Aquatic Toxicology 194 (2018) 140–151

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Differential response between histological and biochemical biomarkers in the apple snail *Pomacea canaliculata* (Gasteropoda: Amullariidae) exposed to cypermethrin

F. Arrighetti[a], E. Ambrosio[b], M. Astiz[c], A. Rodrigues Capítulo[b], S. Lavarías[b,*]

[a] Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" CONICET, CABA, Argentina
[b] Instituto de Limnología de La Plata "Dr. Raúl A. Ringuelet" (ILPLA) CCT CONICET La Plata-Universidad Nacional de La Plata (UNLP), La Plata, Argentina
[c] Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP) CCT CONICET La Plata-Universidad Nacional de La Plata (UNLP), La Plata, Argentina

ARTICLE INFO

Keywords:
Biomarker
Cypermethrin
Histopathological
Mollusc
Oxidative stress

ABSTRACT

To develop effective programs to monitor water quality is necessary to identify sensitive biomarkers in indicator species. The aim of this study was to evaluate different biomarkers in the apple snail *Pomacea canaliculata* exposed to the insecticide Cypermethrin (CYP). Adult male and female snails were exposed to sublethal CYP concentrations (10, 25 and 100 $\mu g\, l^{-1}$) for 1, 4, 7 and 14 days. The recovery of the exposed snails was also studied by a post-exposure assay. The activities of the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), the levels of lipid peroxidation (LPO) and protein oxidation (PC) in digestive gland and gills were studied as biomarkers of exposure. Histopathological changes in target tissues were also evaluated. In digestive gland, CYP caused a significant increase in SOD, CAT and GST activities compared to control ($p < 0.05$) as well as in LPO and PC levels ($p < 0.05$). However, such biochemical effects were neither concentration nor time dependent. Histopatological changes were observed in the exposed groups, such as an increase in the number of basophilic cells, hemocytic infiltration and epithelia atrophy. Additionally, a positive correlation between the surface occupied by pigmented corpuscles and CYP concentrations was observed at all exposure periods. Gills showed greater sensitivity to oxidative damage than digestive gland. CYP caused an acute toxic effect in LPO levels in this respiratory organ. The gill filament of exposed snails, exhibited a reduction or loss of cilia, vacuolization of the columnar cells and an increase in haemocyte content irrespective of the concentration. High concentrations of CYP caused disruptions in the columnar muscle fibers. In general, snails did not show an improvement in their basal state during post-exposure treatment. Apparently, males and females do not have differential sensitivity to the pesticide. The results of this study suggest that histopathological changes are the most sensitive time- and dose-dependent biomarkers of toxicity induced by CYP in *P. canaliculata*.

1. Introduction

Environmental pollutants usually cause multiple toxic effects in exposed organisms, so it is essential to understand the differential biological responses and the underlying mechanisms (Rivadeneira et al., 2013). Among such responses, those at molecular level are usually the first ones to appear and precede those at higher organization levels. It is known that several contaminants cause cellular oxidative stress by disruption of mitochondrial function (Livingstone, 2001). That situation occurs when antioxidant defenses fail to detoxify reactive oxygen species (ROS), causing damage in biomolecules. In aquatic organisms the oxidative status can be estimated by measuring levels of protein and lipid oxidation, as well as the activities of antioxidant enzymes (Monserrat et al., 2007; Lushchak, 2011; Regoli and Giuliani, 2014). On the other hand, histopathological alterations show biochemical and physiological changes caused by toxicant exposure, being useful biomarkers of effect. Water environments are highly vulnerable to the input of anthropogenic pollutants, such as those used for pest control management. In this regard, biomarkers studies are considered valuable tools for the evaluation of general health state of an ecosystem.

Synthetic pyrethroid insecticides are extensively applied in agricultural practices as well as in mosquito control and ectoparasitic disease treatments (Ansari et al., 2011). The pyrethroid cypermethrin (CYP) (α-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl) 2-2-dimethylcyclo- propane carboxylate) has been widely

* Corresponding author at: Instituto de Limnología "Dr. Raúl A. Ringuelet" (ILPLA) CCT CONICET La Plata – Universidad Nacional de La Plata (UNLP), Boulevard 120 y 62 La Plata, 1900, Argentina.Tel.: +54 221 4222775.
E-mail address: sabrinalavarias@ilpla.edu.ar (S. Lavarías).

https://doi.org/10.1016/j.aquatox.2017.11.014
Received 5 October 2017; Received in revised form 21 November 2017; Accepted 22 November 2017
Available online 23 November 2017
0166-445X/

used, mainly to control lepidoptera and coleopteran pest. Nevertheless, this insecticide is toxic for a broad spectrum of aquatic organisms, being fish and invertebrates the most sensitive ones (Friberg-Jensen et al., 2003; Sánchez-Fortún and Barahona, 2005; Carriquiriborde et al., 2007; Sepici-Dincel et al., 2009; Nørum et al., 2010). CYP mainly exerts a neurotoxic action on organisms but it may also cause oxidative stress as a consequence of its metabolism (Shashikumar and Rajini, 2010) Among the diverse symptoms caused by CYP, histological alterations have been observed in aquatic organisms (Korkmaz et al., 2009; Maharajan et al., 2015; Ullah et al., 2015; Wei and Yang, 2015; Arslan et al., 2017; Lavarías et al., 2017).

For biomonitoring freshwater ecosystems contaminated with these pesticides, several biomarkers have been proposed for benthic macroinvertebrates (Nørum et al., 2010; Ray et al., 2013; Antwi and Reddy, 2015; Khazri et al., 2015; Merivee et al., 2015; Khazri et al., 2016). For this purpose, it is necessary to characterize sensitive biomarkers in organisms exposed under laboratory conditions, to identify toxic mechanisms that could be translated to a population level (Faria et al., 2006).

The freshwater snail *Pomacea canaliculata* is a cosmopolitan freshwater mollusc, native from the La Plata basin (Argentina) and able to tolerate several environmental conditions (Seuffert and Martín, 2013; Ferreira and Rodrigues Capítulo,2017). In Asia, this snail is considered harmful to rice and other crops (Lowe et al., 2000) and is associated with the transmission of eosinophilic meningoencephalitis (Lv et al., 2009). Therefore this organism is directly or indirectly target of pesticides.

Although the effect of several pesticides has been studied on this snail, especially those used as molluscides (Giraud-Billoud et al., 2013; Martínez et al., 2017) no data was found about the toxic effect of CYP on different organs. In order to evaluate the most sensitive biomarkers, the aim of the present work was to compare the effect of CYP on biochemical parameters related to oxidative stress and histological alterations in *P canaliculata*. Furthermore, the differences between males and females in such biomarkers were analyzed.

2. Materials and methods

2.1. Sample collection

Adult males and females of *Pomacea canaliculata* were collected in Zapata stream, a tributary of Río de la Plata estuary, Argentina (34°59′19″S, 57°42′59″W) during the pre-reproductive season (end of winter). They were adapted to laboratory conditions in dechlorinated tap water ($CaCO_3$ hardness, 160 mg l^{-1}, pH between 6.6 and 6.9, and dissolved oxygen between 4.5 and 5 mg l^{-1}) at 22 ± 2 °C, and 12:12 h L:D photoperiod for at least two weeks before the experiments. Individuals were fed *ad libitum* with fresh lettuce and supplemented weekly with carp food pellets only during acclimation period (Giraud-Billoud et al., 2011).

The specimens were selected according to their weight (16 ± 4.5 g) and size (30.2 ± 7.4 mm total shell length). The differentiation between males and females was performed taking into account the external shape of the operculum (Cazzaniga, 1990), later confirmed during organ dissection. All experiments were performed according to guidelines of the Institutional Animal Care and Use Committee of National University of La Plata (UNLP).

2.2. Sublethal toxicity assays

It should be clarified that previously to sublethal bioassays, assays to determine the sensitivity of this snail to CYP determining lethal doses as end point were performed. However, no mortality was observed at 400 µg l^{-1} of CYP exposure for 4 days, estimating that 96-h LC_{50} values are greater than this concentration. Due to the maximum concentration detected in water contaminated with CYP from the streams inhabited by *P. canaliculata* was close to 100 µg l^{-1} (Mugni et al., 2011), this concentration was selected.

Bioaccumulation assays were performed exposing the individuals to sublethal CYP concentrations (below NOAEL) 10, 25 and 100 µg l^{-1}, during 1, 4, 7 and 14 days. In order to study recovery response in snails, a biodepuration assay was performed as follow: after 4 days of CYP exposure at the same concentrations used for bioaccumulations assays, the snails were transferred into CYP free water for during 10 days. A stock solution of 2.5 g l^{-1} CYP (Glextrin 25 formulated-solution containing 25% of active principle purchased from GLEBA S.A. La Plata, Argentina) was prepared in absolute ethanol (grade p.a.) and maintained in the dark at 4 °C. The subsequent working stock solutions were prepared by diluting the main stock with absolute ethanol. The final ethanol concentration remained below 0.001% for all treatments (Giraud-Billoud et al., 2013). The control group was kept with ethanol but without CYP, additionally another solvent-free control group was included. For the experiments, adult snails (n total = 360) were placed into glasses aquarium containing 2.5 l of test solution. Three males and 3 females were placed in separate individual containers. The assays were made by triplicate, at 20–22 °C, and 12:12 h L:D photoperiod. Dissolved oxygen and pH were measured in all containers. Test solution was daily replaced. Snails were not fed for 2 days before the assay and during the exposure period. All experiments were performed according to guidelines of the Institutional Animal Care and Use Committee of National University of La Plata (UNLP).

To determine the effective water concentration in the test solutions, CYP was quantified by GC-ECD following the method described in Lavarías et al. (2017).

2.3. Preparation of tissues samples

At 1, 4, 7 and 14 days of CYP exposure and at 10 days recovery after 4 days CYP exposure snails were anesthetized on ice for 10 min and different tissues were dissected. For histopathological analysis, small sections of digestive gland, foot and gill were immediately fixed in Bouiń's solution for 6-h, subsequently washed and stored in 70% ethanol. The remaining of digestive gland and gill were stored at −80 °C for biochemical determinations.

2.4. Oxidative stress parameter measurements

2.4.1. Preparation of tissue homogenate

The tissue was weighed and homogenized (1:6 w/v for digestive gland and 1:5 w/v for gills) in 125 mM Tris-base cold buffer solution, pH 6.8 using a Teflon homogenizer. Homogenates were centrifuged at 10,000 *xg* at 4 °C for 15 min and the supernatant were used for biochemical determinations. Total protein concentration was determined as described by Bradford (1976) using bovine serum albumin as standard.

