

Assessment of biomarkers in the neotropical fish *Brycon amazonicus* exposed to cypermethrin-based insecticide

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Abstract The effects of cypermethrin-based insecticide (CBI), commonly used in aquaculture and agriculture, were evaluated in matrinxa (*Brycon amazonicus*) exposed to sub-lethal concentration (20% of LC50) for 96 h. Physiological and biochemical effects were studied through biomarkers: lipid peroxidation (LPO), glutathione (GSH), and ascorbic acid concentrations; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glucose-6-phosphate dehydrogenase (G6PDH) assays in the liver and gills. Besides, ions Na^+ , Cl^- , and K^+ ; protein and glucose concentrations were measured in the plasma. Red blood cells count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), and hematimetric parameters were evaluated in the total blood. The NKA (Na^+/K^+ ATPase) activity was assayed in the gills. The histopathological effects of CBI were also investigated in the gills. The liver and gill LPO increased 62 and 100%, respectively. The tripeptide GSH concentration reduced in the liver and increased in the gill of exposed fish. The SOD and CAT activities increased in the liver, whereas CAT reduced in the gill. Liver also presented an increase in G6PDH activity. Plasma Na^+ and Cl^- and glucose concentrations increased in the exposed fish. Levels of Ht, Hb and RBC were significantly increased. The gill NKA activity also increased. Exposed fish exhibited histological alterations in the gills such as hypertrophy and proliferation of chloride cells, blood vessels dilation, aneurysms and hemorrhage of the lamella. The histological index indicated moderate to heavy

damage to the gills. CBI provokes liver and gills oxidative stress, gill structural damages, and ionic imbalance. A multi-biomarker approach allows us to see that *B. amazonicus* was unable to cope with CBI exposure.

Keywords Aquatic pollution · Pyrethroid · Hematology · Histology · Osmoregulation · Oxidative stress

Introduction

Cypermethrin ([RS]-alpha-cyano-3-phenoxybenzyl [1RS, 3RS; 1RS, 3SR]-3-[2,2-dichlorovinyl]-2,2-dimethylcyclopropanecarboxylate) is a type-II pyrethroid sold under multiple trademarks. The potent effect and low toxicity of pyrethroids to mammals are responsible for its growing use as an insecticide (Elliott 1976; Soderlund et al. 2002). These compounds are widely used against crop pests, domestic insects, and in aquaculture, against ectoparasites in farmed fish (Hart et al. 1997; SEPA 1998; EMEA 2003; ANVISA 2007; US EPA 2006). The recommended concentration of CBI against sea lice in salmon fish farms is a bath for 1 h ranging 5–15 $\mu\text{g L}^{-1}$ (SEPA 1998; EMEA 2003; Haya et al. 2005).

Despite cypermethrin has been reported to present extremely low potential to move in the soil and it is unlikely able to contaminate groundwater because it binds tightly to soil particles, cypermethrin is stable in sunlight (NPIC 1998). High environmental concentrations of cypermethrin have been reported in aquatic environments, such as 111 $\mu\text{g L}^{-1}$ in Cintra stream, Botucatu, Sao Paulo, Brazil (Belluta et al. 2010), and 194 $\mu\text{g L}^{-1}$ in Del Medio stream, an

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Arrecifes tributary to Parana river, Pampa Ondulada, Argentina (Marino and Ronco 2005).

The basic toxicity mechanisms of pyrethroids in fish are not totally clear but the nervous system is a target of pyrethroids and the xenobiotic metabolism can explain some physiological responses (Coats 2008). Most fish retain pyrethroids for periods longer than other animals, indicating that fish seem to be deficient in pyrethroid-hydrolyzing carboxylesterases (Haya 1989; Coats 2008). Furthermore, these chemicals can be accumulated in fish eleutheroembryos stage (Tu et al. 2014). Pyrethroids alter the permeability of voltage-dependent Na^+ -channels of nerve cells, resulting in repetitive discharges, synaptic disruption, and hyper excitability (Narahashi 1996; Soderlund et al. 2002).

