



The pyrethroid λ -cyhalothrin induces biochemical, genotoxic, and physiological alterations in the teleost *Prochilodus lineatus*

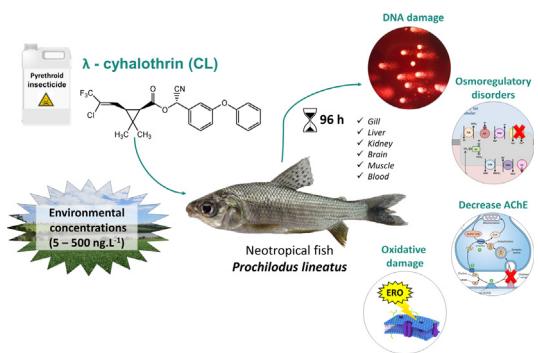
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HIGHLIGHTS

- We evaluated acute effects of a λ -cyhalothrin (CL) formulation on the fish *Prochilodus lineatus*.
- CL promoted oxidative damage in different fish tissues.
- CL promoted a decreased in the muscle acetylcholinesterase activity.
- CL also promoted osmoregulatory disorders.
- DNA damage was detected in erythrocytes of fish exposed to CL.

GRAPHICAL ABSTRACT



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ABSTRACT

The λ -cyhalothrin (CL) is a globally used pyrethroid insecticide that has been detected in different water bodies worldwide. However, studies on the effects of CL on freshwater fishes are still incipient. In this context, we evaluated the acute effects of a commercial formulation containing CL (Karate Zeon® CS 50) in juveniles of the teleost *Prochilodus lineatus* exposed for 96 h to four concentrations of the active ingredient (5, 50, 250 and 500 ng.L^{-1}). Biochemical, physiological, and genotoxic biomarkers were evaluated in different organs of the fish. Exposure to CL induced significant changes in the enzymatic profiles of *P. lineatus*, with specific alterations in biotransformation enzymes and antioxidant defence in different tissues. Lipid peroxidation was observed in fish gills and kidney. Increases in esterases were observed in the liver of fish exposed to all CL concentrations evaluated, whereas acetylcholinesterase activity decreased in the muscles of fish at all concentrations. CL also promoted osmoregulatory disorders, with decreases in calcium and magnesium gill ATPases, with consequent hypocalcaemia, in addition an increase in sodium-potassium ATPase activity was observed in the gills of fish exposed to the highest CL concentration, probably in order to compensate a reduction in plasma sodium. Besides, increases in DNA damage were observed in the erythrocytes of fish exposed to all CL concentrations. Thus, despite the low CL concentrations and the short exposure time, this pyrethroid caused hematological adjustments, oxidative stress, osmoregulatory disorders, and DNA damage in *P. lineatus*, showing that the species is highly sensitive to the deleterious effects of CL.

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1. Introduction

Over the past three decades, the worldwide pattern of insecticide use has been changing, and a decline in the production of high-toxic compounds to mammals, such as organophosphates and carbamates, has been the most notable modification (Kumar et al., 2014). Meanwhile, the use of pyrethroid insecticides is on the rise on a worldwide scale since they are potent and efficient insect control agents and have a rapid action and selective toxicity that is relatively harmless to mammals and birds (Soderlund and Bloomquist, 1989; Goulding et al., 2013; Haverinen and Vornanen, 2014). However, several studies have shown that pyrethroids are among the most toxic pesticides for aquatic organisms, including fish (US EPA, 2001; Saxena and Seth, 2002; Pimpão et al., 2007; Gu et al., 2007; Carriquiriborde et al., 2009; Ansari et al., 2011; Muranli and Güner, 2011; Werner and Young, 2018). Due to their lipophilic character, pyrethroids have a high rate of absorption through fish gills, which may partly explain the high sensitivity of this group to pyrethroid exposure (Polat et al., 2002), and can bioaccumulate in fish (Corcellas et al., 2015; Brander et al., 2016; Clasen et al., 2018). In addition, fish seem to have a deficient enzyme system to hydrolyse these compounds (Viran et al., 2003), leading to slower rates in their metabolism and elimination compared with birds and mammals (Bradbury and Coats, 1989; Fishel, 2005; Brander et al., 2016).

As a result of agricultural runoff, forest spraying, and direct spray procedures in water bodies, pyrethroids can enter aquatic environments and accumulate in sediments due to their high adsorption coefficient (Werner and Young, 2018). This characteristic can cumulatively increase the concentrations of these compounds in sediments of water bodies, thus increasing the risk for freshwater fish, especially the benthic species (Marino and Ronco, 2005; Velmurugan et al., 2007; Kutluyer et al., 2015).

Although pyrethroids are widely used around the world, there is limited information concerning environmental concentrations of these insecticides in surface waters (Weston et al., 2009; Domagalski et al., 2010; Weston and Lydy, 2012; Jabeen et al., 2015; Stehle and Schulz, 2015). Concentrations of λ -cyhalothrin (CL) in surface waters ranging from 346 ng.L⁻¹ in rivers of Greece (Tsaboula et al., 2016) to 797 ng.L⁻¹ in agricultural regions of the southern United States (Anderson et al., 2013) were already reported in the literature. Additionally, in tropical regions CL has been detected in sediments at concentration of 983 ng.L⁻¹ in agro-ecosystems of Costa Rica (Carazo-Rojas et al., 2018), and in Brazilian rivers at concentrations of 1.32 ng.g⁻¹ (Hunt et al., 2016), 1 ng.g⁻¹ to 5 ng.g⁻¹ (Miranda et al., 2008), and 19.7 ng.g⁻¹ to 60.0 ng.g⁻¹ (Possavatz et al., 2014).

The insecticidal activity and toxicity of the pyrethroids are dependent on the stereochemistry of the compound. Cis isomers are usually more toxic than trans isomers, and the introduction of the α -cyano group increases the toxicity of the molecule for both insects and mammals (Casida et al., 1983; Brander et al., 2016). There are two groups of pyrethroids with distinctive poisoning symptoms, distinguished by an alpha-cyano group in their structure. While those without an alpha-cyano group (type I pyrethroids, e.g., cismethrin, permethrin, bifenthrin) exert their neurotoxicity primarily through interference with sodium channel function in the nervous system, type II pyrethroids (with alpha-cyano group, e.g., cyhalothrin, deltamethrin, cypermethrin) can affect additional ion-channel targets such as chloride and calcium channels (Werner and Young, 2018).

