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RESEARCH ARTICLE

Stress oxidative and genotoxicity in *Prochilodus lineatus* (Valenciennes, 1836) exposed to commercial formulation of insecticide cypermethrin

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ABSTRACT

The use of toxic pesticides has become a world problem because they can contaminate streams and rivers, producing an adverse impact on non-target aquatic biota, including fishes. Cypermethrin is one of the most important insecticides to control ectoparasites in wide-scale. The aim of this study was to evaluate the effect of commercial formulations of cypermethrin, SHERPA O (0.0, 0.075, 0.15, and 0.3 μg/L of cypermethrin) in fish *Prochilodus lineatus* for 96 h in semi-static condition, using biomarkers of genotoxicity: micronucleus frequency (MNF) in erythrocytes and biomarkers of oxidative damage: lipid peroxidation (TBARS) and antioxidant defenses, catalase (CAT) and glutathione (GSH) in liver tissue. Our results showed a significant decrease ($p < 0.05$) of CAT at pesticide concentrations of 0.150 and 0.300 μg/L, but no significant difference was observed in TBARS or GSH in any exposed group ($p > 0.05$) in comparison to the control. A significant increase was observed in the MNF in the group exposed to 0.3 μg/L of cypermethrin compared to negative control ($p < 0.05$). Finally, *P. lineatus* proved to be a sensitive species to the commercial formulations of cypermethrin and that CAT and MNF are effective indicators of these toxic effects.

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Fish; liver tissue; biomarkers; micronucleus frequency; catalase

Introduction

Pesticides have become an essential product in agriculture because they improve the production, protect stored crops, and control the vectors of diseases. The agriculture has intensified rapidly, in special in Argentina, and the use of large amounts of pesticides in farm areas contributes to the presence of toxic substances in the environment. In particular, pyrethroids are the main class of broad-spectrum organic insecticides, which have largely replaced organophosphates and organochlorines in the last two decades, due to its lower persistence in the environment. They are recognized as potent neurotoxic insecticides and low mammalian toxicity. However, several studies have reported that these compounds are extremely toxic to fish and other aquatic organisms (Parma *et al*. 2007, Simoniello *et al*. 2009, Jin *et al*. 2011). Recently, also pyrethroid bioaccumulation in wild river fish has been described (Corcellas *et al*. 2015, Ullah *et al*. 2018).

Cypermethrin, a synthetic pyrethroid insecticide and its commercial formulations are used to control many pests, such as moth pests attacking cotton, fruit and vegetable crops, including structural pest control, or landscape maintenance (Uner *et al*. 2001, Polat *et al*. 2002, Aydin *et al*. 2005, Shi *et al*. 2011, Vani *et al*. 2012, Adeyemi *et al*. 2013, Marigoudar *et al*. 2013, Murthy *et al*. 2013, Poletta *et al*. 2013, Yonar 2013, Ullah and Zorriehzahra 2015). This has resulted in its discharge into the aquatic environment (Etchegoyen *et al*. 2017) and consequently, several laboratory studies have been performed, which have shown that cypermethrin is extremely toxic to non-target fish and aquatic invertebrates at very low concentrations (Taju *et al*. 2014, Majumder and Kaviraj 2017, Moraes *et al*. 2018). Fish sensitivity to pyrethroids may be explained by their relatively slow metabolism and elimination of these compounds (David *et al*. 2004, Paravani *et al*. 2018). However, few studies have been carried out on the toxicological effects of commercial of cypermethrin in freshwater fish.

Various pesticides act as pro-oxidants in multiple organs, modify the response of antioxidant defenses, produce accumulation of reactive oxygen species (ROS), damage to proteins, DNA, and may also interact with the cell plasma membrane to generate lipid peroxidation (LPO), resulting in phospholipid degradation, damage to membranes and impaired functionality (Limón-Pacheco and Gonsebatt 2009). ROS at excess level also produce alterations in the activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) (Nwani *et al*. 2015). In addition, pesticides can produce genotoxic effects in fish and the micronuclei frequency (MNF) has often been used to monitor aquatic pollutants displaying mutagenic features and for genotoxicity assessment of chemical and physical agents after direct or indirect exposure

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(De Flora *et al*. 1993, Saotome and Hayashi 2003, Pantaleao *et al*. 2006, Seriani *et al*. 2011, Obiakor and Okonkwo 2012). It has been a powerful tool for *in situ* biomonitoring in several studies aimed at detecting genotoxic agents and applicable to ecosystems fresh and saltwater fish (Hayashi *et al*. 1998; Grisolia *et al*. 2005).