2.4.2. Antioxidant enzyme activities

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured as described by Marklund and Marklund (1974). The method is based on the inhibition of the auto-oxidation of pirogallol (26 mM, pH 2) followed spectrophotometrically at 420 nm. The reaction was carried out in 50 mMTris-cacodilate buffer (pH 8.8) in 1 ml final volume. One SOD unit was defined as the amount of enzyme necessary to inhibit 50% of autocatalytic pyrogallol oxidation min^{-1}. Specific activity was expressed as units of SOD per mg of total protein.

Catalase (CAT, EC 1.11.1.6) activity was determined by following the decrease in absorbance at 240 nm due to H_2O_2 (10 mM) decomposition (Aebi, 1984). The reaction mixture was 1 ml of 50 mM potassium phosphate buffer (pH 7). One CAT unit was defined as the amount of enzyme catalyzing 1 µmol of H_2O_2 min^{-1}. Specific activity was expressed as units of CAT per mg of total protein.

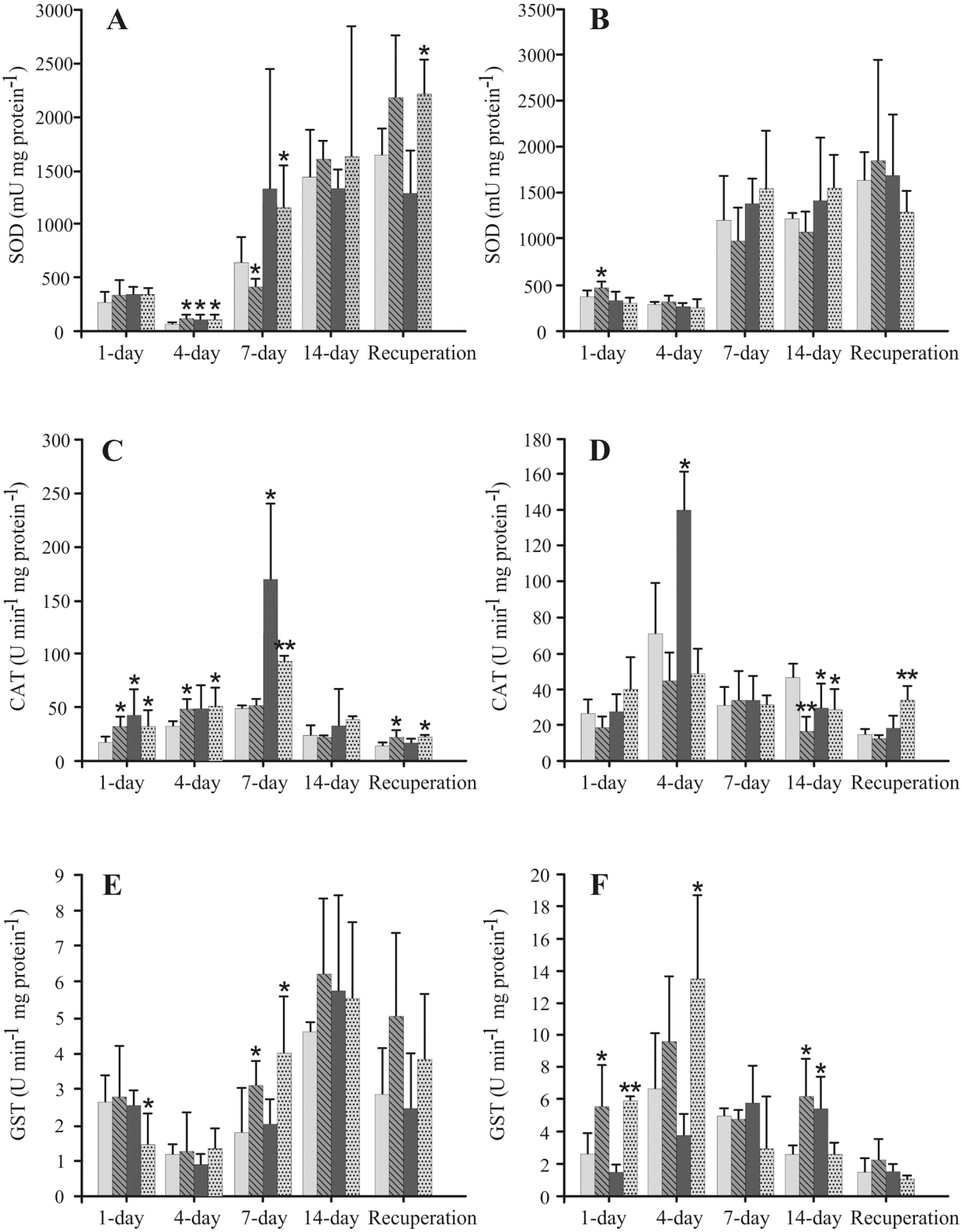

Fig. 1. Effect of sublethal concentrations of CYP on enzymatic activities of SOD, CAT and GST in digestive gland of *P. canaliculata*.
Data are shown as mean ± SD (n = 6). SOD: Superoxide dismutase activity in males (A) and females (B). CAT: Catalase activity in males (C) and females (D). GST: Glutathione S-transferase measured in males (E) and females (F). Statistical differences from the corresponding control are indicated as *($p < 0.05$); **($p < 0.001$). Control 10 µg l^{-1} 25 µg l^{-1} 100 µg l^{-1}

2.4.3. Antitoxic defense

Glutathione S-transferase (GST, EC 2.5.1.18) was measured in accordance to the protocol of 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 in 1 ml final volume. One GST was defined as the amount of enzyme required to conjugate GSH with 1 µmol of CDNB min^{-1} determined at 340 nm. Specific activity was expressed as units of GST per mg of total protein.

2.4.4. Oxidative damage

Lipid peroxidation level was measured according to Buege and Aust (1978) using the formation of thiobarbituric acid-reactive substances (TBARs). Samples were added to the reaction mixture containing trichloroacetic acid 15% (w/v), 2-thiobarbituric acid 0.375% (w/v), and 0.147 mM butylhydroxytoluene at a ratio of 1:5 (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). After centrifugation at 5000 xg for 10 min, the supernatant was measured spectrophotometrically at 535 nm. LPO was expressed as nmol TBARs complexes per mg of wet tissue weight.

Protein oxidation was assessed by the method described by Reznick and Packer (1994) with minor modifications. Briefly, carbonyl concentration (PCs) was quantified spectrometrically at 505 nm. The homogenized sample was incubated with 2,4-dinitrophenylhydrazine (DNPH) 10 mM in HCl 2N during 15 min in dark, followed by neutralization with NaOH 1N. Sodium pyruvate was used as standard. Results were expressed as µg protein carbonyl per mg of total protein.

2.5. Histological studies

Fixed samples stored in 70% ethanol were dehydrated at increasing ethanol concentrations and then embedded in resin (Leica Historesin®). Blocks were sectioned at 4 µm using an automatic microtome (Leica® RM2155) with tungsten knives and mounted on microscope slides. The slides were stained with hematoxilyn-eosin and observed using a light microscope (AXIPLAN 2 Zeiss®). Histopathological changes in gill, foot and digestive gland were described and classified according to the frequency of appearance of such alteration with the following criteria: – = none (no slide presented alteration), + = mid (alteration presents in < 25% of the slides analyzed), + + = moderate (alteration presents in 25%–75% of the slides analyzed) and + + + = severe (alteration presents in > 75% of the slides analyzed). A total of 60 slides were analyzed per treatment (n = 8, males and females snails analyzed per treatment).

To quantify the health status of the digestive gland, the number of pigmented corpuscles was evaluated in 2 sections (100 µm distances from each other) from each snail of both sexes. Five images were randomly taken at 20× magnification and analyzed using an automatic image analysis system (Axiovision Rel 4.4). A segmentation procedure was applied to quantify the area occupied by pigmented corpuscles in the digestive gland. The results were expressed as the average epithelial area filled with corpuscles: $Aec = Ae/Ac \times 100$, where Ae = area of epithelium (0.371 mm^2), and Ac = total area of corpuscles found in the epithelium section.

2.6. Statistical analyses

Statistical comparison of different treatments was done by one-way ANOVA after checking for normality and homogeneity of variances. Student-Newman-Keuls was used as the post-test to determine which treatments differed significantly from the control ($p < 0.05$). Results of biomarker analysis are shown as mean ± standard deviation (SD). Data were analyzed using Instat v. 3.01.

3. Results

Cypermethrin did not cause mortality in snails during the whole exposure phase. Also, no mortality was observed in the control organisms. No significant differences were observed between control groups with and without ethanol. Therefore, the control group with ethanol was used to assess the effects of CYP exposures.

3.1. Effects of CYP on oxidative stress parameters

3.1.1. Enzymatic activities

Fig. 1 shows CYP effect on enzymatic activities in digestive gland of adult snails. SOD activity was significantly higher (with values ranging between 65%–80%) compared to the controls ($p < 0.05$) in males at all pesticide concentrations essayed on day 4 of exposure and at 100 µg l^{-1} of CYP on day 7 of exposure by 80% (Fig. 1A). These results might indicate an acute and short-term response in males. In females, this parameter was only significantly increased ($p < 0.05$) at 10 µg l^{-1} of CYP on day 1 of treatment by 25% showing a low sensitivity of SOD activity to pesticide exposure (Fig. 1B). On the other hand, SOD activity was notably increased in all snails in both genders from day 7 of treatment comparing to those on days 1 and 4 indicating that fasting could have affected this enzyme (Fig. 1A–B).

CAT activity was significantly higher ($p < 0.05$) in males at different CYP concentrations on days 1, 4 and 7 as compared to controls with values ranging between 50%–240%, evidencing an acute response (Fig. 1C). In females, this parameter significantly increased ($p < 0.05$) only on day 4 at 25 µg l^{-1} of CYP by 97%, and showed significant decreases ($p < 0.05$) at all CYP concentrations essayed on day 14 (40%–65%), suggesting a chronic response (Fig. 1D). GST activity did not show a clear concentration-dependent response to CYP exposure in treated organisms when compared to controls. In males, this parameter significantly increased ($p < 0.05$) on 7-day treatment at 10 and 100 µg l^{-1} by 72% and 122%, respectively (Fig. 1E). On 1-day, GST activity measured in males was significantly lower ($p < 0.05$) at 100 µg l^{-1} by 45% (Fig. 1E). Similar to the effect observed in SOD, GST activity was remarkably increased in all organisms on 14-day, although no significant differences were found between the control and different treatments (Fig. 1E). These results might indicate that fasting could also have affected GST activity in male snails but this effect was not observed in females (Fig. 1F). GST activity in females were significantly higher compared to controls ($p < 0.05$) at 10 and 100 µg l^{-1} on 1-day treatment (110% and 127%, respectively) as well as at the highest CYP concentration on 4-day exposure by 104% (Fig. 1F). While on day 14 the enzymatic activity significantly increased ($p < 0.05$) at 10 and 25 µg l^{-1} of CYP exposure (135% and 107%, respectively). However, on 7-day GST activity showed a tendency to decrease at 100 µg l^{-1} of CYP although there were no significant differences with the control (Fig. 1F).