Albeit pyrethroids are considered nontoxic to birds and mammals, these chemicals seem to be highly toxic to fish. For example, neotropical fish pacu *Piaractus mesopotamicus* and matrinxá *Brycon amazonicus* are some reported species very sensitive to pyrethroids, with LC_{50} below 0.01 mg L^{-1} (Moraes et al. 2013; Bacchetta et al. 2014). Despite this, we have few studies about pyrethroid sub-lethal effects in fish. Deleterious effects in fish exposed to sub-lethal concentrations of pyrethroids were reported recently, and include hematological and osmoregulatory alterations (Suvetha et al. 2010; Al-Ghanbousi et al. 2012), embryonic development disorders (Dawar et al. 2016), gill histological injuries (Korkmaz et al. 2009; Al-Ghanbousi et al. 2012; Arslan et al. 2017), behavioral changes (Bonansea et al. 2016), and alterations in cell oxidant status (Sayeed et al. 2003; Ansari et al. 2011; Piner and Üner 2012; Ensibi et al. 2013; Dawar et al. 2016; Mu et al. 2017). A set of biological responses of fish can be an early indicator of pollution and damages to organism. These biological responses, called biomarkers, occur from molecular to organismal levels, including molecular, biochemical, physiological and structural alterations (Pickering and Pottinger 1995).

It is known that cypermethrin can be dangerous to aquatic organisms, and its potential for aquatic contamination is recognized from direct and non-direct applications. Nevertheless, it was not possible to quantify the current risks due to lack of available data and acceptable models (US EPA 2006). In this way, additional information is needed for understanding the effects of pyrethroids in fish, and to design strategies for preventing of the adverse effects, especially in farmed species. Investigations on sub-lethal effects of CBI in *B. amazonicus*, a prominent farmed fish in South America, were carried on by examining a range of biochemical and physiological responses related to oxidative stress, osmoregulation, hematology, and histopathology.

Materials and methods

Experimental animals

Juveniles matrinxá (*B. amazonicus*; $45.8 \pm 17.8 \text{ g}$ and $15.14 \pm 1.78 \text{ cm}$) were obtained from a local fish farm Polettini (Mogi-Mirim, SP, Brazil) and maintained in 2000L tanks with running, filtered, aerated ($\text{P}_{\text{O}_2} = 5.7 \pm 0.4 \text{ mg L}^{-1}$) water at $25.5 \pm 0.5 \text{ }^\circ\text{C}$, $\text{pH} 7.4 \pm 0.3$, alkalinity ($28.5 \pm 0.3 \text{ mg L}^{-1} \text{ CaCO}_3$), hardness ($18.0 \pm 0.1 \text{ HCO}_3^-/\text{CO}_3^{2-}$), and ammonia ($0.8 \pm 0.2 \text{ mg L}^{-1}$). The fish were fed with commercial pellets under 12 h dark: 12 h light photoperiod and let for 1 month to be acclimated.

Chemicals

Galgotrin[®] (250 g L^{-1} cypermethrin as the only active ingredient, CAS number 52315-07-8) was supplied by Milenia Agrociencias S.A. (Londrina, PR, Brazil). The other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Experimental design

Thirty acclimated fish were randomly divided into six 250 L fiberglass tanks. Twenty-four hours before exposing to cypermethrin based-insecticide (CBI), feeding was discontinued and the fish were kept fasted until over the experimental span. The fish were exposed to $7.2 \mu\text{g L}^{-1}$ of CBI (20% LC_{50} ; 96 h) (Moraes et al. 2013) against a CBI-free water (control) for 96 h in a static system. This CBI concentration is close of reported environmental concentration (Marino and Ronco 2005; Belluta et al. 2010), and that used in salmon fish farms against sea lice, in which is recommended a bath for 1 h into $5\text{--}15 \mu\text{g L}^{-1}$ CBI solution (SEPA 1998; EMEA 2003; Haya et al. 2005). The CBI exposure was a single-pulse type into a sublethal concentration, which simulates what usually occurs in the environment. The degradation and stability of pyrethroids depend on the environmental conditions. WHO (1989) reported that persistence (half life) of cypermethrin in natural water is 14 days. For Vieira et. al. (2007), the persistence of cypermethrin is 5 days.