Various organisms, including fish species have been used to monitor aquatic pollutants displaying mutagenic features and for genotoxicity assessment of chemical and physical agents after direct or indirect exposure (De Flora *et al*. 1993, Saotome and Hayashi 2003, Obiakor and Okonkwo 2012). Fish are considered organisms of special interest for the assessment of aquatic pollutants *in vivo*, and they are excellent subjects for the study of the mutagenic and carcinogenic potential of contaminants present in water. This interest is because they can metabolize, accumulate pollutants concentrate, which makes them sensitive to even low concentrations of genotoxicants (El-shehawi *et al*. 2007, Simoniello *et al*. 2009, Loteste *et al*. 2013, Poletta *et al*. 2013). *P. lineatus* is one of the most abundant fish species and is considered the main resource in commercial fisheries in the Middle Paraná River in Argentina (Rossi *et al*. 2007). In addition, this species is found throughout the Jacuí, Paraíba do Sul, Paraná, Paraguay and Uruguay river basins (Castro and Vari 2004). Their detritivorous ecological feature allows them to be an appropriate species for environmental monitoring, as it is in contact with xenobiotics in water and sediment (Camargo and Martinez 2006; Vanzella *et al*. 2007; Carvalho and Fernandes 2008, Cazenave *et al*. 2009, Cavalcante *et al*. 2008). Thus, the aim of this study was to evaluate the effect of commercial formulations of cypermethrin in fish *P. lineatus* using biomarkers of genotoxicity, oxidative damage, and antioxidant defense.

Methods

Animals

Juveniles fish *P. lineatus* were collected from a pristine environment next to Santa Fe city (Argentina) (31° 39′ 36″ L and 60° 35′ 26″ W) and maintained in tanks of capacity 180 L with aerated dechlorinated water for 5 days for acclimation. A total of 60 juveniles (length: 41.7 ± 21.7 mm g and weight: 113.31 ± 19.49 g) were used, which were placed in containers of polyurethane at 5 fish per tank and were fed one daily. The temperature was maintained at 25 °C, photoperiod of 12:12 h and oxygen concentration between 5.7 and 6.8 mg/L. All procedures were in accordance with the ethical standards of the Institutional Ethics Committee (Facultad de Bioquímica y Ciencias Biológicas – Universidad Nacional del Litoral), and all experiments were conducted in this university.

Experimental design and treatments

Juvenile fish ($n = 5$ fish per group and three replicates per concentration) were exposed to concentrations of 0.075, 0.150, and 0.300 μg/L of cypermethrin contained in the commercial formulation "SHERPA O" (containing 25% of cypermethrin as active ingredient). Cypermethrin was dissolved in 0.1% acetone (Cicarelli®, Bs. As. Argentina). The control group was maintained only in tap water, without the pyrethroid. Cypermethrin in water, under normal environmental temperatures and pH, is stable to hydrolysis with a half-life greater than 50 days and to photodegradation with a half-life greater than 100 days (Kamrin 1997). To maintain a relatively constant concentration of the compound, the assay was semi-static, with a duration of 96 hours, renewing 50% of the solution every 24 hours. Mortality and behavioral alterations (frequency and speed of swimming and frequency of erratic movements) in fish were registered. At the end of the experiment, the animals were euthanized, and peripheral blood samples were obtained with heparinized micropipette by dissection of the caudal peduncle. Then, smears were stained with Giemsa to be analyzing later for the determination of genotoxicity (MNF). In addition, the fish liver was dissected, washed in saline solution, and immediately frozen at −80 °C for biochemical analysis.