In order to analyze the time effect at all CYP concentrations, the percentages variation of treatments as compared with 100% of their respective controls was calculated (Supplemental material).

Time-dependent trend in SOD activity was found mainly in females, whereas in males this parameter tends to decrease on 14-day (Supplemental material Fig. 1A–B). No clear time-dependent responses were observed in both genders neither in CAT nor GST activities (Supplemental material Fig. 1C–F).

3.1.2. Oxidative damage in digestive gland and gill

Toxic effect of different CYP concentrations on lipids (TBARs) and proteins (PCs) oxidation in digestive gland of adult snail is shown in Table 1.

Notably, TBARs levels in digestive gland of males were not sensitive to CYP exposure. Significantly decreases as compared to controls ($p < 0.05$) at 25 and 100 µg l^{-1} on 4-day of exposure (64% and 67%, respectively) as well as at 100 µg l^{-1} on 7-day by 67%, were observed (Table 1). These results would suggest that the pesticide exposure not induce LPO. In females TBARs levels showed a highly significant increase ($p < 0.001$) on 7-day treatment at 25 and 100 µg l^{-1} of CYP by 50% and 82%, respectively (Table 1). Nevertheless, this parameter was significantly lower ($p < 0.05$) at 25 µg l^{-1} on day 1 and at all CYP concentrations on day 14, with values ranging between 11%-22% respect to controls (Table 1).

In general, CYP caused increasing in PCs levels in digestive gland of

Table 1
Effect of sublethal concentrations of CYP on lipid peroxidation levels (TBARs) and protein oxidation damage (PCs) in digestive gland and gills of males and females of *P. canaliculata*.

			Digestive Gland				Gills			
			Control	10 μg l^{-1}	25 μg l^{-1}	100 μg l^{-1}	Control	10 μg l^{-1}	25 μg l^{-1}	100 μg l^{-1}
TBARs	1-day	M	26.5 ± 7.8	31.1 ± 3.3	28.4 ± 9.4	27.3 ± 4.4	20.2 ± 0.6	25.5 ± 3.8*	24.3 ± 1.1**	23.4 ± 2.5*
		F	33.7 ± 2.4	33.0 ± 5.0	29.8 ± 0.3*	29.4 ± 8.7	22.2 ± 1.2	30.2 ± 4.6*	33.9 ± 2.9**	34.5 ± 3.7**
	4-day	M	43.2 ± 17	29.5 ± 18.2	15.6 ± 4.7*	14.1 ± 3.1*	25.5 ± 4.3	19.2 ± 2.9*	24.5 ± 3.3	19.7 ± 5.4
		F	ND	ND	ND	ND	16.9 ± 2.0	20.6 ± 6.3	35.7 ± 5.7**	18.4 ± 6.8
	7-day	M	18.6 ± 6.4	15.3 ± 3.6	13.0 ± 2.6	6.2 ± 3.1*	14.0 ± 4.8	17.6 ± 8.2	25.4 ± 15.6	22.0 ± 9.9
		F	25.3 ± 0.9	23.5 ± 6.5	37.9 ± 1.5**	46.0 ± 5.7**	18.4 ± 3.7	32.7 ± 2.1**	36.9 ± 7.5**	23.6 ± 4.4*
	14-day	M	28.8 ± 5.3	26.7 ± 6.4	29.6 ± 4.9	25.7 ± 3.1	25.2 ± 9.6	11.9 ± 6.3*	43.0 ± 9.1*	42.3 ± 15.2*
		F	33.1 ± 2.5	25.8 ± 0.4**	26.3 ± 1.1**	29.6 ± 0.7*	21.5 ± 6.4	36.7 ± 5.2*	34.6 ± 4.0*	39.9 ± 8.7*
	Recuperation	M	26.3 ± 3.3	39.0 ± 3.2**	33.3 ± 3.0*	46.0 ± 8.6**	32.7 ± 5.0	68.0 ± 7.6**	54.3 ± 11.2*	43.4 ± 5.9*
		F	25.6 ± 3.4	26.2 ± 3.8	30.2 ± 5.9	36.1 ± 9.1*	45.3 ± 10.8	24.4 ± 3.0*	50.3 ± 12.1	33.8 ± 5.1*
PCs	1-day	M	0.25 ± 0.13	0.24 ± 0.11	0.30 ± 0.17	0.52 ± 0.01**	0.25 ± 0.05	0.33 ± 0.10	0.73 ± 0.38*	0.67 ± 0.13**
		F	0.11 ± 0.05	0.34 ± 0.10**	0.39 ± 0.04**	0.31 ± 0.08**	0.37 ± 0.04	0.44 ± 0.21	1.09 ± 0.07**	0.69 ± 0.50
	4-day	M	0.46 ± 0.20	0.90 ± 0.28*	0.73 ± 0.25	0.68 ± 0.22	0.23 ± 0.03	0.11 ± 0.03**	0.16 ± 0.01*	0.20 ± 0.06
		F	0.53 ± 0.23	0.73 ± 0.37	0.47 ± 0.03	0.46 ± 0.12	0.39 ± 0.04	0.50 ± 0.10*	0.66 ± 0.12*	1.05 ± 0.19**
	7-day	M	0.74 ± 0.08	0.41 ± 0.07**	3.16 ± 1.17**	1.06 ± 0.29*	0.22 ± 0.02	0.32 ± 0.03**	0.27 ± 0.04*	0.29 ± 0.05*
		F	0.34 ± 0.17	0.29 ± 0.24	0.63 ± 0.36	0.52 ± 0.25	1.50 ± 0.42	2.25 ± 0.45*	2.14 ± 0.76	2.80 ± 0.93*
	14-day	M	0.65 ± 0.13	0.93 ± 0.32	0.69 ± 0.30	1.06 ± 0.57	0.31 ± 0.29	0.42 ± 0.03	0.58 ± 0.26	1.61 ± 0.34**
		F	1.09 ± 0.46	0.58 ± 0.09*	1.21 ± 0.29	0.88 ± 0.57	0.29 ± 0.19	0.54 ± 0.35	0.86 ± 0.21**	0.80 ± 0.40*
	Recuperation	M	0.95 ± 0.21	1.10 ± 0.18	0.58 ± 0.20*	1.21 ± 0.43	0.23 ± 0.14	0.34 ± 0.20	0.83 ± 0.69	0.57 ± 0.34*
		F	0.83 ± 0.25	0.68 ± 0.53	0.58 ± 0.16	0.61 ± 0.22	0.27 ± 0.09	0.59 ± 0.23*	1.21 ± 0.26**	0.57 ± 0.31*

Data are shown as mean ± SD (n = 6). TBARs units as nmol mg^{-1}. wet weight. PCs units as mg carbonyl mg^{-1}. protein. F: females; M: males. Statistical differences from the corresponding control *($p < 0.05$); **($p < 0.001$). ND: not determined.

exposed organisms as compared with their respective controls (Table 1). On 1-day, PCs values in males significantly increased ($p < 0.001$) at 100 μg l^{-1} by 107%. The same was observed on 4 days of exposure at 10 μg l^{-1} ($p < 0.05$) by 97%, as well as on 7 days of treatment at 25 μg l^{-1} ($p < 0.001$) and 100 μg l^{-1} ($p < 0.05$) by 325% and 43%, respectively. In females, PCs levels significantly increase ($p < 0.001$) at all CYP concentrations on 1-day treatment, with values ranging between 181%–256%.

Gills were also damaged by CYP exposure by lipids and proteins oxidation (Table 1). Males showed significantly higher TBARs values ($p < 0.05$) at all CYP concentrations on 1 day with values ranging between 15%–26%, as well as at 25 and 100 μg l^{-1} on 14 days of exposure (70% and 68%, respectively). In females, TBARs levels in gills were higher as compared to controls at all CYP concentrations and all treatments, with significant increase ($p < 0.05$) at 10 μg l^{-1}, 25 μg l^{-1} and 100 μg l^{-1} on 1, 7 and 14 days of exposure with values ranging between 28% and 101%. On 4-day, TBARs levels showed a significantly increase ($p < 0.001$) only at 25 μg l^{-1} by 111%.

PCs levels measured in gills of exposed snails of both genders were higher at all CYP concentrations and exposure periods compared to controls, but on 4-day treatment they were significantly lower in males (Table 1). It was observed in males a significantly increase ($p < 0.05$) at 25 and 100 μg l^{-1} on 1-day of exposure, as well as at all CYP concentrations on 7-day and at 100 μg l^{-1} on 14 day, with values ranging between 23% and 422%. In females, significantly higher values on PCs ($p < 0.05$) corresponded to 1-day (25 μg l^{-1}), 4-day (all CYP concentrations), 7-day (10 and 100 μg l^{-1} CYP) and 14-day (25 and 100 μg l^{-1} CYP) treatments, with values ranging between 29% and 195%.

Time-dependent analyses of oxidative damage showed that there was no clear time-dependent response in both genders neither in TBARs and PCs levels in digestive gland (Supplemental material Fig. 2) nor in gills of *P. canaliculata* (Suplemental material Fig. 3).

3.1.3. Recuperation trial

In order to analyze if post-exposure treatment affects the parameters evaluated during CYP exposure, another assay where snails were exposed for 4 days and then transferred to clean water for 10 days, was carried out. Oxidative stress parameters in males and females from the recuperation period were compared with their respective controls. Although in digestive gland, SOD activity of exposed snails showed a tendency to increase, it significantly increased compared to the control ($p < 0.05$) only in males at 100 μg l^{-1} by 34% (Fig. 1A–B).

CAT activity showed similar behavior, with significant increases ($p < 0.05$) in males at 10 and 100 μg l^{-1} (58% and 61%, respectively) and in females showed an increase of 128% at 100 μg l^{-1} of CYP, compared to control (Fig. 1C–D). No significant differences between control and exposed snails were observed in GST activity for either males or females (Fig. 1E–F).