The CL is a type II α -cyano pyrethroid current use as an insecticide for a wide range of target insects, it has also been adopted for the management of pests or in public health campaigns in the

control of disease vectors (Muranli and Güner, 2011). The main action mode of pyrethroids is the change in the permeability of voltage-dependent sodium channels of nerve cells. Pyrethroids modify the activation and inactivation kinetics of the sodium channels, resulting in the prolonged opening of individual channels, causing membrane depolarization, repetitive discharges, and synaptic disturbances and leading to the symptoms of intoxication by hyperexcitability (Vijverberg and vanden Bercken, 1990; Palmquist et al., 2012). In Brazil, 253 pyrethroid compounds are registered to commercial formulations, with CL being the pyrethroid with the highest number of registrations ($n = 72$) (BRASIL, 2018).

The freshwater fish *Prochilodus lineatus* (Valenciennes, 1836) has been used as a suitable biological model to study the effects of pesticides because it is sensitive to herbicides (Langiano and Martinez, 2008; Modesto and Martinez, 2010a, 2010b; Santos and Martinez, 2012; Pereira et al., 2013; Moreno et al., 2014) and insecticides (Maduenho and Martinez, 2008; Bacchetta et al., 2011; Poletta et al., 2013; Loteste et al., 2013; Vieira et al., 2018). In this context, our objective is to evaluate the potential toxic effects of an λ -cyhalothrin formulation in biochemical, physiological, and genotoxic biomarkers of the fish *P. lineatus* after acute exposure to environmentally relevant CL concentrations.

2. Material and methods

2.1. Experimental design

Juveniles of *P. lineatus* ($n = 40$; weight, 21.57 ± 3.86 g; length, 11.15 ± 0.70 cm) were obtained from the Fish Hatchery Station of the State University of Londrina. They were acclimated for seven days in 300-L tanks containing clean, dechlorinated water and with constant aeration, under a 12:12-h light/dark photoperiod. During this period, commercial feed (Guabi®, 36% protein content) was given to the fish every 48 h, and the feed was interrupted 24 h before the tests and during the exposure. The physical and chemical parameters of the water were monitored throughout the acclimation and exposure using a multiparameter water quality meter (HORIBA U-52, Japan) (temperature = 22.86 ± 0.071 °C; pH = 7.88 ± 0.023 ; dissolved oxygen = 7.26 ± 0.028 mg O₂.L⁻¹; conductivity = 0.102 ± 0.004 mS cm⁻¹).

After the acclimation period, the fish were randomly divided into five groups ($n = 8$ fish/group) and kept in glass aquaria containing 80 L of dechlorinated water. One group was kept under controlled conditions only in clean water (CL₀), and the other four groups were exposed to different nominal CL concentrations from the commercial formulation Karate Zeon® CS (Syngenta S.A., Brazil), as follows: 5 (CL₅), 50 (CL₅₀), 250 (CL₂₅₀), and 500 (CL₅₀₀) ng.L⁻¹. The nominal concentrations of CL were calculated considering the content of the active ingredient (CL) in the commercial formulation (5% a.i.). Fish were exposed to the different treatments over 96 h, under semi-static conditions, with a complete renewal of the test solution every 24 h, once pyrethroids can be degraded by chemical processes, mainly hydrolysis and photolysis, and via aerobic and anaerobic biodegradation (Laskowski, 2002).

The lethal concentration (LC₅₀) values over the 96 h for different fish species varied between 0.21 and 2.3 μ g.L⁻¹ (Maund et al., 1998), presenting high toxicity to these organisms. Thus, we sought to use safe CL concentrations for *P. lineatus* that would not cause mortality, starting from a minimum concentration of 5 ng.L⁻¹ and increasing in a geometric progression of 10 to the maximum concentration of 5000 ng.L⁻¹. However, as there was 100% mortality at the highest concentration after 24 h, an intermediate concentration was added to the experiment (250 ng.L⁻¹).

After the exposure period, the fish were anaesthetized with

benzocaine (0.1 g.L^{-1}) to withdraw blood through the caudal vein. Immediately after sampling blood samples were used to determine hematological parameters and an aliquot of blood was stored in fetal bovine serum and refrigerated until performing the comet assay. The animals were then killed by medullary section to remove liver, gills, kidney, brain, and muscle. The organs were stored in an ultra-freezer (-80°C) for biochemical analyses. These procedures were approved by the Ethics Committee on the Use of Animals of the State University of Londrina under process number 10493.2014.27.

2.2. Physiological biomarkers

To determine the hematocrit (Hct), blood was centrifuged ($1200 \times g$, 5 min) in a micro-capillary centrifuge (Luguimac S.R.L., Model LC 5, Argentina) and readings were performed using a standardized chart. The number of red blood cells per mm^3 blood (RBC) was counted using an improved Neubauer hemocytometer under a light microscope. The hemoglobin content (Hb) was estimated using the cyanmethemoglobin colorimetric method (commercial kit, Labtest Diagnóstica, Brazil); readings were performed at 540 nm using a spectrophotometer (Libra S32, Biochrom, United Kingdom). Blood samples were centrifuged ($1870 \times g$, 10 min), and glucose levels were determined using the glucose oxidase method with a commercial kit (Doles Reagentes Ltda, Brazil) at 505 nm (Victor TM³, PerkinElmer, USA). The concentrations of sodium and potassium were determined in plasma samples using a flame photometer (Digimed DM-62, Brazil). Plasma concentrations of chloride were determined using a commercial test kit (Labtest Diagnóstica, Brazil) at 470 nm (Victor TM³, PerkinElmer, USA) and calcium and magnesium concentrations were measured using the flame atomization method with an atomic absorption spectrometer (Perkin Elmer A700).