To analyze oxidative stress parameters in fish liver, homogenates were made according to Sabatini *et al*. (2011). Liver (30 mg) were taken for each sample and mixed with KCl buffer and protease inhibitors (PMSF and benzamidine). Then, they were centrifuged at 900 *g* for 10 minutes and the supernatant was used for the determinations of antioxidant defense (CAT and GSH) and oxidative damage (TBARS). A commercial kit (Wiener lab®) was used to determine the concentration of proteins in each homogenate.

Catalase activity in liver tissue

Catalase activity (CAT) activity was measured by the method of Aebi (1984). The method measures the decrease of H_2O_2 at 240 nm (at 25 °C) for 60 seconds, and then CAT was calculated as the difference in absorbance within 60 seconds of reaction. All determinations were performed in triplicate, and results were expressed as U/mg of protein.

Concentration of glutathione in liver tissue

Glutathione (GSH) was determined by a modification of the technique of Ellman (1959), using DTNB (dithio-bis-nitrobenzoate) in phosphate buffer, pH 8.0. The GSH content was recorded at 412 nm spectrophotometrically, and its concentration was calculated from a standard curve and expressed as μmol GSH/mg of protein.

Lipid peroxidation by TBARS in liver tissue

LPO was performed indirectly by the technique of Buege and Aust (1978) that measures malondialdehyde (MDA) by determining the reactive substances with thiobarbituric acid (TBARS). Trichloroacetic acid as a reagent and thiobarbituric acid was employed in the presence of butylated hydroxytoluene (BHT), which after being mixed with the homogenate was heated for 45 minutes at 95 °C in glycerin bath and then measuring the absorbance at 535 nm. Results of TBARS concentration in liver tissue were expressed as mol/g protein.

Micronucleus frequency in erythrocytes

The MNF was conducted according to the technique described by Grisolia and Cordeiro (2000). Each sample was performed in duplicate and was stained with Giemsa (1:10) for 10 minutes, previously centrifuged and filtered to reduce any lump of dye, which could interfere with the identification of MN. From each fish, 1000 (500 cells from each slides) were scored under an optical microscope with a magnification of 1000×. MNF was recorded as the number of cells with MN per 1000 cells counted.

Statistical analysis

For statistical analysis, the software SPSS 14.0 for Windows was used. Data are expressed as the mean ± standard error (SE). KolmogorovSmirnov test was used for testing normality, and homogeneity of variances between groups was verified by Levene test. Significant differences ($p < 0.05$) in the groups were analyzed by the Kruskal Wallis test followed by Mann–Whitney test.

Results

Comparison showed that sublethal concentrations of cypermethrin not caused mortality or behavioral alterations in the fish exposed. When was evaluate CAT, GSH and TBARS in liver tissue exposed of cypermethrin (Figure 1(a–c)), our results showed a diminution in the CAT activity in the groups exposed to the highest concentrations of cypermethrin (0.15 μg/L and 0.30 μg/L), compared to the negative control ($p < 0.05$, Figure 1(a)). However, there were no differences in GSH and TBARS at any treatment ($p > 0.05$, Figure 1(b,c)), demonstrating reproducibility of results in this study, under this experimental schedule. In relation to the genotoxicity of cypermethrin has been observed in erythrocytes of *P. lineatus* exposed *in vivo* under laboratory-controlled conditions, reproducibility in separate experiments, a significant increase in the MNF only in the group exposed to 0.3 μg/L of cypermethrin (2.43 ± 0.48) in comparison to the control group (0.8 ± 0.25, $p < 0.05$). The groups exposed to lowest cypermethrin concentrations (0.15 and 0.075 μg/L) showed no increase in the MNF (1.35 ± 0.32 and 1.83 ± 0.54, respectively) in comparison to the negative control ($p > 0.05$, Figure 1(d)).