A test solution of CBI was prepared with water from experimental tanks. This solution was diluted directly in the experimental system with acclimated fish. The cypermethrin concentration was calculated according to active ingredient presented in a commercial formulation. Each experimental conditional (control and exposed group) were performed in triplicates. It means that three tanks of control had five fish each, and three tanks of exposed group had five fish each,

totalizing fifteen fish per experimental condition. Water quality for control group was $P_{O_2} \sim 5.74 \pm 0.06 \text{ mg L}^{-1}$, $25.6 \pm 0.4 \text{ }^\circ\text{C}$, $\text{pH } 7.4 \pm 0.04$, alkalinity ($25.2 \pm 2.2 \text{ mg L}^{-1} \text{ CaCO}_3$), hardness ($19.6 \pm 1.2 \text{ HCO}_3^-/\text{CO}_3^{2-}$), and ammonia ($0.6 \pm 0.2 \text{ mg L}^{-1}$); and for exposed group was: $P_{O_2} \sim 5.90 \pm 0.15 \text{ mg L}^{-1}$, $25.0 \pm 0.5 \text{ }^\circ\text{C}$, $\text{pH } 7.2 \pm 0.1$, alkalinity ($22.0 \pm 3.1 \text{ mg L}^{-1} \text{ CaCO}_3$), hardness ($19.6 \pm 1.9 \text{ HCO}_3^-/\text{CO}_3^{2-}$), and ammonia ($0.8 \pm 0.2 \text{ mg L}^{-1}$).

After exposure, fish were anesthetized in 40 mg L^{-1} eugenol bath (Inoue et al. 2003), blood was withdrawn from the caudal vein with heparinized syringes, and fish were killed by pinching the spinal cord. Liver and gills were excised, quickly frozen in liquid nitrogen, and kept at $-80 \text{ }^\circ\text{C}$ for biochemical assays. Three branchial arches of four fish per experimental group were sampled for morphological analysis.

Tissue preparation

Blood samples were divided into aliquots to hematological determinations or centrifuged at $13,400 \times g$ for 3 min and the supernatant plasma was separated for biochemical analyses.

Liver and gill pieces nearly 50 mg were homogenized in 0.5 mL of 0.1 M $\text{K}_2\text{HPO}_4/0.25 \text{ M}$ sucrose buffer (1:2) pH 7.0, under ice-bath, with a Turrax[®] rotative motor driven homogenizer by two strokes at 5000 rpm for 30 s under ice-bath. Homogenates were centrifuged at $15,000 \times g$ for 10 min at $4 \text{ }^\circ\text{C}$ and supernatant was used as crude enzyme source. Aliquots of crude enzyme source were mixed 1:1 into 12% TCA, centrifuged at $5000 \times g$ for 10 min and 0.1 mL of supernatants were used to LPO determination.

Branchial filaments were homogenized in pH 7.4 Sacarose-EDTA-Imidazole (SEI) buffer with a Turrax[®] homogenizer as reported above, and the homogenates were centrifuged at $10,000 \times g$ for 5 min at $4 \text{ }^\circ\text{C}$ and the supernatants were used to assay Na^+/K^+ ATPase.

Ascorbate was quantified in acidic tissue extracts as follows. Tissue pellets of liver and gill were disrupted into 5% TCA at 1:20 and 1:40 (tissue: acid) ratio, respectively, in a Turrax[®] homogenizer as reported above. The tissue extracts were centrifuged at $13,500 \times g$ for 3 min at $4 \text{ }^\circ\text{C}$, and the supernatants were used as tissue extracts. Reduced glutathione concentration was determined in neutral extracts made as reported above but replacing 5% TCA by 0.2 M Na_2HPO_4 buffer pH 7.0 and a tissue: buffer ratio of 1:20.

Three branchial arches of four fish per experimental group were dipped into 2.5% glutaraldehyde, buffered with 0.1 M sodium phosphate pH 7.3, to be fixed for histological preparations.

Oxidative stress biomarkers

Lipid peroxidation was assayed in liver and gills through FOX - Fe^{2+} oxidation in the presence of xylenol orange (Jiang et al. 1992). Hydro-peroxide concentrations were determined at 560 nm against a 100 nmol standard solution of cumene hydro peroxide (CHP) and expressed in μmol per gram of wet tissue.

Superoxide dismutase (SOD) activity was determined in liver homogenates through auto-oxidation of pyrogallol, which is inhibited in the presence of SOD. Reactions were followed at 420 nm for 2 min. One unit (1.0 IU) of SOD corresponded to the enzyme amount needed to inhibit the auto-oxidation of 50% of pyrogallol (Beutler 1984).

Catalase (CAT) activity was estimated in liver and gill homogenates through continuous decay of H_2O_2 concentration at 230 nm (Beutler 1984). An aliquot of enzyme crude source was previously treated with 95% ethanol (1:50) to avoid reversion of enzyme activity. The optical density was follows for 1 min at 230 nm. Molar Extinction Coefficient (MEC) of H_2O_2 was $\epsilon_o = 0.071 \text{ (mM}\cdot\text{cm)}^{-1}$ and enzyme activity was expressed in units per mg of protein. One unit (1.0 IU) of CAT was defined as the enzyme amount needed to reduce 1.0 μmol of H_2O_2 per minute.