The gill filaments were homogenized in a homogenization buffer (100 mM Tris, 500 mM sucrose, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged ($10000 \times g$, 25 min, 4°C). The supernatant was used to measure ATPase activity. Na^+/K^+ -ATPase (NKA) activity was determined according to Quabius et al. (1997). In this method, the enzyme activity is indirectly measured by the production of inorganic phosphate from the ATP breakdown in samples incubated with KCl or ouabain (NKA inhibitor). A 0.65 mM phosphorus solution (Sigma) was used as a standard. The assays were performed on a microplate reader (620 nm, ELX 800, Biotek, USA).

Ca^{2+} -ATPase (CaATP) and Mg^{2+} -ATPase (MgATP) enzyme activities were measured according to Vijayavel et al. (2007) with modifications. First, the samples were incubated in a reactive solution (189 mM NaCl, 5 mM MgCl₂, 20 mM Tris, 5 mM CaCl₂, 2 mM ouabain, pH 7.6) without ATP to determine the basal concentration of inorganic phosphate (Pi) in the sample. The samples were then incubated in a reactive solution (CaATP: 189 mM NaCl, 5 mM MgCl₂, 20 mM Tris, 5 mM CaCl₂, 2 mM ouabain, pH 7.6; MgATP: 189 mM NaCl, 5 mM MgCl₂, 20 mM Tris, 14 mM KCl, 0.2 mM EDTA, 2 mM ouabain, pH 7.6) containing ATP (3 mM). CaATP and MgATP activities were determined by the absorbance difference between the samples with ATP and the samples without ATP by quantifying the Pi released in the sample using a staining solution. The readings were performed on a microplate reader (ELX 800, Bio-Tek Instruments) at 620 nm.

2.3. Biochemical biomarkers

Individual tissue (liver, gills, kidney, brain and muscle) samples were homogenized (1:10 w/v) in a phosphate buffer solution

(0.1 M; pH 7.0 or 7.5 for AChE analysis). Homogenates were centrifuged ($15,000 \times g$, 20 min, 4°C) and the supernatants were stored at -80°C for subsequent biochemical analysis. For all biochemical biomarkers, the protein content was determined according to Bradford (1976).

Glutathione-S-transferase (GST) activity was determined according to Keen et al. (1976), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, at 340 nm. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the reduction rate of cytochrome c by the superoxide radical in a spectrophotometer at 550 nm (McCord and Fridovich, 1969). Catalase (CAT) activity was determined by the decomposition rate of hydrogen peroxide (H_2O_2) by the enzyme, and the absorbance decrease was quantified in a spectrophotometer at 240 nm (Beutler, 1975). Glutathione peroxidase activity (GPx) was estimated indirectly through the NADPH oxidation in the presence of glutathione reductase (GR) and the oxidized glutathione substrate, produced by the GPx action, according to the method of Hopkins and Tudhope (1973). The glutathione (GSH) concentration in the liver was determined according to the method of Beutler (1963), through reaction of the $-SH$ groups with the staining reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form thiolate (TNB), which was quantified at 412 nm. The TBARS assay (thiobarbituric acid reactive substances, including malondialdehyde [MDA]) was used for evaluating lipoperoxidation (LPO), according to the protocol described by Camejo et al. (1998).

Esterase activity levels in the liver and plasma were quantified according to the method of Li and Fan (1997) with some adaptations, through the consumption of α -naphthyl and β -naphthyl substrates by α -EST and β -EST, which transform the substrates into α -naphthol and β -naphthol, respectively. A standard curve for α -naphthol and β -naphthol (0–10 $\mu\text{g mL}^{-1}$) was built for the calculation of converted substrate concentrations in the samples.

Subsamples of muscle and cerebral tissues were used for the analysis of acetylcholinesterase activity (AChE), according to a method described by Ellman et al. (1961) and adapted by Alves-Costa et al. (2007). AChE activity was determined at 415 nm.

2.4. Genotoxic biomarker

The alkaline comet assay with erythrocytes was performed according to Singh et al. (1988), with some modifications. An aliquot of cellular suspension was also mixed with FBS (1:100), and then mixed in low melting point agarose (0.5%), placed on slides pre-coated with normal (1%) melting point agarose, covered with coverslips and kept under refrigeration (10°C) for 40 min. The slides were subjected to: a) lysis: 2 h at 4°C , protected from light, in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 10% DMSO, 1 mL Triton X-100, pH 10.0); b) DNA denaturation: 30 min in the dark in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13, at 4°C); c) electrophoresis: 20 min, 300 mA, 25 V, 0.7 e 0.8 V cm^{-1} ; and d) neutralization: three rinses for 5 min each with buffer (0.4 M Tris, pH 7.5). Slides were fixed with absolute ethanol and kept under refrigeration until analyses. Slides were stained with gelRed and analysed under a Leica microscope (DM 2500) adapted for fluorescence/epifluorescence at 1000 \times magnification. All slides were blind-reviewed. The extent of DNA damage was quantified by the length of the tail formed by the migration of DNA fragments and classified into four comet classes, according to Vieira et al. (2018). DNA damage score was obtained by multiplying the number of cells in each class by the damage class and ranged from 0 (all undamaged) to 300 (all maximally damaged).

2.5. Statistical analysis

After checking data for normality (Shapiro Wilk test) and homoscedasticity (Levene's test) mean values for the different treatments, for each organ analysed, were cross-compared using parametric (ANOVA) or non-parametric (Kruskal-Wallis) analysis of variance, followed by the Student-Newman-Keuls (SNK) or Dunn's test, respectively, when necessary. A significance level of 0.05 was used in all analyses. The data shown in the graphs represent means \pm SEM.

3. Results

The results of the different physiological parameters measured in *P. lineatus* after CL exposure are presented in Table 1. Regarding CL₀, significant increases ($p = 0.022$) were observed in hematocrit at CL₅ and CL₅₀. Increases ($p < 0.001$) in glycemia were observed at CL₅₀, CL₂₅₀, and CL₅₀₀ in relation to CL₀. Ionic concentration changes indicated decreases in Na⁺ concentrations at CL₂₅₀ and CL₅₀₀ ($p < 0.001$) and in Ca²⁺ concentrations at CL₅₀₀ compared with CL₀ ($p = 0.028$).