TBARs levels in digestive gland of both males and females were higher on recuperation treatments than controls (Table 1). Significant increases ($p < 0.05$) of this parameter were registered in males at all CYP concentrations with values ranging between 27%–75%, but only were observed in females at the highest concentration (41%). Although a clear trend to decrease in PCs levels in this organ was observed in both genders, only males showed significant differences ($p < 0.05$) at 25 μg l^{-1} CYP by 40%, as compared to control (Table 1).

In gills, the recuperation treatment caused significant increases in TBARs levels ($p < 0.05$) at all CYP concentrations in males, with values ranging between 33%–108%. In contrast, females showed significant decreases ($p < 0.05$) of this parameter at 10 and 100 μg l^{-1} (46% and 25%, respectively) (Table 1). In general, PCs levels in gills of both males and females were higher at all treatments as compared with their respective controls. A significant increase ($p < 0.05$) of this parameter was observed at all CYP concentrations in females, with values ranging between 113% and 354% (Table 1).

Taken into account that organisms were exposed during 4 days to CYP, it would be expected that the recuperation treatment would showed an improvement in altered parameters as compared to such treatments. Although in general there was no clear trend in modification of oxidative stress parameters evaluated in this study, TBARs and PCs levels were increased in gills during depuration treatment. These results may indicate a change in oxidative metabolism of one of the first target organ of toxicant input.

3.1.4. Comparison between males and females

To evaluate whether during the pre-reproductive period males and females present differences, statistically significant effects on

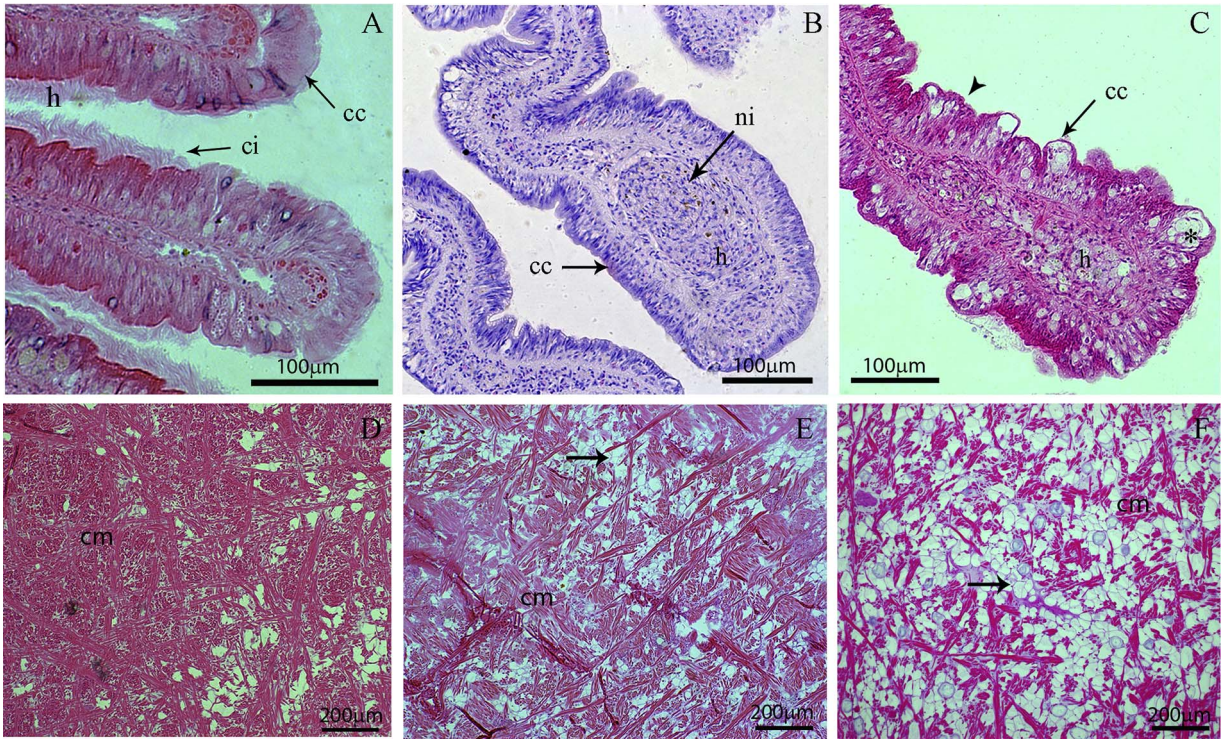

Fig. 2. Micrographs of gill and foot of *P. canaliculata*. A. Detail of a gill filament from control snail showing columnar cells (cc) with long apical cilia (ci) and narrow hemolymph spaces (h). B, C. Gills filaments of snails exposed to CYP indicating: loss of cilia (arrowhead), wider hemolymph spaces (h) and columnar cells vacuolization (asterisk). Note the presence of nodular-type inflammation in C. D. Foot transverse section of control snail showing columnar muscle cells (cm). E, F. Foot transverse section of exposed snails to CYP showing disruption of columnar muscle fibers and increasing amount of spaces in between them (arrow).

antioxidant defense system at each treatment were evaluated (Supplemental material Table 1). It is interesting to note that males and females of *P. canaliculata* showed statistical differences ($p < 0.05$) in SOD activities at all CYP concentrations on 4-day treatment as well as at 10 µg l^{-1} of CYP on 1, 7 and 14 day exposure. CAT activity showed statistical differences ($p < 0.05$) between genders at all CYP concentrations on 7-day of exposure. GST levels showed significant differences ($p < 0.05$) between males and females at all CYP concentrations on 1 and 4 days of treatments as well as at 10 and 25 µg l^{-1} on 7-day and 100 µg l^{-1} on 14-day of exposure. On recuperation trial, main statistical differences ($p < 0.05$) on SOD, CAT and GST activities corresponded to the highest CYP concentration.

Statistical differences between males and females on oxidative damage to proteins and lipids in digestive gland and gills were analyzed (Supplemental material Table 2). TBARs levels measured in digestive gland showed statistical differences ($p < 0.05$) at all CYP concentrations on 7-day as well as at 100 µg l^{-1} on day 14 in both genders. The main statistical differences ($p < 0.05$) on PCs levels were found at 25 µg l^{-1} (on 4, 7 and 14 days) and 100 µg l^{-1} (on 1 and 7 days). On recuperation trial, statistical differences between males and females were found on TBARs levels at 10 µg l^{-1} CYP ($p < 0.0001$) and PCs levels at 100 µg l^{-1} CYP ($p < 0.05$). TBARs levels measured in gills showed statistical differences ($p < 0.0001$) between genders at higher CYP concentrations (25 and 100 µg l^{-1}) on 1 and 4 days treatment, whereas at longer exposure periods (7 and 14 days) were also found at the lowest CYP concentration. The main significantly differences ($p < 0.0001$) on PCs levels were registered at all CYP concentrations on both 4 and 7 days treatment. On recuperation treatments, no significant differences were found between males and females on protein oxidation measured in gills, whereas TBARs levels showed differences ($p < 0.05$) at 10 and 100 µg l^{-1} CYP.

Although these results demonstrate some significant differences between males and females to CYP exposure, did not show a clear trend in the sensitivity level of any of the genders to the pesticide.

3.2. Effects of CYP on histophatological alterations

Histological alterations were observed in gill, foot and digestive gland in snails exposed to CYP compared to control treatment. The quantification of the histopathologial effects is show in Table 2. No differences were observed in all analyzed organs between genders for any of the CYP concentrations and exposure times.

3.2.1. Gill

Gill of the control snails are formed by a central axis with numerous filaments extended along one side. The gill filament epithelium contained ciliated columnar cells and mucus-secreting cells (Fig. 2A). Numerous hemocytes were found in the hemolymph space. No histopathological alteration was found on 1-day of CYP exposure; however after such period both concentration- as well as time-dependent changes were observed. The most remarkable filament alteration was the presence of wider hemolymph space with severe hemocyte infiltration after 25 and 100 µg l^{-1} CYP exposure for 4, 7 and 14 days (Fig. 2B). Other epithelial alteration observed was a reduction in length or, in some cases, loss of cilia at 100 µg l^{-1} CYP on 4 and 7-day of exposure as well as at 25 and 100 µg l^{-1} CYP on 14-day (Fig. 2B, C). Additionally, at higher CYP concentrations (25 and 100 µg l^{-1}), the apical portion of the columnar cells exhibited swelling and some cells degenerate producing large intercellular spaces (Fig. 2C), with more severe alterations on 7 and 14 days of exposure. On recuperation trial, no histopathological alterations were observed at 10 µg l^{-1} of CYP exposure. Although, at the highest CYP concentration (100 µg l^{-1}) a severe columnar cell vacuolization was still observed, the hemolymph space was reduced compared to treatment at 4 days of exposure.

3.2.2. Foot

The foot tissue of control snails mainly contained columnar muscle cells (Fig. 2D). No histological alteration was observed after 1-day of exposure. The most conspicuous histolopathological effect was the