Glutathione peroxidase (GPx) activity was determined in liver and gill homogenates in a reaction coupled with GR activity and followed by extinction of reduced β -NADPH at 340 nm (Beutler 1984). Optical extinction of β -NADPH was followed at 340 nm for 1 min and MEC of β -NADPH was $\epsilon_o = 6.2 \text{ (mM}\cdot\text{cm)}^{-1}$. Specific GPx activity was expressed in units per mg of protein and one unit (1.0 IU) of the enzyme was defined as the enzyme amount required to oxidize 1.0 μmol of β -NADPH per minute.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined in liver and gill homogenates throughout NADP^+ reduction followed at 340 nm (Beutler 1984). Extinction of NADP^+ was followed at 340 nm for 2 min and the specific activity of G6PDH was expressed in units per mg of protein. One unit (1.0 IU) of G6PDH was defined as the enzyme amount required for reducing 1.0 μmol of NADP^+ per minute.

Ascorbate was determined in liver and gill TCA extracts. Ascorbate concentration was read at 524 nm against a standard ascorbic acid solution (Carr et al. 1983) and expressed in μmol per gram of tissue.

Reduced glutathione concentration was determined in neutral extracts of liver and gills through the anion thionitrobenzoate from dinitrothiobenzoate (DTNB) as a reagent (Beutler 1984).

Hematology

Microhematocrit (Ht%) was determined with glass capillaries; total hemoglobin (Hb g dL⁻¹) was determined according to Drabkin (1948), and Red Blood Cells (RBC 10⁶ cells mm⁻³) were counted in a Neubauer chamber. The hematimetric parameters: mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Wintrobe (1934).

Plasma metabolites and osmoregulatory biomarkers

Plasma glucose concentration was colorimetrically determined in microplates at 525 nm, through glucose oxidase LabTest Kit (Trinder 1969).

Tissue total protein and plasma protein were colorimetrically determined at 620 nm in microplates with Bradford reagent (Kruger 1994).

Sodium and potassium concentrations were determined in plasma diluted in distilled water (1:100 v/v) against 140 mEq Na⁺ and 5.0 mEq K⁺ standard solutions (ref. DM-S 13A) in a flame photometer Digimed, model DM-61. Plasma chloride concentration was read at 480 nm (APHA 1980) against a standard solution 1.0 mM of NaCl after plasma dilution in water at 1:100 v/v.

Na⁺/K⁺ ATPase (NKA) activity was determined in branchial filaments. The enzyme activity was read at 620 nm and expressed in $\mu\text{M Pi mg protein}^{-1} \text{ h}^{-1}$ (Quabius et al. 1997).

Gill histology

Gill samples fixed in glutaraldehyde were dehydrated in ascending series of ethanol (70-95%) for 1 h and then embedded in ethanol 95% plus historesin (Leica) for 4 h. The samples were left overnight in pure historesin (Leica) to inclusion. Histological sections (3 μm thick) were performed in a microtome (HM 360 MICRON), stained with toluidine blue and examined under a light microscope (BX 51, Olympus, Denmark). The histopathological analyses were done using a randomized blind method in which a trained investigator did not know if the histological sections were from control or exposed group. So, the alterations were evaluated through the Histopathological alteration Index (HI), according to Poleksic and Mitrovic-Tutundzic (1994). It follows the formula: $\text{HI} = 10^0 \sum\text{I} + 10^1 \sum\text{II} + 10^2 \sum\text{III}$, where $\sum\text{I}$, $\sum\text{II}$, and $\sum\text{III}$ refer the sum of total alterations in the stages I, II, and III, respectively. Stage I is not severe injury; Stage II is moderate to severe injuries that can affect the organ function; and Stage III is very severe and irreversible injuries that alter the organ function. The potential factors (10⁰, 10¹ e 10²) are related to the severity

of injury (Poleksic and Mitrovic-Tutundzic 1994). The average HI value was classified into five categories: HI (1–10) normal function of gills; HI (11–20) slightly to moderately damaged gills; HI (21–50) moderately to heavily damaged gills; HI (51–100) severely damaged gills; and (HI > 100) irreversibly damaged gills.