The results of the gill ATPases are shown in Fig. 1. Decreases ($p = 0.004$) in CaATP activity were observed at CL₅₀, CL₂₅₀, and CL₅₀₀ compared with CL₀. MgATP responded similarly, and decreases ($p = 0.004$) in enzyme activity were observed at CL₂₅₀ and CL₅₀₀ compared with CL₀. In contrast, an increase in NKA activity was observed at CL₅₀₀ compared with the remaining groups ($p < 0.001$).

Relative to CL₀, increases ($p < 0.001$) in GST activity (Fig. 2-A) were observed in the liver, gills, brain, and muscle of fish exposed to CL₅₀₀. SOD activity presented a decrease ($p = 0.032$) in the gills at the highest concentration in comparison to CL₀ (Fig. 2-B). CAT activity (Fig. 2-C) was decreased ($p = 0.031$) in the liver at CL₅, CL₅₀, CL₂₅₀, and CL₅₀₀ compared with CL₀, whereas in the muscle, there were increases ($p < 0.001$) in the enzymatic activity at CL₅₀, CL₂₅₀, and CL₅₀₀ in relation to CL₀. Regarding GPx activity (Fig. 2-D), the liver showed an increase ($p < 0.001$) in the enzymatic activity at CL₅₀₀ compared to CL₀, whereas in the gills and kidney, decreases ($p < 0.001$) in the enzyme activity were observed at the highest CL concentration. No significant differences were observed in GSH concentration (Fig. 2-E) between the different groups in any organ analysed. Significant LPO increases (Fig. 3) were observed in the gills ($p < 0.001$) and kidney ($p = 0.002$) at CL₅₀₀ compared with CL₀.

The α -EST activity (Fig. 4-A) increased ($p < 0.001$) in the fish liver in all groups compared with CL₀. Similarly, β -EST activity (Fig. 4-B) increased in the liver ($p < 0.001$) at all CL concentrations compared to CL₀ and also increased in the plasma ($p < 0.001$). AChE activity (Fig. 4-C) in the muscle was decreased ($p = 0.002$) at all CL concentrations compared with CL₀. No significant changes in AChE activity were detected in the brain of the different experimental groups.

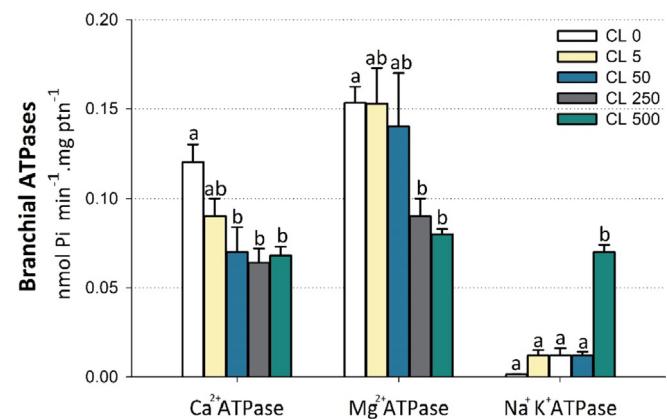


Fig. 1. Calcium (Ca²⁺ATPase), magnesium (Mg²⁺ATPase) and sodium-potassium (Na⁺K⁺ATPase) ATPases activity in gills of *P. lineatus* kept under the control condition (CL₀) or exposed to CL formulation at 5, 50, 250, and 500 ng L⁻¹. Data are mean \pm SEM, n = 8. Different letters indicate significant differences between groups ($p < 0.05$).

3.1. DNA damage

The results of the comet test showed that CL was able to promote an increase in DNA damage score in *P. lineatus* erythrocytes at all concentrations tested (Fig. 5) compared with CL₀ ($p < 0.001$).

4. Discussion

In this study, we show that CL formulation presented high toxicity to *P. lineatus* and that the low concentrations used were able to promote oxidative and genetic damage and neurological and osmoregulatory alterations in different tissues of the fish.

The fish physiological parameters were affected after acute exposure to CL. There were increases in hematocrit at CL₅ and CL₅₀ compared with CL₀. CL also promoted hyperglycemia in fish in almost all experimental groups. Altogether, these two parameters may indicate a situation of physiological stress induced by CL. Under the action of stress hormones such as catecholamines, increases in hematological parameters, including RBC, Hb, and Hct, may occur as a result of splenic contraction (Wenderlaar Bonga, 2011), with the aim of providing greater oxygen supply to metabolically active tissues. The rapid release of glucose, the main energetic substrate of animals, due to hepatic glycogenolysis is also a typical effect of a stress situation (Wenderlaar Bonga, 2011), with the aim of supplying the metabolic demands of animals. Thus, the increases in Hct and hyperglycemia in *P. lineatus*, observed in almost all exposure groups, seem to indicate a stress situation promoted by CL. However, the other analysed hematological parameters were not altered, and the increase in Hct was not accompanied by an increase in the number of circulating

Table 1

Physiological parameters of *P. lineatus* kept under the control condition (CL₀) or exposed to CL formulation (nominally: 5, 50, 250, and 500 ng L⁻¹).

Physiological parameters	CL ₀	CL ₅	CL ₅₀	CL ₂₅₀	CL ₅₀₀
Hemoglobin (mg. dL ⁻¹)	9.07 \pm 0.54	9.44 \pm 0.20	10.45 \pm 0.38	10.43 \pm 0.37	10.35 \pm 0.39
Hematocrit (%)	37.14 \pm 2.03 ^a	47.25 \pm 2.67 ^b	48.00 \pm 2.03 ^b	41.00 \pm 0.95 ^{ab}	43.00 \pm 0.79 ^{ab}
RBCs ($\times 10^6$ cel. mm ³)	2.37 \pm 0.14	2.75 \pm 0.14	3.05 \pm 0.15	2.59 \pm 0.07	2.66 \pm 0.18
Glucose (mg.dL ⁻¹)	35.51 \pm 1.63 ^a	41.10 \pm 2.65 ^a	48.40 \pm 2.00 ^b	49.28 \pm 2.24 ^b	46.63 \pm 2.25 ^b
Na ⁺ (mM)	148.88 \pm 4.14 ^a	145.02 \pm 2.13 ^a	145.75 \pm 3.40 ^a	128.65 \pm 2.48 ^b	120.28 \pm 5.60 ^b
K ⁺ (mM)	7.46 \pm 0.96	7.90 \pm 1.40	7.93 \pm 0.82	8.57 \pm 1.97	10.10 \pm 1.66
Cl ⁻ (mM)	107.52 \pm 4.28	105.98 \pm 2.97	109.02 \pm 4.78	102.65 \pm 4.54	104.23 \pm 3.87
Ca ²⁺ (mM)	2.65 \pm 0.09 ^a	2.53 \pm 0.06 ^{ab}	2.65 \pm 0.03 ^a	2.73 \pm 0.03 ^a	2.16 \pm 0.23 ^b
Mg ²⁺ (mM)	0.60 \pm 0.02	0.59 \pm 0.03	0.63 \pm 0.02	0.59 \pm 0.01	0.58 \pm 0.09

