodology, Writing - original draft. **R. Najle:** Resources. **S.M.L. Lavarías:** Conceptualization, Validation, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Ahirrao, D.V., Phand, D.L., 2015. Impact of cypermethrin on biochemical contents of a freshwater snail *Bellamya bengalensis*. J. Exp. Biol. Agric. Sci. 3 (1), 10–14.
Arrighetti, F., Ambrosio, E., Astiz, M., Rodrigues Capítulo, A., Lavarías, S., 2018. Differential response between histological and biochemical biomarkers in the apple snail Pomacea canaliculata (Gasteropoda: Amullariidae) exposed to cypermethrin. Aquat. Toxicol. 194, 140–151.
Assef, Y.A., Horak, C.N., Boquet, M.G., 2014. Characterisation of the multixenobiotic resistance (MXR) mechanism in the freshwater snail Physa acuta from Patagonia (Argentina). N. Z. J. Mar. Freshw. Res. 48 (1), 86–96.
Ayad, M.A., Fdil, M.A., Mouaba, A., 2011. Effects of cypermethrin (pyrethroid insecticide) on the valve activity behavior, byssal thread formation, and survival in air of the marine mussel Mytilus galloprovincialis. Arch. Environ. Contam. Toxicol. 60, 462–470.
Bhagat, J., Ingole, B.S., Singh, N., 2016. Glutathione S-transferase, catalase, superoxide dismutase, glutathione peroxidase, and lipid peroxidation as biomarkers of oxidative stress in snails: a review. Invertebr. Surviv. J. 13, 336–349.
Brooks, S.J., Harman, C., Hultman, M.T., Berge, J.A., 2015. Integrated biomarker assessment of the effects of tailing discharges from an iron ore mine using blue mussels (*Mytilus* spp.). Sci. Total Environ. 524–525, 104–114. https://doi.org/10.1016/j.scitotenv.2015.03.135.
Carriquiriborde, P., Díaz, J., Mugni, H., Bonetto, C., Ronco, A.E., 2007. Impact of cypermethrin on stream fish populations under field-use in biotech-soybean production. Chemos 68, 613–621.
CASAFE, 2013. Cámara de Sanidad Agropecuaria y Fertilizantes. Buenos Aires, Argentina. http://www.casafe.org/publicaciones/estadisticas.
Cengiz, E.I., Yildirim, M.Z., Otludil, B., Ünlü, E., 2005. Histopathological effects of Thiodan® on the freshwater snail, *Galba truncatula* (gastropoda, pulmonata). J. Appl. Toxicol. 25, 464–469.
Cuevas, N., Zorita, I., Costa, P.M., Franco, J., Larreta, J., 2015. Development of histopathological indices in the digestive gland and gonad of mussels: integration with contamination levels and effects of confounding factors. Aquat. Toxicol. 162, 152–164.
Damásio, J., Navarro-Ortega, A., Tauler, R., Lacorte, S., Barceló, D., Amadeu, M.V.M., Soares, A.M.V.M., Miguel Angel López, M.A., Riva, M.C., Barata, C., 2010. Identifying major pesticides affecting bivalve species exposed to agricultural pollution using multi-biomarker and multivariate methods. Ecotoxicology 19, 1084–1094.
El-Gendy, K.S., Radwan, M.A., Gad, A.F., Khamis, A.E., Eshra, E.H., 2019. Use of multiple endpoints to investigate the ecotoxicological effects of abamectin and thiamethoxam on *Theba pisana* snails. Ecotoxicol. Environ. Saf. 167, 242–249.
Ellman, G.L., 1959. Tissue sulfhydril groups. Arch. Biochem. Biophys. 82, 70–77.
Estebenet, A., Cazzaniga, N., Pizani, N., 2002. The natural diet of the Argentinean endemic snail *Chilina parchappii* (Basommatophora: Chilinidae) and two other Coexisting pulmonate gastropods. Veliger 45 (1), 71–78.
Faggio, C., Tsarpali, V., Dailianis, S., 2018. Mussel digestive gland as a model tissue for

assessing xenobiotics: an overview. Sci. Total Environ. 636, 220–229.
Finney, D.J., 1971. Probit Analysis, third ed. Cambridge University Press, London, UK.
Guerlet, E., Ledy, K., Giambérini, L., 2006. Field application of a set of cellular biomarkers in the digestive gland of the freshwater snail *Radix peregra* (Gastropoda, Pulmonata). Aquat. Toxicol. 77, 19–32.