Discussion

The toxicity of pyrethroids has received much attention in recent years, especially cypermethrin and its commercial formulations, which is one of the most common pollutants in ecosystems (Hussien *et al.* 2013). In this study, a significant

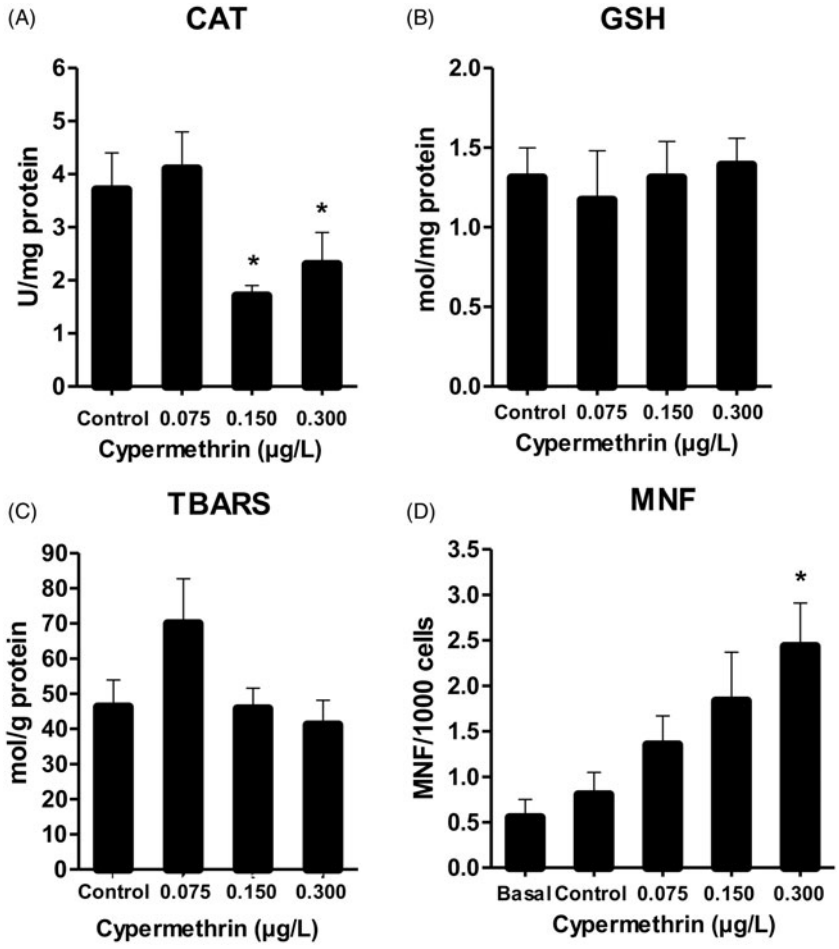

Figure 1. Activity of CAT, GSH content, and lipid peroxidation in the liver of *P. lineatus* exposed to sublethal concentrations (0.0, 0.075, 0.15, and 0.3 μg/L) of cypermethrin. (A) catalase (CAT) activity, (B) Reduced glutathione (GSH) concentration, (C) TBARS concentration, (D) Frequency of micronuclei/1000 erythrocytes. Data expressed as mean ± standard error of the mean (SEM). *Statistically significant with respect to the negative control ($p < 0.05$; Mann–Whitney test).

decrease in the activity of CAT enzyme in fish liver was observed to the highest concentrations of cypermethrin, which suggest a direct effect on antioxidant system, observed after 4 days of exposure. Considering that SOD and CAT are the key enzymes for the removal of ROS formed during bioactivation of xenobiotics in the liver tissues (Moraes *et al*. 2009), a diminution of CAT could be an indicator of effects of free radicals on proteins, lipids, and nucleic acids, causing cell damage in the fish. Similar results of decrease in the activity of CAT enzyme were obtained with other pesticides on CAT activity in *Oncorhynchus mykiss* exposed to Carbaryl, after 96 hours of exposure (Ferrari *et al*. 2007) and in *Danio rerio* exposed to deltamethrin pyrethroid for 16 days (Sharma and Ansari 2013). In addition, Tripathi and Bandooni (2011) also reported that activity CAT declined significantly in liver of *Clarias batrachus* after treatment with alphamethrin pyrethroid for 14 days. Decrease in CAT activity also has been observed in organs different: brains, gills, livers, and skeletal muscles of *Channa punctatus* exposed to pyrethroids Alphamethrin (Tripathi and Singh 2013) and sperm *Oncorhynchus mykiss* exposed to cypermethrin (Kutluyer *et al*. 2016). In addition, the molecular mechanism of effect of cypermethrin is little known, however, this pesticide could binding to CAT or by inhibiting CAT synthesis similar to other pyrethroids (deltamethrin) as reported by Sayeed *et al*. (2003).