Statistics

Each experimental condition (exposed and control) was performed in triplicate (3 control tanks and 3 exposure tanks) with five fish per tank, totalizing 15 fish per experimental condition. The normality of data was evaluated through the Kolmogorov-Smirnov test followed by the parametric test *t* of Student or the non-parametric test of Mann-Whitney as applicable. Differences between means were accepted as significant at $P < 0.05$. The parameters are expressed as mean \pm standard error of the mean (SEM) for $n = 15$. The histological alterations index is presented as mean value \pm SEM for $n = 4$.

Results

Hepatic LPO in fish exposed to the CBI was 62% greater than control and two-fold higher in the gills (Table 1). Antioxidant defenses displayed alteration in consequence of CBI exposure. The increase of CAT, SOD and G6PDH activities and the decrease of GSH were observed in the liver ($P < 0.05$). The activity of GPx and concentration of ascorbic acid in liver were apparently unresponsive to CBI (Table 1). In the gills, decrease of CAT activity ($P < 0.05$) was observed, but GPx and G6PDH were kept steady. Concentration of ascorbic acid remained constant but GSH increased 25% in the gills of fish exposed to CBI (Table 1).

Histopathological alterations were observed in the gills of *matrinxa* exposed to CBI (Fig. 1). Histological alterations of stage I and II were the most pronounced (Table 2). Hemorrhages with rupture of epithelium (Fig. 1e), hypertrophy and proliferation of chloride-rich cells (Fig. 1g), blood vessels dilation (Fig. 1h) and aneurysms (Fig. 1i and j), were frequently observed. Alterations of stage III were not observed. Moderate to heavy damages (HI 25.5 ± 5.8) were observed in gills of fish exposed to CBI in comparison with normal structures (HI 9.5 ± 0.5) ($P < 0.05$).

Concentrations of sodium, chloride and glucose increased in the plasma of fish exposed to CBI, but the levels of potassium and protein did not change significantly ($P < 0.05$). The activity of NKA in the gills increased 30% in the exposed fish ($P < 0.05$) (Table 1). The blood variables Ht, Hb, and RBC also increased in fish exposed to CBI (Table 3) ($P < 0.05$).

Table 1 Biochemical variables of liver and gill of *Brycon amazonicus* exposed to sub-lethal concentration (7.2 µg L⁻¹) of CBI (Cypermethrin-Based Insecticide) Galgotrin® for 96 h

Variable	Liver		Gill	
	Control	CBI	Control	CBI
LPO	1.0 ± 0.06	1.6 ± 0.18*	0.3 ± 0.03	0.7 ± 0.12*
CAT	311.4 ± 13.9	347.7 ± 15.8*	20.3 ± 0.7	17.9 ± 0.4*
GPx	1.0 ± 0.11	0.9 ± 0.09	0.05 ± 0.005	0.05 ± 0.005
G6PDH	0.030 ± 0.0007	0.038 ± 0.002*	0.034 ± 0.002	0.029 ± 0.002
SOD	2.22 ± 0.07	2.45 ± 0.07*	–	–
GSH	8.17 ± 0.43	5.95 ± 0.20**	2.73 ± 0.15	3.61 ± 0.20*
Ascorbic acid	0.87 ± 0.02	0.90 ± 0.02	0.76 ± 0.03	0.80 ± 0.04
NKA	–	–	0.15 ± 0.01	0.2 ± 0.01*

The data are represented as mean ± SEM for $n = 15$. The LPO is represented in µmol cumene hidroperoxide-CHP (g of tissue)⁻¹; CAT (Catalase), GPx (glutathione peroxidase), G6PDH (glucose-6-phosphate dehydrogenase) and SOD (superoxide dismutase) are expressed in IU (mg protein)⁻¹; GSH (Glutathione) and ascorbic acid concentrations are expressed in µmol (g of tissue)⁻¹; NKA (Na⁺/K⁺ ATPase) activity is expressed in µM Pi (mg protein · h)⁻¹; (*) indicates differences between control and exposed group at $P < 0.05$ or (**) $P < 0.001$

Discussion

There has been an alarming increase in the use of chemotherapeutical agents in fish farming, including pyrethroids. Indiscriminate use of them can lead to several unpredictable consequences to non-target organisms. This study has evaluated a range of biomarkers in the freshwater fish *B. amazonicus* exposed to a sub-lethal concentration of cypermethrin-based insecticide.