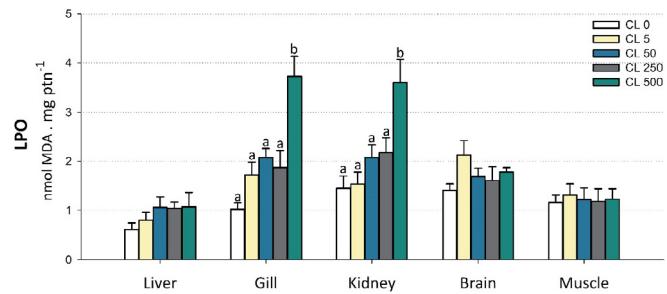
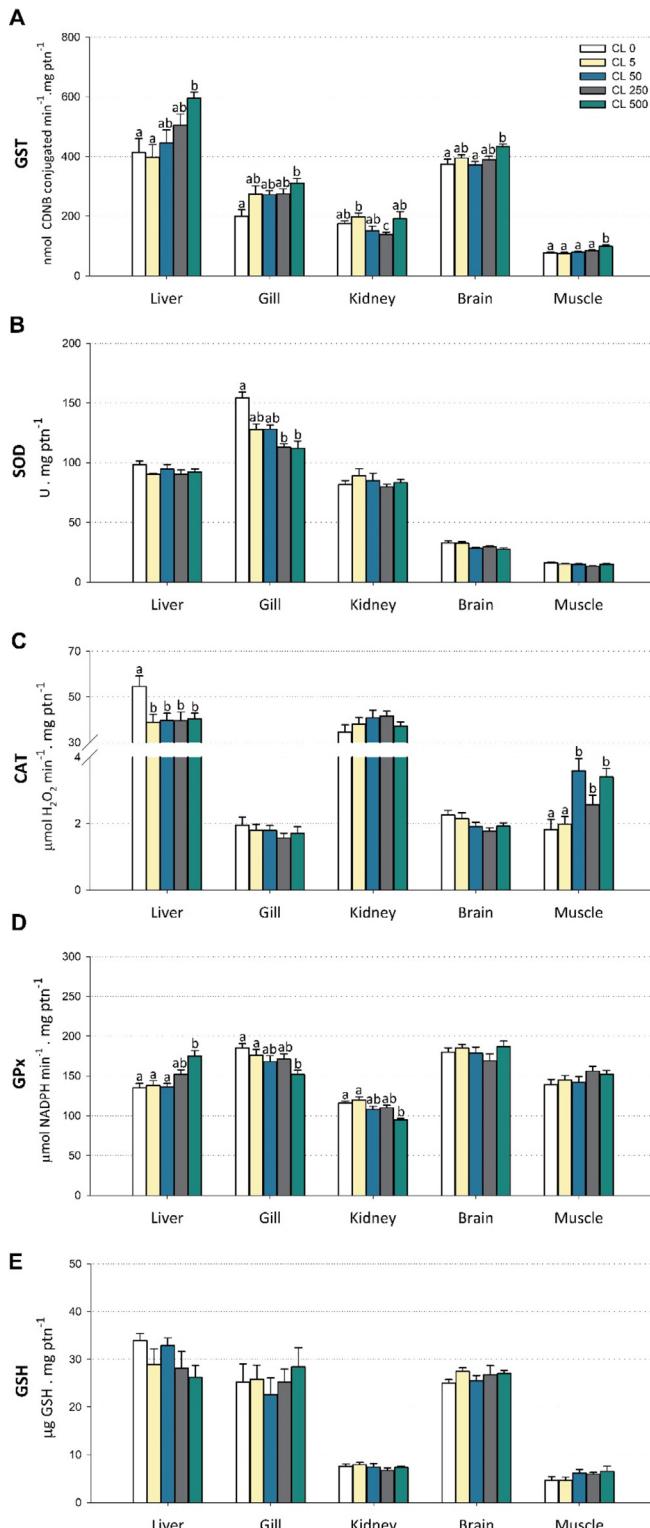


Fig. 3. Lipid peroxidation (LPO) in the liver, gill, kidney, brain, and muscle of *P. lineatus* kept under the control condition (CL0) or exposed to CL formulation at 5, 50, 250, and 500 ng L⁻¹. Data are mean ± SEM, n = 8. Different letters indicate significant differences between groups ($p < 0.05$).

erythrocytes. In addition, we found no differences in Hct at the two highest CL concentrations. The results of hematological parameters seem to have been influenced by the osmoregulatory imbalances promoted by CL. Decreases in the plasma concentrations of Na^+ at CL₂₅₀ and CL₅₀₀, along with a decrease in Ca^{2+} at the highest CL concentration, may indicate that hemodilution had occurred in the fish, leading to Hct decreases at these concentrations. Thus, the possible increases of Hct, RBC, and Hb at the highest CL concentrations were possibly masked by animal water gain.

In the literature, variations in blood parameters in fish exposed to pyrethroids are controversial and seem to depend on the species, compound, and concentrations used. No significant differences were observed in the blood parameters of *Piaractus mesopotamicus* exposed to 0.7 $\mu\text{g L}^{-1}$ of CL for 96 h (Bacchetta et al., 2014). In contrast, CL promoted decreases in Hct, Hb, and RBC of *Clarias gariepinus* (Yeeken et al., 2013). Other type II pyrethroids, such as deltamethrin, promoted significant increases in the hematological parameters of *Ancistrus multispinis* (Pimpão et al., 2007), as did cypermethrin in the teleost freshwater species *Brycon amazonicus* (Moraes et al., 2018) and *Rhamdia quelen* (Borges et al., 2007; Montanha et al., 2014).