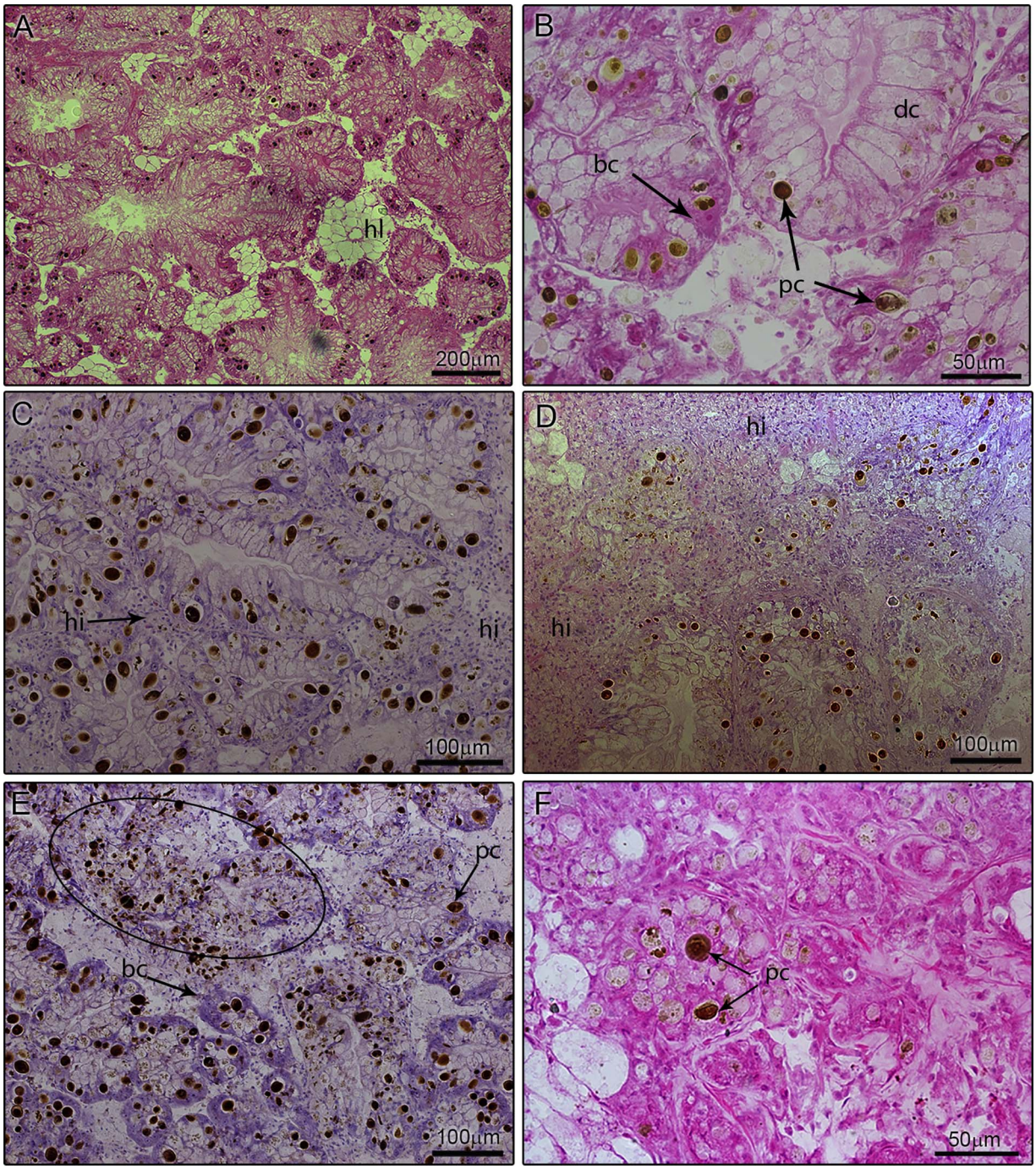

Fig. 3. Micrographs of digestive gland of *P. canaliculata*. A. Tubules of the digestive gland of control snail showing digestive cells (dc) and basophilic cells (bc) and pigmented intracellular corpuscles (pc). B. A haemocytic infiltration (hi) around digestive tubules of an exposed snail. C. Digestive gland tubules of treated snail showing numerous pigmented corpuscles in the epithelia. D. Necrosis of the digestive gland is evident, although some pigmented corpuscles are evident.

disruption of columnar muscle fibers (Fig. 2E). This change slightly occurred on 4-day of 100 µg l^{-1} CYP exposure, becoming more severe on 7 and 14 days at 100 µg l^{-1} of CYP exposure. Also, to those more extreme CYP treatments spaces and gaps between the muscle fibers were observed (Fig. 2F). At the recuperation period slight disruption of the columnar muscle fibers was still observed.

3.2.3. Digestive gland

The digestive gland of control snails consisted of numerous blind-ended tubules composed of abundant digestive cells and scarce basophilic cells (Fig. 3A, B). The basophilic cells were triangular with a big central nucleus while the digestive cells were columnar with a basal nucleus. Pigmented intracellular corpuscles of different sizes and shapes were found along the digestive gland epithelium (Fig. 3B). Another histological characteristic of the digestive gland control was a hemolymphatic space and connective tissue between the tubules. Significant histopathological alterations were observed in the digestive gland at different CYP concentrations and time of exposure (Table 2). In all snails exposed at 10 µg l^{-1} and 25 µg l^{-1} CYP for 7 and 14 days, a moderate increase of hemocytes in areas between the tubules were observed (Fig. 3C). The same symptom occurred at 100 µg l^{-1} CYP, irrespective of the time exposure, but was more intensity on 14-days (Fig. 3D). At 100 µg l^{-1} CYP on 4-day and at all CYP concentrations on 7 and 14 days of exposure, an increase number of basophilic cells were

Table 2
Histopathological effects of sublethal CYP concentrations in gill, foot and digestive gland of *P. canaliculata*.

			1-day			4-day			7-day			14-day			Recuperation		
			CYP (μg l^{-1})														
	Histopathological Effects	Control	10	25	100	10	25	100	10	25	100	10	25	100	10	25	100
Gill	Cilia alterations	–	–	–	–	–	–	+	–	–	++	–	+	+++	–	–	+
	Wider hemolymph space	–	–	–	–	–	+	+++	–	++	+++	–	++	+++	–	–	+
	Columnar cells vacuolization	–	–	–	–	–	+	+++	–	++	+++	–	+++	+++	–	+	+++
Foot	Lipid vacuoles	–	–	–	–	–	–	–	–	–	++	–	+	+++	– –	–	–
	Columnar muscle fibers atrophy	–	–	–	–	–	–	+	–	+	++	+	+	+++	–	–	+
Digestive gland	Hemocytic infiltration	–	–	–	++	–	–	++	++	++	++	++	++	+++	–	–	++
	Increase number of basophilic cells	–	–	–	–	–	–	++	+	++	+++	+	++	+++	–	–	+
	Atrophy of the epithelia	–	–	–	–	–	–	+	–	–	+	+	+	++	–	–	+
	Necrosis	–	–	–	–	–	–	–	–	–	+	–	–	++	–	–	–

Score value:–: no histopathology, +: mid histopathology (present in < 25% of the slides), ++: moderate histopathology (present in 25%–75% of the slides) and +++ = severe histopathology (present in > 75% of the slides). N = 60 slides analyzed per treatment (n = 8). For more details see M&M.

observed (Fig. 3E). Epithelial damage or atrophy was observed at 100 μg l^{-1} CYP on 4 and 7-day of exposure with more intensity at 14-day (Fig. 3E). The first necrotic changes occurred at the highest sublethal concentration of CYP (100 μg l^{-1}) on 7 and 14-day (Fig. 3F). During the recuperation period, hemocyte infiltration, a high number of basophilic cells and a slight epithelial atrophy were still observed.

3.2.3.1. Quantitative evaluation of corpuscles in digestive gland. Fig. 4 shows the percentage of area occupied by corpuscles in the digestive gland epithelium in males and females of *P. canaliculata*. In general, concentration-dependent responses to CYP exposure were observed in all treated snails compared to controls. On days 1 and 7 the area occupied by corpuscles in males significantly increased ($p < 0.05$) only at the highest concentration of CYP (100 μg l^{-1}) by 80% and 98% respectively (Fig. 4A). But this parameter was significantly increased ($p < 0.05$) at all CYP concentrations essayed on days 4 and 14 with values ranging between 74% and 251% (Fig. 4A). On 1-day, females showed an acute toxic effect in the area occupied by corpuscles evidenced by the significant increase ($p < 0.05$) at the three CYP concentrations essayed (18%–51%) (Fig. 4B). Nevertheless at longer exposure times (4, 7 and 14 days), this parameter significantly increased ($p < 0.05$) only at the highest CYP concentrations (25 and 100 μg l^{-1}) (84%–195%), while at 10 μg l^{-1} of CYP the values were similar to controls showing a less sensitive response.

In contrast, during the recuperation period the percentage of corpuscles increased considerably ($p < 0.001$) and such levels were above those observed on 4 days of CYP exposure in both genders (Fig. 4A, B).

It is interesting to note that males and females of *P. canaliculata* showed statistical differences between them in corpuscle content only on 14-day in all CYP concentrations essayed (Supplemental material Table 3). From all these results it is clear that this parameter is very sensitive to CYP exposition and could reflect chronic effect mainly in males than females (Fig. 4).

The analysis of the effect of time on corpuscle content in digestive gland, at any CYP concentration essayed showed a time-dependent trend mainly of males except on day 7 where the values decreased in both genders (Supplemental material Fig. 4).

4. Discussion

Synthetic pyrethroids are effective pesticides against insects but they are considered to be safe for mammals and birds. Thus these insecticides have been used worldwide in agricultural programs as well as in public health (Palmquist et al., 2012). However, there is enough evidence that shows that aquatic organisms are very sensitive to their toxic effects. CYP, the most frequently used type II pyrethroid, is highly toxic to fish and invertebrates causing alterations in several metabolic and physiological pathways (Palmquist et al., 2012). The principal neurotoxic effect of CYP on the target organism is that acts as a

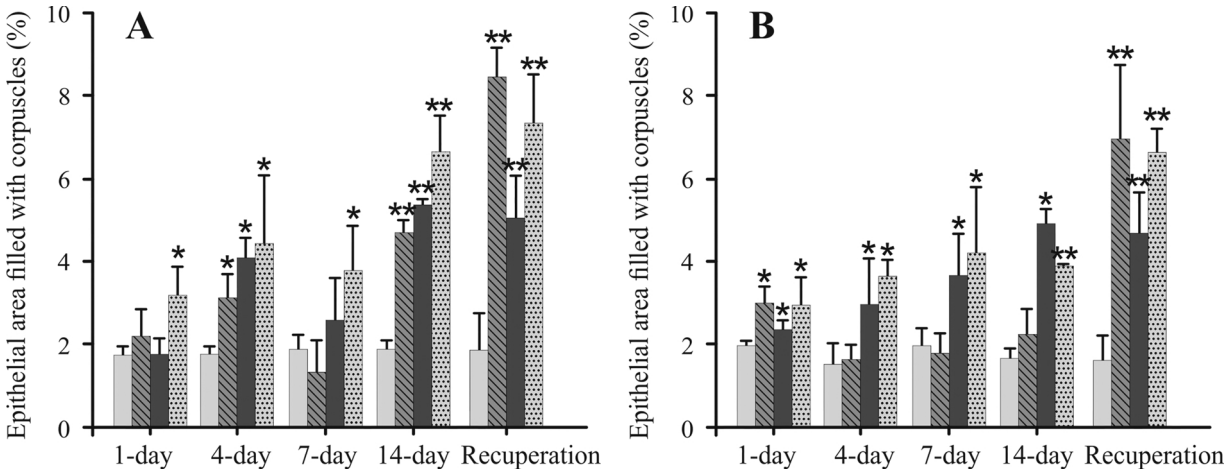

Fig. 4. Effect of sublethal concentrations of CYP on average epithelial area filled with pigmented corpuscles in digestive gland of *P. canaliculata*.
Data are shown as mean ± SD (n = 4). Males (A) and females (B). Statistical differences from the corresponding control are indicated as *($p < 0.05$); **($p < 0.001$).