Habig, W.B., Pabst, M.J., Jakoby, W.B., 1974. Glutathione Transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Hamed, S., Abdelmeguied, N., Essawy, A., Radwan, M.A., Hegazy, A.E., 2007. Histological and ultrastructural changes induced by two carbamate molluscicides on the digestive gland of *Eobania vermiculata*. J. Biol. Sci. 7, 1017–1037.
Hladik, M.L., Smalling, K.L., Kuivila, K.M., 2009. Methods of analysis-determination of pyrethroid insecticides in water and sediment using gas chromatography/mass spectrometry. U.S. Geol. Surv. Tech. Methods 5-C2.
Hartnik, T., Sverdrup, L.E., Jensen, J., 2008. Toxicity of the pesticide alpha-cypermethrin to four soil nontarget invertebrates and implications for risk assessment. Environ. Toxicol. Chem. 27, 1408–1415.
Hinton, D.E., Baumann, P.C., Garner, G.R., Hawkins, W.E., Hendricks, J.D., Murchelano, R.A., Okihiro, M.S., 1992. Histopathologic biomarkers. In: Hugget, R.J., Kimerie, R.A., Mehrle, P.M., Bergman, H.L. (Eds.), Biomarkers Biochemical, Physiollogical, and Histological Markers of Anthropogenic Stress. Lewis Publishers, Chelsea, USA, pp. 155–159.
Howcroft, C., Amorim, M., Gravato, C., Guilhermino, L., Soares, A., 2009. Effects of natural and chemical stressors on *Enchytraeus albidus*: can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environ. Int. 35, 318–324.
Jergentz, S., Mugni, H., Bonetto, C., Schulz, R., 2005. Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. Chemos 61, 817–826.
Kakko, I., Toimela, T., Tahti, H., 2003. The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. Chemos 51, 475–480.
Khalil, A., 2015. Toxicological effects and oxidative stress responses in freshwater snail, *Lanistes carinatus*, following exposure to chlorpyrifos. Ecotoxicol. Environ. Saf. 116, 137–142.
Khazri, A., Sellami, B., Dellali, M., Corcellas, C., Eljarrat, E., Barceló, D., Mahmoudi, E., 2015. Acute toxicity of cypermethrin on the freshwater mussel Unio gibbus. Ecotoxicol. Environ. Saf. 115, 62–66.
Khazri, A., Sellami, B., Dellali, M., Corcellas, C., Eljarrat, E., Barceló, D., Beyrem, H., Mahmoudi, E., 2016. Diastereomeric and enantiomeric selective accumulation of cypermethrin in the freshwater mussel *Unio gibbus* and its effects on biochemical parameters. Pestic. Biochem. Physiol. 129, 83–88.
Klobučar, G.I.V., Lajtner, J., Erben, R., 1997. Lipid peroxidation and histopathological changes in the digestive gland of a freshwater snail *Planorbarius corneus* L. (gastropoda, pulmonata) exposed to chronic and sub-chronic concentrations of PCP. Bull. Environ. Contam. Toxicol. 58, 128–134.
Klotz, L., Steinbrenner, H., 2017. Cellular adaptation to xenobiotics: interplay between xenosensors, reactive oxygen species and FOXO transcription factors. Redox Biol. 13, 646–654.
Köprücü, K., Yonar, S.-M., Seker, E., 2010. Effects of cypermethrin on antioxidant status, oxidative stress biomarkers, behavior, and mortality in the freshwater mussel *Unio elongatulus eucirrus*. Fish. Sci. 76, 1007–1013.
Kumar, S., Pandey, R.K., Das, S., Das, V.K., 2012. Acute toxicity and behavioral responses of a freshwater mussel, *Lamellidens marginalis*, exposed to Cypermethrin. J. Appl. Biosci. 38, 98–100.
Lavarías, S., Arrighetti, F., Siri, A., 2017. Histopathological effects of cypermethrin and *Bacillus thuringiensis var. israelensis* on midgut of *Chironomus calligraphus* larvae (Diptera: Chironomidae). Pestic. Biochem. Physiol. 139, 9–16.
Leomanni, A., Schettino, T., Calisi, A., Gorbi, S., Mezzelani, M., Regoli, F., Lionetto, M., 2015. Antioxidant and oxidative stress related responses in the Mediterranean land snail *Cantareus apertus* exposed to the carbamate pesticide Carbaryl. Comp. Biochem. Physiol., C 168, 20–27.
Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.
Lowe, D.M., 1988. Alterations in cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. Mar. Ecol. Prog. Ser. 46, 91–100.
Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.
Mahmoud, N., Dellali, M., Aissa, P., Mahmoudi, E., 2012. Environ Monit Assess. Acute toxicities of cadmium and permethrin on the pre-spawning and post-spawning phases of *Hexaplex trunculus* from Bizerta Lagoon. Tunisia 184, 5851–5861.
Majumder, R., Kaviraj, A., 2015. Variation in acute toxicity between technical grade and commercial formulation of cypermethrin to some non- target freshwater organisms. Int. J. Curr. Res. 7, 16755–16759.
Manduzio, H., Rocher, B., Durand, F., Galap, C., Leboulenger, F., 2005. The point about oxidative stress in molluscs. ISJ 2, 91–104.
Manjunatha, B., Tirado, J., Salas, P., Ortiz, D., Mohiddin, G., Reddy, V., Raju, M., Selvanayagam, M., 2015. Enzyme activities of Nile tilapia (Oreochromis niloticus) during lethal and sublethal exposure to cypermethrin. Der Pharma Chem. 7 (7), 279–283.
Marigómez, I., Soto, M., Kortabitarte, M., 1996. Tissue-level biomarkers and biological effect of mercury on sentinel slugs *Arion ater*. Arch. Environ. Contam. Toxicol. 31, 54–62.
Marigómez, I., Baybay-Villacorta, L., 2003. Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquat. Toxicol. 64, 235–257.
Marigómez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., Cajaraville, M.P., 2013. Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill "mussel Watch. Ecotoxicology 22, 486–505.
Maund, S.J., Campbell, P.J., Giddings, J.M., Hamer, M.J., Henry, K., Pilling, E.D., Warinton, J.S., Wheeler, J.R., 2012. Ecotoxicology of synthetic pyrethroids. Top. Curr. Chem. 314, 137–166.
Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Lopes Leães Pinho, G., Soares Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 221–234.
Moore, M.N., 1988. Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscan digestive cells to environmental and experimental exposure to xenobiotics. Mar. Ecol. Prog. Ser. 46, 81–89.
Moore, M.N., Allen, J.I., Somerfield, P.J., 2006. Autophagy: role in surviving environmental stress. Mar. Environ. Res. 62, 420–425.
Mugni, H., Demetrio, P., Marino, D., Ronco, A., Bonetto, C., 2010. Toxicity persistence following an experimental cypermethrin and chlorpyrifos application in Pampasic surface waters (Buenos Aires, Argentina). Bull. Environ. Contam. Toxicol. 84, 524–528.
Mule, M.B., Lomte, V.S., 1992. Cypermethrin (synthetic pyrethroid) toxicity to freshwater snail *Thiara tuberculate*. Pollut. Res. 11, 225–229.
Nebbia, C., Dacastro, M., Soffietti, M.G., Rasero, R., Principato, G.B., Di Simplicio, P., 1993. Inhibition of hepatic xenobiotic metabolism and of glutathione-dependent enzyme activities by zinc ethylene-bis-dithiocarbamate in the rabbit. Pharmacol. Toxicol. 73, 233–239.
Nuñez, V., Gutiérrez Gregoric, D.E., Rumi, A., 2010. Freshwater gastropod provinces from Argentina. Malacologia 53, 47–60.
Otludil, B., Cengiz, E.I., Yildirim, M.Z., Ünver, Ö., Ünlü, E., 2004. The effects of endosulfan on the great ramshorn snail Planorbarius corneus (Gastropoda, Pulmonata): a histopathological study. Chemos 56 (7), 707–716.
Palmquist, K., Salatas, J., Fairbrother, A., 2012. Pyrethroid insecticides: use, environmental fate, and ecotoxicology. In: Perveen, F. (Ed.), Insecticides - Advances in Integrated Pest Management. InTech, Rijeka, Croatia, pp. 251–278.
Perrat, E., Couzinet-Mossion, A., Fossi Tankoua, O., Amiard-Triquet, C., Wielgosz-Collin, G., 2013. Variation of content of lipid classes, sterols and fatty acids in gonads and digestive glands of Scrobicularia plana in relation to environment pollution levels. Ecotoxicol. Environ. Saf. 90, 112–120.
Poletta, G.L., Gigena, F., Loteste, A., Parma, M.J., Kleinsorge, E.C., Simoniello, M.F., 2013. Comet assay in gill cells of *Prochilodus lineatus* exposed in vivo to cypermethrin. Pestic. Biochem. Physiol. 107, 385–390.