Increases in the GST activity in the liver, gills, brain, and muscle of *P. lineatus* were observed at the highest CL concentration. In addition to being a phase II biotransformation enzyme, GST plays a key role in the prevention of oxidative stress. GST complements the GPx role in the hydroperoxide reduction and protects cells from the toxic LPO end-products (Hubatsch et al., 1998; Yang et al., 2001; Sharma et al., 2004). GST does not appear to play a role in pyrethroid metabolism in vertebrates (Vontas et al., 2002). The mechanisms of pyrethroid metabolism in fish will be discussed later. In this work, the GST increase at the highest CL concentration seems to have had a substantial protective effect against LPO occurrence in these organs, even with the decreases in the activity of some antioxidant enzymes. Thus, GST would not only complement GPx activities in preventing LPO by reducing hydroperoxides but would also protect cells from toxic LPO end-products. GST increases were also observed in *Oreochromis niloticus* exposed to CL (Piner and Uner, 2012), in *Carassius auratus* (Dinu et al., 2010), and in *Cyprinus carpio* exposed to deltamethrin (Ensibi et al., 2014).

Alterations in the antioxidant defence enzymes were also observed in *P. lineatus* after acute exposure to CL and presented distinct responses in the different organs of the fish. In the liver, CAT decreases were observed at all CL concentrations, while GPx activity was increased at the highest CL concentration. In the gills, there were decreases in SOD at CL₂₅₀ and CL₅₀₀ and a decrease in GPx activity at the highest CL concentration. Similarly, the posterior kidney also showed a decrease in GPx activity at CL₅₀₀. No changes

Fig. 2. Glutathione S-transferase (A), superoxide dismutase (B), catalase (C), glutathione peroxidase (D) activity and reduced glutathione concentration (E), in the liver, gill, kidney, brain, and muscle of *P. lineatus* kept under the control condition (CL0) or exposed to CL formulation at 5, 50, 250, and 500 ng L⁻¹. Data are mean ± SEM, n = 8. Different letters indicate significant differences between groups ($p < 0.05$).

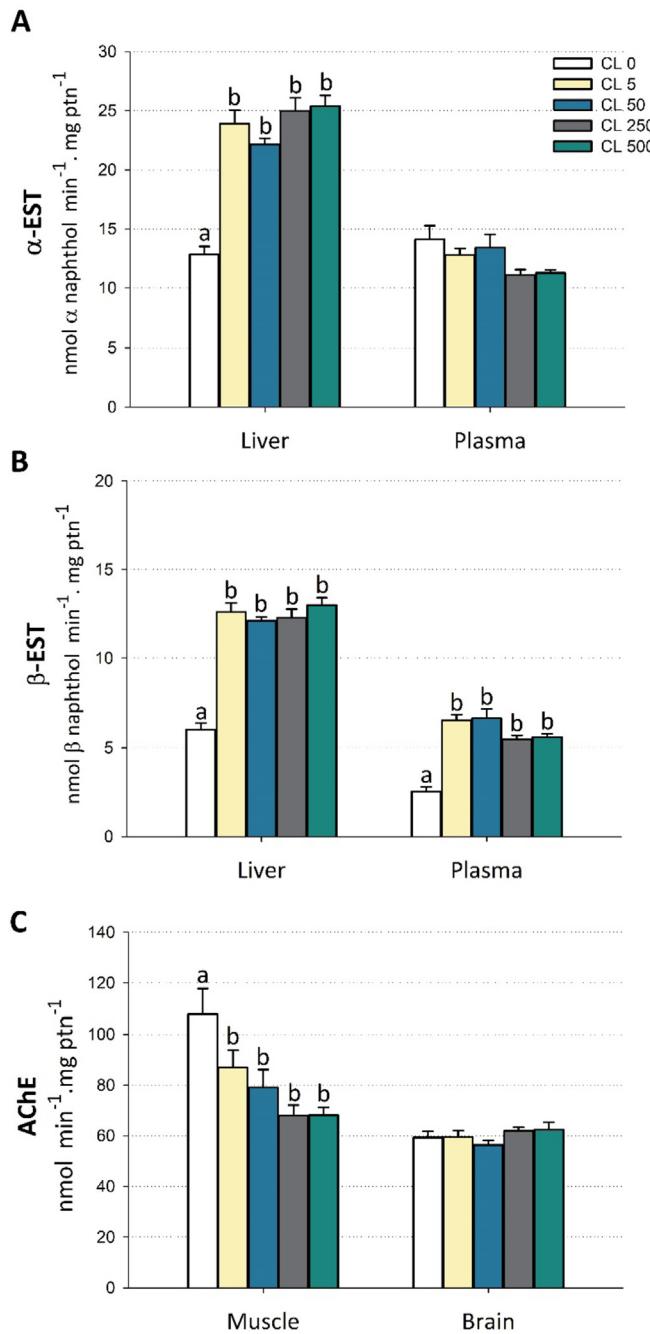


Fig. 4. α -esterases (A) and β -esterases (B) activity in liver and plasma, and acetylcholinesterase (C) activity in muscle and brain of *P. lineatus* kept under the control condition (CL0) or exposed to CL formulation at 5, 50, 250, and 500 ng L^{-1} . Data are mean \pm SEM, $n = 8$. Different letters indicate significant differences between groups ($p < 0.05$).

were observed in the brain for any of the antioxidants evaluated. Finally, the muscles showed increases in CAT activity at CL₅₀, CL₂₅₀, and CL₅₀₀. In addition to the variations in the activity of these enzymes, LPO was detected in the gills and kidneys. The possible adaptive mechanisms of each organ will be discussed below.