disruptor of the voltage-gated sodium channels in nerve cells, prolonging the time during which such channels are open. This results in stimulus-dependent sustained depolarization promoting convulsions, paralysis and eventually death (Soderlund and Bloomquist, 1989). In addition, CYP showed other toxic effects on cells, promoting oxidative damage and disturbing cell membrane structure due to its hydrophobic nature (Grajeda-Cota et al., 2004). CYP catabolism releases cyanohydrins, which are decomposed to cyanide and aldehyde. These compounds together with other lipophilic conjugates may lead to ROS generation which produces DNA damage, lipid peroxidation and protein carbonylation, modifying the normal cellular functions (Shashikumar and Rajini, 2010). ROS, such as H_2O_2, superoxide (O_2-) and hydroxyl radical (OH−), are constantly generated as a consequence of aerobic metabolism, therefore organisms hold both enzymatic and non-enzymatic antioxidant defensive systems to neutralize them. Enzymatic defenses like SOD and CAT, decompose (O_2-) and H_2O_2 respectively to produce less toxic metabolites (Livingstone, 2001). By up-regulating the cell activity of these antioxidant enzymes, organisms are able to adapt to higher ROS production due to xenobiotic exposure. This defensive mechanism is promoted by enhancing the enzymatic activity expression through transcriptional and post-translational regulations (Regoli and Giuliani, 2014). SOD and CAT activities have been often proposed as biomarkers of pollutant contamination in aquatic systems (Valavanidis et al., 2006; Monserrat et al., 2007; Regoli and Giuliani, 2014). It should be noted that even glutathione peroxidases and peroxiredoxins are components of the antioxidant system, and perform the same function of CAT. These antioxidant enzymes have recently received much attention from the scientific community, and also their expression has recently been characterized in response to environmental stress (Sattin et al., 2015; Tolomeo et al., 2016). The components of the antioxidant system have been conserved throughout deep evolutionary time, confirming the importance of the role played by these molecules in all eukaryotic organisms, from unicellular organisms to vertebrates (Santovito et al., 2012; Ferro et al., 2013, 2015), although some of them are less studied in invertebrates.

Some studies about pyrethroid effects on molluscs reported an increase in SOD and CAT activities in the digestive gland of freshwater mussel *Unio gibbus* exposed to CYP (Khazri et al., 2015) and marine clams *Ruditapes descussatus* (Sellami et al., 2014) and *Venerupis decussate* (Sellami et al., 2015) exposed to permethrin. In the freshwater snail *P. canaliculata*, both concentration- as well as time-dependent responses in the activity of these enzymes resulted remarkable dissimilar at CYP exposition. In some cases, a significant increase of activities between the treatments and their respective controls was observed, whereas some others cases showed significant decrease. Mahmoud et al. (2012) observed that in digestive gland of marine gastropod *Hexaplex trunculus*, permethrin caused concentration- dependent increase in CAT activity, however at high concentrations this activity decreased. The authors explain that this pattern could be related to the energetic status of this organism caused by pyrethroid exposure. On the other hand, Regoli and Giuliani (2014) suggested that this biphasic trend of CAT activity could be associated with a regulatory mechanism by which low ROS levels, increase CAT activity while higher levels promotes it inactivation. Superoxide radicals are proposed to be the main specie responsible for CAT inhibition, causing excessive H_2O_2 production which finally inhibits SOD activity as well (Wei and Yang, 2015). ROS may also affect enzymatic activities impairing protein synthesis, as suggested by the authors.

Although SOD and CAT constitute the first defense line against ROS, there are many reports showing that both enzymes do not respond in the same manner. In digestive gland of *P. canaliculata* exposed to tributyltin, CAT activity was highest than controls but SOD was no different (Martínez et al., 2017). In this study, *P. canaliculata* showed only in a few cases that both enzymes were significantly different from controls at the same time. On the other hand, it was observed in the nematode *Caenorhabditis elegans*, that SOD activity decreased while CAT and GST activities increased after CYP exposure (Shashikumar and Rajini 2010). Differential responses between enzymes that are part of the same metabolic pathway could be attributed to different reasons. It is important to take into account that enzymatic activity depends on kinetic parameters (e.g. *Km*, Vmax, optimum pH and temperature), regulatory mechanisms (e.g. modulators, transcription factors), their stability against stressors (e.g. ROS) and the physiology of the organism.

The biotransformation enzyme of phase II metabolism, GSTs, is the most studied in aquatic organisms. It main role is detoxification of electrophiles, such as pesticides metabolites, by conjugation with reduced glutathione to facilitate their excretion (Livingstone, 2001). Xenobiotics could induce GST activity inducing transcription or through stress-activated signaling cascades (Richardson et al., 2009). Some reports suggest that GST is also involved in CYP metabolism in aquatic organism (Xu and Huang, 2017).

The digestive gland is the major site of both phase I and II detoxification in mollusk. GST increases its activity when the enzymes of phase I are unable to protect the cells (Sellami et al., 2015). However, in *P. canaliculata* exposed to CYP, the GST activity was rarely affected indicating that this snail has little capacity to metabolize CYP.

It was observed in gills and digestive gland of the freshwater mussel *U. gibbus*, that the activities of antioxidant enzymes were significant increased by CYP exposure, however, did not prevent lipid oxidative damage (Khazri et al., 2015; Khazri et al., 2016). This phyretroid caused similar responses in *P. canaliculata* but it was observed neither a concentration- nor a time-dependent linear response in LPO. In gills of *P. canaliculata,* CYP only caused a very short- and long-term effect on lipid oxidation evidenced by significant high TBARs levels. Nevertheless in clams *R. decussatus* and *V. decussate*, the digestive gland showed to be the most sensitive organ target to phyretroid toxicity compared to gills (Sellami et al., 2013; Sellami et al., 2015). These tissue-specific responses could be due to the function of the digestive gland as the main site of pesticide detoxification that generates high ROS production (Sellami et al., 2015). However, gill is a site for gas exchange, ion regulation, and excretion of catabolic products, moreover, it constitutes one of the first routes of input dissolved toxicants from the water (Wei and Yang, 2015). Thus, this organ is susceptible to be damaged by ROS, as was observed in *P. canaliculata*. CYP, such as other xenobiotics, has the ability to induce lipid peroxidation affecting membrane fluidity as well as their biomolecules integrity (Wei and Yang, 2015).

Lipid peroxidation products can promote protein oxidation by carbonyl groups formation (aldehydes and ketones). These moieties are also generated by amino acid oxidation by glycation and glycoxidation reactions (Dalle-Donne et al., 2003). PCs derivatives are chemically stable and are the biomarker of choice to evaluate oxidative damage in proteins (Dalle-Donne et al., 2003). In freshwater crayfish *Procambarus clarkii*, PCs levels were very sensitive to CYP exposition and showed a good correlation with ROS and with histological damage in gills (Wei and Yang, 2015). However, it was not the case of *P. canaliculata* since PCs levels showed significant differences only in some treatments, as the others biochemical parameters evaluated in this study. As Lushchak (2011) explained, the measurement of protein carbonyl groups is a dynamic parameter that could be affected by different factors. Thus, other techniques could be more appropriate to evaluate oxidative damage to protein in snails exposed to CYP. On the other hand, additional components could be playing an important role in oxidative balance in *P. canaliculata*. It was observed that this snail stores uric acid in specialized tissue that can function as soluble antioxidant during estivation and arousal periods (Giraud-Billoud et al., 2011) and it may be involved in the CYP toxicity process.

There are evidences that measurements assessing antioxidant responses and oxidative damage in some aquatic invertebrates are poor biomarkers (Monserrat et al., 2007). So is important to evaluate others alternative or complementary biomarkers that help to evaluate the

health status of exposed organisms.

The analysis of histopahtological alterations offer information about the general health status of species, because reflects cellular and subcellular morphologic changes. Therefore, are sensitive biomarkers, responding to a diversity of xenobiotics and natural stressors (Marigómez et al., 2004). There is scarce evidence that phyrethroids induce histopathological changes in aquatic invertebrates. In *P. canaliculata*, all tissues studied in this work (gill, digestive gland and foot) showed histological changes, but this alterations were more conspicuous in gill and digestive gland, in particular at higher CYP concentrations. The effects of the pesticide started to be observed from day-4 of exposure, showing mid-term response. As mentioned above, histopathological biomarkers are a result of profound changes at biochemical and physiological levels (Hinton et al., 1992), so these latter are expected to be more sensitive.

As ampullariids, *P. canaliculata* presents both gills and lungs that allow it adaptation to different environmental conditions (Hayes et al., 2015). During CYP exposure it was observed that the snails preferred aquatic breathing (personal observation), thus gill is probably the respiratory organ more affected by the pesticide. In this study, the most remarkable effects observed in gill of *P. canaliculata* exposed to CYP were the widening of hemolymph spaces, due to hemocytes accumulation, and the reduction or loss of cilia in the apical portion of the columnar cells. The increment in hemocytes may indicate an inflammatory reaction while the loss of cilia may reduce the oxygen consumption and disturb the osmoregulation and hemodynamic functions. Similar pathological changes were observed by Kruatrachue et al. (2011) and Dummee et al. (2012, 2015) after exposure to heavy metals. The same occurred to other freshwater snail, *Marisa cornuarietis,* (Sawasdee et al., 2011) exposed to heavy metals.

In foot, the histopathological changes were less pronounced than in the rest of the tissues, although some changes were observed such as a disruption of muscle fibers. This disruption may generate gaps between the cells affecting the normal function of the foot.

Because the digestive gland is the main detoxification organ, it is expected to show histopathological sensitivity to CYP exposure. In digestive gland of *P. canaliculata*, an increased number of basophilic cells, hemocytic infiltration, an increment in the area occupied by pigmented corpuscles and atrophy of the epithelia were observed. All this alterations seems to be dose dependent and correlated to the time of exposure.

Digestive cells are usually more abundant than basophilic cells, but under stress conditions an increment in the number of basophilic cells occurs, a process called “cell-type replacement” (Marigómez et al., 1998; Soto et al., 2002; Zaldibar et al., 2008). In *P. canaliculata* this cell-type replacement occurred, especially on 7 and 14 days of CYP exposure. In the experimental groups exposed to CYP during 4 days and to a recovery period of 10 days, it was observed that, this process is reversible. This effect was also observed in the slug *Arion ater,* transferred from an abandoned zinc mine to an unpolluted site and the other way round (Zaldibar et al., 2008). However, in snails this process was not enough to reverse tissue damage.

