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

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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

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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.

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