In the liver, induction of GPx and GST activity may have been responsible for protecting the hepatic tissue against oxidative stress by metabolizing organic hydroperoxides (ROOH), based on the non-alteration of LPO levels in this organ. Overall, inhibition of CAT activity has been related to the binding of toxic substances to

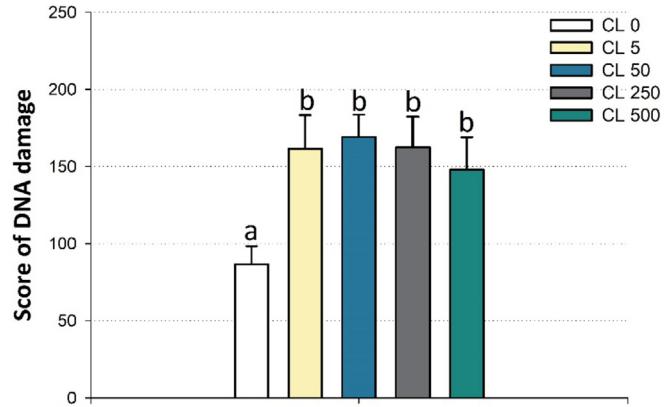


Fig. 5. Score of DNA damage in erythrocytes of *P. lineatus* kept under the control condition (CL0) or exposed to CL formulation at 5, 50, 250, and 500 ng L^{-1} . Data are mean \pm SEM, $n = 8$. Different letters indicate significant differences between groups ($p < 0.05$).

enzyme-SH groups and increases in H_2O_2 and/or superoxide radical (Ruas et al., 2008), although the SOD activity remained unchanged. However, some studies indicate that increased H_2O_2 levels resulting from CAT inhibition may ultimately inhibit SOD (Kono and Fridovich, 1983). When there is a CAT decrease, the organism depends on the GPx to eliminate H_2O_2 , which may have occurred in this situation, given the GPx increase at the highest CL concentration. Our results indicate the efficiency of the antioxidant system in combating the oxidative damage in the liver of *P. lineatus*. In contrast, Piner and Üner (2012) found that CL caused an increase in the total glutathione and GSH content and in the GST activity in the liver of *O. niloticus* juvenile specimens, although these defences were not sufficient to combat LPO. Alak et al. (2013) showed that the antioxidant enzyme activity in the hepatic tissue of rainbow trout was significantly increased after CL exposure. Dinu et al. (2010) found results similar to ours, with decreases in SOD and CAT and increases in GST, GPx, and LPO in the liver of *C. auratus* exposed to deltamethrin.

The gills were one of the organs most affected by CL exposure. Because the gills are in direct contact with the contaminants in the water, and due to the high lipophilicity of pyrethroids, high absorption of these compounds occurs through the gill surface (Mishra et al., 2005). Thus, this organ is highly sensitive to LPO induced by xenobiotics, and its antioxidant potential is weak in comparison to the other organs (Sayed et al., 2003). The GPx decrease in this organ at the highest CL concentration may indicate a deficiency in the defence against reactive oxygen species (ROS), resulting in H_2O_2 accumulation, which in turn can inhibit SOD activity, resulting in superoxide radical accumulation. Thus, these excess radicals could readily oxidize the membrane lipids, which culminated in the expressive LPO increase observed at CL₅₀₀. The GST increase at the highest CL concentration may be due to a higher ROOH production during LPO. Decreases in gill antioxidant defences were also observed in *C. punctatus* exposed to deltamethrin (Sayed et al., 2003), in *Labeo rohita* exposed to fenvalerate (Prusty et al., 2011), and in *B. amazonicus* exposed to cypermethrin (Moraes et al., 2018). Increased LPO has also been reported in the gills of fish exposed to deltamethrin and cypermethrin (Kaur et al., 2011; Amin and Hashem, 2012; Moraes et al., 2018).

Similar to the gills, the kidney is one of the first affected organs regarding exposure to contaminants since it receives most of the blood coming from the respiratory organs (Hinton, 1990). As observed in the gills, the kidney of *P. lineatus* exposed to CL₅₀₀ exhibited a GPx decrease with a concomitant LPO increase,

indicating that deficiency in the H₂O₂ neutralization led to the occurrence of oxidative stress in this organ.

The brain may also be subject to oxidative stress, given the high rate of oxidative metabolic activities and the high content of polyunsaturated fatty acids in its membranes (Hai et al., 1997; Fetoui et al., 2008). However, this organ did not present alterations in the antioxidant enzymes or LPO after CL exposure. The GST increase in the brain at the highest CL concentration seemed to have been enough to prevent LPO. Similar results were observed in juveniles of *P. lineatus* exposed to the insecticide imidacloprid, where increased GST at the highest concentrations of the neonicotinoid prevented LPO at the same concentrations (Vieira et al., 2018).

Although detoxification functions primarily occur in the liver, the skeletal muscles are also involved in these processes. Skeletal muscle cells have been shown to express different types of xenobiotic metabolizing enzymes, including cytochrome P450 and GST (Hussey et al., 1991; Bainy et al., 1999). Skeletal muscles of *P. lineatus* can be affected by CL exposure. Adaptive responses such as increased GST at CL₅₀₀ and increased CAT at the three highest CL concentrations due to an increase in ROS production prevented LPO in the skeletal muscles. In *C. carpio* kept in an irrigated rice cultivation system in southern Brazil for 100 days, CL accumulation, LPO, and protein oxidation were observed in the muscles, in addition to liver and gill damage (Clasen et al., 2018). Thus, the muscular tissue of fish can accumulate pyrethroids and consequently suffer biochemical changes due to a redox imbalance.

Pyrethroids are highly non-polar compounds and can thus accumulate in lipid-rich tissues. The most efficient way to increase the solubility of a carboxylic ester such as CL is by its hydrolysis catalysed by carboxylesterases (CbE) in alcohol and carboxylic acid molecules. These metabolites are more soluble in water and can be eliminated by urine. However, some authors suggest a deficiency in the enzymatic system of pyrethroid hydrolysis in fish, related to the small CbE amounts in these organisms and that bile would represent the main route for the excretion of hydroxylated pyrethroid conjugated products (Demoute, 1989; Bradbury and Coats, 1989; Haya, 1989; Wheelock et al., 2005). Studies conducted by Glickman et al. (1981) and Edwards et al. (1986) corroborate the hypothesis that in fish, pyrethroid metabolism occurs mainly through phase I reactions. However, in the present study, acute exposure to CL promoted increases in β-esterases in the liver and plasma of *P. lineatus* at all concentrations. These results may indicate the participation of these esterases in CL metabolism in *P. lineatus*, with particular importance for CbE. Fraga (2010) studied the distribution and characterization of CbE activity levels in three species of Neotropical fishes, including *Piaractus mesopotamicus*, *Leporinus macrocephalus*, and *P. lineatus*, and verified the presence of this enzyme at high quantities in the liver and plasma of the latter species. Therefore, our results suggest that esterases in *P. lineatus* may represent an important pathway in CL metabolism.