The presence of hemocytic infiltration in the digestive gland, at long-term exposures, is characteristic during an acute inflammatory process and might reflect a physiological adaptation to stress. The accumulation of hemocytes in the connective tissues between the tubules was also observed for the pulmonate freshwater snails *Lymnaea stagnalis* and *Galba truncatula* exposed to sublethal concentrations of endosulfan (Ünlü et al., 2005 and Cengiz et al., 2005 respectively). The atrophy of the digestive tubules is an initial degenerative process that ends with epithelia necrosis indicating systemic damage. These studies showed that atrophic tubules were rarely observed in short-time exposure, however, atrophy gradually increased even showing necrosis after longer exposures. Histological alterations may affect biochemical pathways leading to failure of the digestive gland function and as a final consequence the organism death. This inflammatory reaction and degenerative changes in the tubules were found to be irreversible.

The other conspicuous change caused by CYP exposure was the increase of the area occupied by pigmented corpuscles, when compared to the digestive gland of control snails. According to Vega et al. (2005, 2006) this corpuscles are a prokaryotic symbiont, and seems to reproduce within the digestive gland and expelled in the feces. Results of this work revealed that the average area occupied by corpuscles in exposed snails was higher than in controls and was dose-dependent. The increase in the observed area seems to be mainly caused by an individual growth of each corpuscle than by its reproduction. It was observed that the pool of corpuscles is maintained and the rest are eliminated in the feces (Vega et al., 2005). Besides, Vega et al. (2012) observed under experimental conditions that the corpuscles could bioconcentrate heavy metals, so these corpuscles could be involved in a detoxification process by feces excretion (Vega et al., 2012). This fact could explain the high tolerance of *P. canaliculata* to different environmental contaminants such as CYP.

In the freshwater snail *Bellamya bengalensis* exposed to CYP during reproductive, pre and post −reproductive periods, although the concentration of the antioxidant ascorbic acid was affected in a similar manner, the oxidative status was different between such periods (Ahirrao and Phand, 2015). This observation could be due to differential metabolic rates during those periods. On the other hand, there is little data available about sex-dismorphic response to pyrethroid exposure, especially in aquatic invertebrates. Differences in the activities of the antioxidant enzymes observed in rat exposed to permethrin could are associated with hormonal status (Wang et al., 2016). Taking into account that the present study was carried out during the pre-reproductive period, differences between *P. canaliculata* genders were expected. Some differences between males and females in almost all parameters evaluated were observed specially in CAT and GST activities. Similar dose-response behavior was observed in both genders, although females responded at earlier times than males in activation of these enzymes (at 4 and 7 days respectively). Nevertheless, whether one gender is more sensitive than the other to pesticide exposure is difficult to be deduced. Considering that the metabolism in females is more active during the reproductive period, it is usually recommended to measure biomarkers in males.

It was observed that mussels can bioaccumulate CYP at high concentrations (Gowland et al., 2002; Khazri et al., 2015) being the gills the main routes of uptake. In tadpoles *Rana nigromaculata* a rapid uptake of CYP with a time-dependent concentration was observed, reaching a maximum level on day-14 of exposure (Xu and Huang, 2017). The authors also reported that during the depuration period CYP was rapidly eliminated from the tadpoles. Although CYP concentrations were not measured in *P. canaliculata*, the pesticide could be accumulated in lipid compartments of tissues causing morphological alterations and oxidative damage. To compare between exposure and depuration treatment, in general there were no clear modifications in oxidative stress parameters and in some histophatological symptoms in *P. canaliculata*. However, the symbiotic corpuscles increased considerably in the recuperation period showing a high capacity of regeneration. So, it is possible that the snails need a recovery period more than 10 days for the altered parameters by CYP reaching the basal levels as observed in the freshwater fish *Labeo rohita* (Adhikari et al., 2004).

As mentioned earlier, biomarkers are usually considered as useful tools to anticipate damage at higher biological organization levels. However, many reports included the present one suggest that a single biomarker is not sufficient to evaluate toxicants effects on organisms. Thus it could be recommendable to analyze a battery of complementary biomarkers in order to understand the organism responses to the pollution in a given area, as suggested by Vieira et al. (2016).

5. Conclusions

These data indicated that CYP affected several biomarkers in the

apple snail *Pomacea canaliculata*, but histopathological changes appear to be the most sensitive ones. Although there were some significant differences between males and females in some of the biomarkers analyzed, is not clear whether they are related to a differential sensitivity to CYP exposure. It could be interesting to evaluate other biomarkers in addition to classical oxidative stress determinations in order to integrate them into more adequate monitoring strategies to evaluate the environmental risk by pyrethroids.

Acknowledgements

This work was partially supported by grants from Agencia Nacional de Promoción Científica y Técnica, Argentina (PICT 2014-0810 and PICT 2015-0669) to Dr. A. Rodrigues Capítulo and Dr. F. Arrighetti respectively, and grant from CONICETPIP 0022 to Dra. F. Arrighetti, and grant from FCNyM-UNLP N° 738. We appreciate the kind help of Dr. Alfredo Castro-Vazquez for interpreting the digestive gland histology. We are grateful to Dr. Fernando Spaccesi, Roberto Jensen, and Dr. Hernán Benitez for their help in sample collection, to Sergio Mijailovsky for chemical analysis and to Norma Tedesco for language revision. We also thank Mario Ramos and Luciana De Tezanos for graphic design assistance. This paper is Scientific Contribution N° 1010 of the Institute of Limnology "Dr. Raúl A. Ringuelet" (ILPLA, CCT-La Plata CONICET, UNLP).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.11.014.

References

Ünlü, E., Cengiz, E.I., Yildirim, M.Z., Otludil, B., 2005. Histopathological effects in tissues of snail *Lymnaea stagnalis* (Gastropoda, Pulmonata) exposed to sublethal concentration of Thiodan® and recovery after exposure. J. Appl. Toxicol. 25, 459–463.

Adhikari, S., Sarkar, B., Chatterjee, A., Mahapatra, C.T., Ayyappan, S., 2004. Effects of cypermethrin and carbofuran on certain hematological parameters and prediction of their recovery in a freshwater teleost, *Labeo rohita* (Hamilton). Ecotoxicol. Environ. Saf. 58, 220–226.

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*. JEBAS 3, 10–14.

Ansari, R.A., Rahman, S., Kaur, M., Anjum, S., Raisuddin, S., 2011. In vivo cytogenetic and oxidative stress-inducing effects of cypermethrin in freshwater fish, *Channa punctata* Bloch. Ecotoxicol. Environ. Saf. 74, 150–156.

Antwi, F.B., Reddy, G.V.P., 2015. Toxicological effects of pyrethroids on non-target aquatic insects. Environ. Toxicol. Pharmacol. 40, 915–923.

Arslan, H., Özdemir, S., Altun, S., 2017. Cypermethrin toxication leads to histopathological lesions and induces inflammation and apoptosis in common carp (*Cyprinus carpio* L.). Chemosphere 180, 491–499.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–309.

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. Chemosphere 68, 613–621.

Cazzaniga, N.J., 1990. Sexual dimorphism in *Pomacea canaliculata* (Gastropoda: Ampullariidae). Veliger 33, 384–388.

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.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.

Dummee, V., Kruatrachue, M., Trinachartvanit, W., Tanhan, P., Pokethitiyook, P., Damrongphol, P., 2012. Bioaccumulation of heavy metals in water, sediments, aquatic plant and histopathological effects on the golden apple snail in Beung Boraphet reservoir, Thailand. Ecotoxicol. Environ. Saf. 86, 204–212.

Dummee, V., Tanhan, P., Kruatrachue, M., Damrongphol, P., Pokethitiyook, P., 2015. Histopathological changes in snail, *Pomacea canaliculata*, exposed to sub-lethal copper sulfate concentrations. Ecotoxicol. Environ. Saf. 122, 290–295.

Faria, M.S., Ré, A., Malcato, J., Silva, P.C., Pestana, J., Agra, A.R., Soares, A.M., 2006. Biological and functional responses of in situ bioassays with *Chironomus riparius* larvae to assess river water quality and contamination. Sci. Total Environ. 371, 125–137.

Ferreira, A.C., Rodrigues Capítulo, A., 2017. Growth and survival of juvenile *Pomacea canaliculata* (Gastropoda: Ampullariidae) in plain streams associated to different land uses. Stud. Neotrop. Fauna Environ. 52, 95–102.

Ferro, D., Franchi, N., Mangano, V., Bakiu, R., Cammarata, M., Parrinello, N., Santovito, G., Ballarin, L., 2013. Characterization and metal-induced gene transcription of two new copper zinc superoxide dismutases in the solitary ascidian *Ciona intestinalis*. Aquat. Toxicol. 140–141, 369–379.

Ferro, D., Bakiu, R., De Pittà, C., Boldrin, F., Cattalini, F., Pucciarelli, S., Miceli, C., Santovito, G., 2015. Cu, Zn superoxide dismutases from *Tetrahymena thermophila*: molecular evolution and gene expression of the first line of antioxidant defenses. Protist 166, 131–145.

Friberg-Jensen, U., Wendt-Rasch, L., Woin, P., Christoffersen, K., 2003. Effects of the pyrethroid insecticide, cypermethrin, on a freshwater community studied under field conditions: I. Direct and indirect effects on abundance measures of organisms at different trophic levels. Aquat. Toxicol. 63, 357–371.

Giraud-Billoud, M., Abud, M.A., Cueto, J.A., Vega, I.A., Castro-Vazquez, A., 2011. Uric acid deposits and estivation in the invasive apple snail, *Pomacea canaliculata*. Comp. Biochem. Physiol. Part A 158, 506–512.

Giraud-Billoud, M., Vega, I.A., Wuilloud, R.G., Clément, M.E., Castro-Vazquez, A., 2013. Imposex and novel mechanisms of reproductive failure induced by tributyltin (TBT) in the freshwater snail *Pomacea canaliculata*. Environ. Toxicol. Chem. 32, 2365–2371.

Gowland, B., Webster, L., Fryer, R., Davies, I., Moffat, C., Stagg, R., 2002. Uptake and effects of the cypermethrin-containing sea lice treatment Excis® in the marine mussel, *Mytilus edulis*. Environ. Pollut. 120, 805–811.

Grajeda-Cota, P., Ramírez-Mares, M.V., de Mejía, E.G., 2004. Vitamin C protects against in vitro cytotoxicity of cypermethrin in rat hepatocytes. Toxicol. In Vitro 18, 13–19.

Habig, W., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-Transferases. J. Biol. Chem. 22, 7130–7139.