Raftopoulou, E.K., Dailianis, S., Dimitriadis, V.K., Kaloyianni, M., 2006. Introduction of cAMP and establishment of neutral lipids alterations as pollution biomarkers using the mussel *Mytilus galloprovincialis*. Correlation with a battery of biomarkers. Sci. Total Environ. 368, 597–614.
Raftopoulou, E.K., Dimitriadis, V.K., 2012. Aspects of the digestive gland cells of the mussel *Mytilus galloprovincialis*, in relation to lysosomal enzymes, lipofuscin presence and shell size: Contribution in the assessment of marine pollution biomarkers. Mar. Pollut. Bull. 64, 182–188.
Regoli, F., 1992. Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. Mar. Ecol. Prog. Ser. 84, 63–69.
Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117.
Sacchi, A., Mouneyrac, C., Bolognesi, C., Sciutto, A., Roggieri, P., Fusi, M., Beone, G.M., Capri, E., 2013. Biomonitoring study of an estuarine coastal ecosystem, the Sacca di Goro lagoon, using *Ruditapes philippinarum* (Mollusca: Bivalvia). Environ. Pol. 177, 82–89.
Sellami, B., Khazri, A., Mezni, A., Louati, H., Dellali, M., Aissa, P., Mahmoudi, E., Beyrem, H., Sheehan, D., 2015. Effect of permethrin, anthracene and mixture exposure on shell components, enzymatic activities and proteins status in the Mediterranean clam *Venerupis decussata*. Aquat. Toxicol. 158, 22–32.
Shashikumar, S., Rajini, P.S., 2010. Cypermethrin-induced alterations in vital physiological parameters and oxidative balance in *Caenorhabditis elegans*. Pestic. Biochem. Physiol. 97, 235–242.
Shawa, J.P., Dondero, F., Moore, M.N., Negri, A., Dagnino, A., Readman, J.W., Lowe, D.R., Frickers, P.E., Beesley, A., Thain, J.E., Viarengo, A., 2011. Integration of biochemical, histochemical and toxicogenomic indices for the assessment of health status of mussels from the Tamar Estuary. U.K. Mar. Environ. Res. 72, 13–24.
Soderlund, D.M., Bloomquist, J.R., 1989. Neurotoxic actions of pyrethroid insecticides. Annu. Rev. Entomol. 34, 77–96.
Solé, M., Bonsignore, M., Rivera-Ingraham, G., Freitas, R., 2018. Exploring alternative biomarkers of pesticide pollution in clams. Mar. Pollut. Bull. 136, 61–67.
Tietze, E., De Francesco, C., 2010. Environmental significance of freshwater mollusks in the Southern Pampas, Argentina: to what detail can local environments be inferred from mollusk composition? Hydrobiologia 641, 133–143.
Tang, W., Wang, D., Wang, J., Wu, Z., Li, L., Huang, M., Xu, S., Yan, D., 2018. Pyrethroid pesticide residues in the global environment: an overview. Chemos 191, 990–1007.
Usheva, L.N., Vaschenko, M.A., Durkina, V.B., 2006. Histopathology of the digestive gland of the bivalve mollusk *Crenomytilus grayanus* (Dunker, 1853) from southwestern Peter the great Bay, sea of Japan. Russ. J. Mar. Biol. 32, 166–172.
Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koeheler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol., C 146, 281–300.
Wei, K., Yang, J., 2014. Oxidative damage induced by copper and beta-cypermethrin in

gill of the freshwater crayfish *Procambarus clarkii*. Ecotoxicol. Environ. Saf. 113, 446–453.
Werner, I., Geist, J., Okihiro, M., Rosenkranz, P., Hinton, D.E., 2002. Effects of dietaryexposure to the pyrethroid pesticide esfenvalerate on medaka (Oryzias latipes). Mar. Environ. Res. 54, 609–614.
Xu, P., Huang, L., 2017. Effects of α-cypermethrin enantiomers on the growth, biochemical parameters and bioaccumulation in *Rana nigromaculata* tadpoles of the anuran amphibians. Ecotoxicol. Environ. Saf. 139, 431–438.
Yao, L., Pan, L., Guo, R., Miao, J., 2017. Expression profiles of different glutathione S-transferase isoforms in scallop Chlamys farreri exposed to benzo[a]pyrene and chrysene in combination and alone. Ecotoxicol. Environ. Saf. 142, 480–488.
Zaldibar, B., Cancio, I., Marigómez, I., 2007. Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarker measurements. Aquat. Toxicol. 81, 183–196.