Similar to β-EST, increases in α-EST were observed in the liver at all CL concentrations, although no differences in enzyme activity were detected in the plasma. Among the α-ESTs are the paraoxonases (PONs), which exhibit protective action against cellular oxidative stress (Aviram and Rosenblat, 2004). PONs are a multi-enzyme complex with antioxidant properties that prevent increases in ROS, show protective effects on cell membranes (Aşkar and Büyükleblebici, 2012), and neutralize the effects of LPO.

AChE is another abundant β-EST in fish, responsible for acetylcholine (Ach) hydrolysis. Our results showed that CL formulation promoted reductions in muscle AChE activity at all concentrations evaluated. However, there was no enzymatic alteration in the brain. Inhibition of this enzyme leads to Ach accumulation in the synaptic cleft that may lead to disruption of nerve activity by the

hyperstimulation of postsynaptic ACh receptors. Since the main action site of the pyrethroids is the nervous system, their action may not be restricted only to voltage-dependent Na⁺ channels (Coats, 2008). Other studies have demonstrated AChE inhibition in CL-exposed fish, such as *O. niloticus* (Piner and Üner, 2014) and *C. punctatus* (Kumar et al., 2009), and exposure to other pyrethroids, such as cypermethrin (Reddy and Philip, 1994; Kumar et al., 2009), deltamethrin (Szegletes et al., 1995), and fenvalerate (Mushigeri and David, 2005).

A proposed mechanism of secondary pyrethroid action is related to osmoregulatory disorders since these compounds can affect calcium channels and CaATP and MgATP (Coats, 2008). In this study, all of the gill ATPases were affected by exposure to CL at its highest concentrations. CaATP inhibition was apparent starting from 50 ng·L⁻¹, while MgATP inhibition was observed at CL₂₅₀ and CL₅₀₀. In freshwater teleosts, Ca²⁺ homeostasis occurs in part through Ca²⁺ uptake from the surrounding environment, and the transbranchial movement of this divalent cation is particularly important, as it is believed to occur primarily in all mitochondrial-rich cells (Marshall, 2002; Evans et al., 2005). Although gill CATPase is not a limiting factor for the Ca²⁺ uptake in normal situations (Perry, 1997), the activity of this gill transporter is essential for the maintenance of plasma calcaemia levels. Our results showed that CaATPase inhibition resulted in hypocalcaemia at the highest CL concentration. Similarly, MgATP inhibition occurred at the highest CL concentration, although Mg²⁺ levels remained unchanged. In addition to the food pathway, Mg²⁺ can be actively absorbed in the gills through processes that have not yet been fully understood (Bijvelds et al., 1998; Marshall, 2002).

In contrast, a significant increase in NKA activity was observed in the gills of fish at CL₅₀₀. The sodium-potassium pump is responsible for the generation of the electrochemical gradient that favours Na⁺ entry by the apical membrane of the gill mitochondrial-rich cells (MRC), and a reduction in its activity should result in changes in this ion concentration (Ahern and Morris, 1998; Rogers et al., 2003, 2005). The osmoregulation data obtained after CL exposure allow us to infer that there was an increase in the branchial perfusion in response to stress, leading to higher water inflow, concomitant with ion losses. Ion data and the decrease in hematocrit demonstrated water gain in the animals, which may have contributed to the increase in the branchial NKA activity as an adaptive response, aiming to increase active Na⁺ uptake. Similar results were observed by Moraes et al. (2018) for the fish species *B. amazonicus* exposed to cypermethrin. According to the authors, increased branchial NKA may have been due to MRC proliferation as an effect of increased serum cortisol levels. Fish exposed to pyrethroids demonstrated secondary responses associated with increased cortisol, as observed in *C. gariepinus* exposed to deltamethrin (Datta and Kaviraj, 2003), in *Heteropneustes fossilis* exposed to cypermethrin (Saha and Kaviraj, 2008), in *Channa punctatus* exposed to CL and cypermethrin (Kumar et al., 2012), and in *C. carpio* exposed to cyfluthrin (Sepici-Dinçel et al., 2009).

In addition to all of these effects due to CL exposure, an increase in the DNA damage score in *P. lineatus* erythrocytes was detected at all concentrations, demonstrating the genotoxic effect of this pyrethroid even at low concentrations. Different studies report the genotoxic effects of pyrethroids on fish. Ansari et al. (2011) reported that cypermethrin has a clastogenic potential in *C. punctatus* and associated the genotoxic effect of this insecticide to ROS oxidative damage. DNA damage was evidenced in erythrocytes of *P. lineatus* exposed to cypermethrin by the comet test (Simoniello et al., 2009) and in hepatocytes of *Danio rerio* exposed to the same insecticide (Jin et al., 2011). In combination, these results support the genotoxic potential of pyrethroids in fish. These effects may reflect on carcinogenic diseases and morphological abnormalities, which affect

the physical fitness, adaptability, and survival of animals (Jha, 2008).

Our results indicate that *P. lineatus* was highly sensitive to the effects of a CL formulation. Despite the low CL concentrations and the short exposure time, this pyrethroid caused hematological adjustments, oxidative stress, osmoregulation disorders, and DNA damage in *P. lineatus*. Given the worldwide increase in the use of pyrethroids and considering the characteristics of these xenobiotics, an increase in CL environmental concentrations leading to chronic fish exposure is expected. The ecological consequences of this exposure can be drastic, as the reported shortcomings may be reflected in various ecological parameters related to behaviour, disease resistance, growth, and reproduction, which need to be investigated in future studies. Finally, CL metabolism mechanisms in *P. lineatus* should be better characterized in order to improve the knowledge of the toxic effects of pyrethroids in fish.

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