Glutathione antioxidant system plays an important role in cellular defense against reactive free radicals and other oxidant species. In addition to being a direct free radical scavenger, GSH is known to function as a substrate for glutathione peroxidase and glutathione-S-transferase. Hepatic GSH is related to detoxification of xenobiotics (Haque *et al*. 2003). However, no significant differences were found in GSH level between the groups exposed to cypermethrin concentrations in comparison to the control. Previous studies have found diminished to GSH after 96 hour of the exposition *Cyprinus carpio* to cypermethrin found by David *et al*. (2004). These authors have also demonstrated that GSH content is produced in 24, 48, and 72 hours after exposition with cypermethrin. Ansari *et al*. (2011) also reported an increase of GSH in *Channa punctata*, at the two concentrations of cypermethrin (0.8 and 1.2 μg/L) after 72 hours of exposure. This suggests that the effect of cypermethrin on the GSH content in fish exposed is dependent on the time of analysis, and species-specific effect as a function of concentration.

LPO has also been used to measure pesticides and xenobiotic-induced oxidative stresses in freshwater fishes (Ansari and Ansari 2014). In our study, no significant differences were observed in LPO in fish exposed to any of the concentrations of cypermethrin, which could be due to the short period of exposure (96 hours). Pesticides-exposed in *P. lineatus* organs for a longer time allow the accumulation of LPO as found in the study of Paulino *et al*. (2012), which the LPO concentration was increased in gills of *P. lineatus* after a subchronic exposure (14-days).

ROS generation can cause DNA damage, which could lead to single-strand breaks, mutation and subsequently to chromosomal fragmentation. Micronuclei (MN) presences are considered as indicator of chromosomal damage (Flores-García *et al*. 2011). MN assay has been widely used in studies for the evaluation of genotoxic effects caused by various xenobiotics in aquatic organisms (Vera-Candioti *et al*. 2013). In the present study, a significant increase in the MNF was observed in the group exposed to high concentration of cypermethrin (0.300 μg/L) in comparison to control group. These results could be related to the decrease in antioxidant capacity (CAT activity) determined for the same concentrations in this study. DNA damage has been described in five fish cells exposed to cypermethrin increasing when the concentration of cypermethrin increased from 1.25 ng/ml (Taju *et al*. 2014). In addition, DNA damage to cypermethrim in *P. lineatus* has been observed in other studies (Simoniello *et al*. 2009, Poletta *et al*. 2013). However, the observed effects in *P. lineatus* could be due not only to cypermethrin, additives present in the commercial formulation of cypermethrin "SHERPA O", which are not mentioned in the product information, could increase its toxicity.

In addition, toxic effects of the commercial formulation of cypermethrin on *P. lineatus* fish could decline the population of this species causing changes in the ecosystem. Considering that this species is a detritivorous fish and detritus is a crucial pathway in energy and nutrient fluxes in ecosystems, loss of detritivores could therefore strongly influence ecosystem functioning (Bowen 1983, Flecker 1996, Taylor *et al*. 2006).

Conclusions

In the present study, it was demonstrated diminution of CAT and genotoxicity effect (MNF) in fish *P. lineatus* exposed to higher concentration of cypermethrim. In this context, CAT proved to be the most effective to monitor the effects of commercial formulation "SHERPA O" in *P. lineatus* and the MNF proved to be a sensitive indicator of genotoxicity. In addition, further research in different time of analysis of exposition the formulation of cypermethrin could be performed in order to determine others effects in *P. lineatus*. Finally, *P. lineatus* could be used as a sensitive organism for the evaluation of potentially harmful substances in the aquatic environment.

Disclosure statement

No potential conflict of interest was reported by the authors.

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