Hayes, K.A., Burks, R.L., Castro-Vazquez, A., Darby, P.C., Heras, H., Martín, P.R., Qiu, J.W., Thiengo, S.C., Vega, I.A., Wada, T., Yusa, Y., Burela, S., Cadierno, M.P., Cueto, J.A., Dellagnola, F.A., Dreon, M.S., Frassa, M.V., Giraud-Billoud, M., Godoy, M.S., Ituarte, S., Koch, E., Matsukura, K., Pasquevich, M.Y., Rodriguez, C., Saveanu, L., Seuffert, M.E., Strong, E.E., Sun, J., Tamburi, N.E., Tiecher, M.J., Turner, R.L., Valentine-Darby, P.L., Cowie, R.H., 2015. Insights from an integrated view of the biology of apple snails (Caenogastropoda: Ampullariidae). Malacologia 58, 245–302.

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. Jr., Bergman, H.L. (Eds.), Biomarkers Biochemical, Physiollogical, and Histological Markers of Antropogenic Stress. Lewis Publishers, Chelsea, USA, pp. 155–159.

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.

Korkmaz, N., Cengiz, E.I., Unlub, E., Uysal, E., Yanar, M., 2009. Cypermethrin-induced histopathological and biochemical changes in *Nile tilapia* (Oreochromis niloticus): and the protective and recuperative effect of ascorbic acid. Environ. Toxicol. Pharm. 28, 198–205.

Kruatrachue, M., Sumritdee, C., Pokethitiyook, P., Singhakaew, S., 2011. Histopathological effects of contaminated sediments on golden apple snail (*Pomacea canaliculata*, Lamarck 1822). Bull. Environ. Contam. Toxicol. 86, 610–614.

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.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Lowe, S., Browne, M., Boudjelas, S., De Poorter, M., 2000. 100 of the World's Worst Invasive Alien Species: A Selection from the Global Invasive Species Database. Invasive Species Specialist Group (ISSG), Auckland pp.12.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Lv, S., Zhang, Y., Liu, H.X., Hu, L., Yang, K., Steinmann, P., Chen, Z., Wang, L.Y., Utzinger, J., Zhou, X.N., 2009. Invasive snails and an emerging infectious disease: results from the first national survey on *Angiostrongylus cantonensis* in China. PLOS. Negl. Trop. Dis. 3, 368.

Maharajan, A., Narayanasamy, Y., Ganapiriya, V., Shanmugavel, K., 2015. Histological alterations of a combination of Chlorpyrifos and Cypermethrin (Nurocombi) insecticide in the fresh water crab, *Paratelphusa jacquemontii* (Rathbun). J. Bas. Appl. Zool. 72, 104–112.

Mahmoud, N., Dellali, M., Aissa, P., Mahmoudi, E., 2012. Acute toxicities of cadmium and permethrin on the pre-spawning and post-spawning phases of *Hexaplex trunculus* from Bizerta Lagoon, Tunisia. Environ. Monit. Assess. 184, 5851–5861.

Marigómez, I., Cajaraville, M.P., Soto, M., Lekube, X., 1998. Cell-type replacement, a successful strategy of mollusks to adapt to chronic exposure to pollutants. Cuad. Invest. Biol. 20, 411–414.

Marigómez, I., Soto, M., Orbea, A., Cancio, I., Cajaraville, M.P., 2004. Biomonitoring of environmental pollution along the Basque coast, using molecular, cellular and tissue-level biomarkers: an integrative approach. In: In: Borja, A., Collins, M. (Eds.), Oceanography and Marine Environment of the Basque Country 70. Elsevier Oceanography Series, Amsterdam, pp. 335–364.

Marklund, S., Marklund, G., 1974. Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469–474.

Martínez, M.L., Piol, M.N., Sbarbati Nudelman, N., Verrengia Guerrero, N.R., 2017. Tributyltin bioaccumulation and toxic effects in freshwater gastropods *Pomacea canaliculata* after a chronic exposure: field and laboratory studies. Ecotoxicology 26, 691–701.

Merivee, E., Tooming, E., Must, A., Sibul, I., Williams, I.H., 2015. Low doses of the common alpha-cypermethrin insecticide affect behavioural thermoregulation of the non-targeted beneficial carabid beetle *Platynus assimilis* (Coleoptera: Carabidae). Ecotoxicol. Environ. Saf. 120, 286–294.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Leães, Lopes, 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.

Mugni, H., Ronco, A., Bonetto, C., 2011. Insecticide toxicity to *Hyalella curvispina* in runoff and stream water within a soybean farm (Buenos Aires Argentina). Ecotoxicol. Environ. Saf. 74, 350–354.

Nørum, U., Friberg, N., Jensen, M.R., Pedersen, J.M., Bjerregaard, P., 2010. Behavioural changes in three species of freshwater macroinvertebrates exposed to the pyrethroid lambda-cyhalothrin: laboratory and stream microcosm studies. Aquat. Toxicol. 98, 328–335.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

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.

Ray, M., Bhunia, A.S., Bhunia, N.S., Ray, S., 2013. Density shift, morphological damage, lysosomal fragility and apoptosis of hemocytes of Indian molluscs exposed to pyrethroid pesticides. Fish Shellfish Immunol. 35, 499–512.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.

Richardson, K.L., Gold-Bouchot, G., Schlenk, D., 2009. The characterization of cytosolic glutathione transferase from four species of sea turtles Loggerhead (*Caretta caretta*), green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*), and hawksbill (*Eretmochelys imbricata*). Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 150, 279–284.

Rivadeneira, P.R., Agrelo, M., Otero, S., Kristoff, G., 2013. Different effects of subchronic exposure to low concentrations of the organophosphate insecticide chlorpyrifos in a freshwater gastropod. Ecotoxicol. Environ. Saf. 90, 82–88.

Sánchez-Fortún, S., Barahona, M.V., 2005. Comparative study on the environmental risk induced by several pyrethroids in estuarine and freshwater invertebrate organisms. Chemosphere 59, 553–559.

Santovito, G., Piccinni, E., Boldrin, F., Irato, P., 2012. Comparative study on metal homeostasis and detoxification in two Antarctic teleosts. Comp. Biochem. Physiol. C 155, 580–586.

Sattin, G., Bakiu, R., Tolomeo, A.M., Carraro, A., Coppola, D., Ferro, D., Patarnello, T., Santovito, G., 2015. Characterization and expression of a new cytoplasmic glutathione peroxidase 1 gene in the Antarctic fish *Trematomus bernacchii*. Hydrobiologia 761, 363–372.

Sawasdee, B., Köhler, H.R., Triebskorn, R., 2011. Histopathological effects of copper and lithium in the ramshorn snail, *Marisa cornuarietis* (Gastropoda, Prosobranchia). Chemosphere 85, 1033–1039.

Sellami, B., Louati, H., Dellali, M., Aissa, P., Mahmoudi, E., Coelho, A.V., Sheehan, D., 2013. Effects of permethrin exposure on antioxidant enzymes and protein status in Mediterranean clams *Ruditapes decussatus*. Environ. Sci. Pollut. Res. Int. 21, 4461–4472.

Sellami, B., Khazri, A., Louati, H., Boufahja, F., Dellali, M., Sheehan, D., Aissa, P., Driss, M., Mahmoudi, E., Beyrem, H., 2014. Effects of permethrin on biomarkers in Mediterranean clams (*Ruditapes decussatus*). Bull. Environ. Contam. Toxicol. 92, 574–578.

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.

Sepici-Dincel, A., Caglan Karasu Benli, A., Selvi, M., Sarıkaya, R., Sahina, D., Ayhan Ozkul, I., Erkoc, F., 2009. Sublethal cyfluthrin toxicity to carp (*Cyprinus carpio* L.) fingerlings: biochemical, hematological, histopathological alterations. Ecotoxicol. Environ. Saf. 72, 1433–1439.

Seuffert, M.E., Martín, P.R., 2013. Distribution of the apple snail *Pomacea canaliculata* in Pampean streams (Argentina) at different spatial scales. Limnologica 43, 91–99.

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.

Soderlund, D.M., Bloomquist, J.R., 1989. Neurotoxic actions of pyrethroid insecticides. Ann. Rev. Entomol. 34, 77–96.

Soto, M., Zaldibar, B., Cancio, I., Taylor, M.G., Turner, M., Morgan, A.J., Marigómez, I., 2002. Subcellular distribution of cadmium and its cellular ligands in mussel digestive gland cells as revealed by combined autometallography and X-ray microprobe analysis. Histochem. J. 34, 273–280.

Tolomeo, A.M., Carraro, A., Bakiu, R., Toppo, S., Place, S.P., Ferro, D., Santovito, G., 2016. Peroxiredoxin 6 from the Antarctic emerald rockcod: molecular characterization of its response to warming. J. Comp. Physiol. B 186, 59–71.

Ullah, R., Zuberi, A., Naeem, M., 2015. Toxicity to hematology and morphology of liver: brin and gills during acute exposure of mahseer (Tor putitora) to cypermethrin. Int. J. Agric. Biol. 17, 199–204.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. 64, 178–189.

Vega, I.A., Gamarra-Luques, C., Koch, E., Bussmann, L., Castro-Vazquez, A., 2005. A study of corpuscular DNA and midgut gland occupancy by putative symbiotic elements in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). Symbiosis 39, 37–45.

Vega, I.A., Damborenea, M.C., Gamarra-Luques, C., Koch, E., Cueto, J.A., Castro-Vazquez, A., 2006. Facultative and obligate symbiotic associations of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). Biocell 30, 367–375.

Vega, I.A., Arribére, M.A., Almonacid, A.V., Ribeiro Guevara, S., Castro-Vazquez, A., 2012. Apple snails and their endosymbionts bioconcentrate heavy metals and uranium from contaminated drinking water. Environ. Sci. Pollut. Res. 19, 3307–3316.

Vieira, C.E.D., Costa, P.G., Lunardelli, B., de Oliveira, L.F., da Costa Cabrera, L., Risso, W.E., dos Reis Martinez, C.B., 2016. Multiple biomarker responses in *Prochilodus lineatus* subjected to short-term in situ exposure to streams from agricultural areas in Southern Brazil. Sci. Total Environ. 542, 44–56.

Wang, X., Martínez, M.A., Dai, M., Chen, D., Ares, I., Romero, A., Anadón, A., 2016. Permethrin-induced oxidative stress and toxicity and metabolism. A review. Environ. Res. 149, 86–104.

Wei, K., Yang, J., 2015. Oxidative damage induced by copper and beta-cypermethrin in gill of the freshwater crayfish *Procambarus clarkii*. Ecotoxicol. Environ. Saf. 113, 446–453.

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.

Zaldibar, B., Cancio, I., Soto, M., Marigómez, I., 2008. Changes in cell-type composition in digestive gland of slugs and its influence in biomarkers following transplantation between a relatively unpolluted and a chronically metal-polluted site. Environ. Pollut. 156, 367–379.