Oxidative stress is a complex phenomenon resulting in cell toxicity (Di Giulio and Meyer 2008). When the levels of pro-oxidants increase or antioxidants fall, oxidative stress is ensued that may cause serious cell damages such as peroxidation of unsaturated lipids (Gutteridge 1995; Kelly et al. 1998). The level of LPO has been included into the most consistent oxidative stress biomarkers (Gutteridge 1995; Di Giulio and Meyer 2008). High levels of LPO in the liver and gills of *B. amazonicus* indicate oxidative stress from exposure to CBI. Similar findings have been reported in liver of *Oreochromis niloticus* and *P. mesopotamicus* exposed to lambda-cyhalothrin (Piner and Üner 2012; Bacchetta et al. 2014); and in the liver, gills and kidney of *O. niloticus* exposed to deltamethrin (Abdelkhalek et al. 2015). The high levels of LPO observed in the gills of *B. amazonicus* can be attributed to the initial impact of exposure of such structures. Gills, along with skin and gut, are the first structures affected by environmental chemicals and play an important role in the absorption of xenobiotics (Kleinow et al. 2008). In spite of the responsiveness of gills and liver to the exposure to CBI, the antioxidant defense system in *B. amazonicus* was inefficient to cope with this xenobiotic. The observed level of biochemical responses in matrinxá is worrying, especially considering the low experimental concentration of cypermethrin. Another hypothesis for the antioxidant enzymes be not effectively

responding is that a cell damage caused by LPO, previously to 96 h, was already installed. The cellular toxic effects of LPO at high levels are destabilization of organelles membranes, decay of membrane potential, extrusion of lysosomal enzymes to cytoplasm, increase of cell membrane permeability, fluidity decrease, and inhibition of membrane enzymes (Gutteridge 1995; Di Giulio and Meyer 2008), all of which bring drastic biological consequences. A chronic oxidative stress due to long-term exposure to environmental stressors could influence cellular physiology, leading to impairment of the fitness of animal (Jha 2008).

The cooperative action SOD-CAT may reduce harmful, oxidative effects of O₂^{•-} by forming H₂O and O₂ (Kono and Fridovich 1982). Despite protective mechanism against effects from ROS, damages can come of hydroxyl radicals (OH[•]) from the Fenton and Harber-Weiss reaction. The ROS effects may explain the high levels of LPO observed in the liver of *B. amazonicus* despite enhancement of the SOD-CAT system activity, concurring with the hypothesis that matrinxá was unable to cope sufficiently with CBI exposure.

Although an oxidative stress caused by pesticides is able to over-express enzymes of antioxidant defense mechanisms, it is also responsible for the inactivation of such enzymes and consequently decrease the cell antioxidant potential (Lushchak 2011). An in vitro inhibitory effect of O₂^{•-} over CAT activity has been previously reported (Kono and Fridovich 1982). Antioxidant systems in gills of *B. amazonicus* responded poorly to the CBI. An already established cell damage provoked by LPO or a presumed increase of ROS could have inhibited the CAT activity in the gills of matrinxá. A decrease of CAT activity has also been reported in gills of *C. punctatus* and *O. niloticus* exposed to deltamethrin (Sayeed et al. 2003; Abdelkhalek et al. 2015). In addition, gill GPx was unresponsive in

Fig. 1 Representative sagittal sections of gill filament of *B. amazonicus* exposed to sub-lethal concentration ($7.2 \mu\text{g L}^{-1}$) of Cypermethrin-Based Insecticide (CBI) Galgotrin® for 96 h. **a.** Normal gill structure (see abbreviations); **b.** Black arrows indicate hypertrophy of the lamellar epithelia; **c.** Asterisks indicate hyperplasia of the gill filament epithelium; **d.** Asterisks indicate hyperplasia of the gill lamellar epithelia and black arrow indicates fusion of several lamellae; **e.** Black arrows indicate epithelial lifting of the lamellae; **f.** Black arrows indicate hypertrophy and hyperplasia of mucous cells and red arrow indicates empty mucous cells; **g.** Black arrows indicate hypertrophy and hyperplasia of chloride cells; **h.** Black arrows indicate filament blood vessel enlargement; **i.** Black arrows indicate apical aneurysms; **j.** Black arrow indicates aneurysm. CMar marginal channel, CPV pavement cell, E erythrocytes, CPi pillar cell, CC chloride cell, L lamellae, F filament. Scale bar = 20 μm

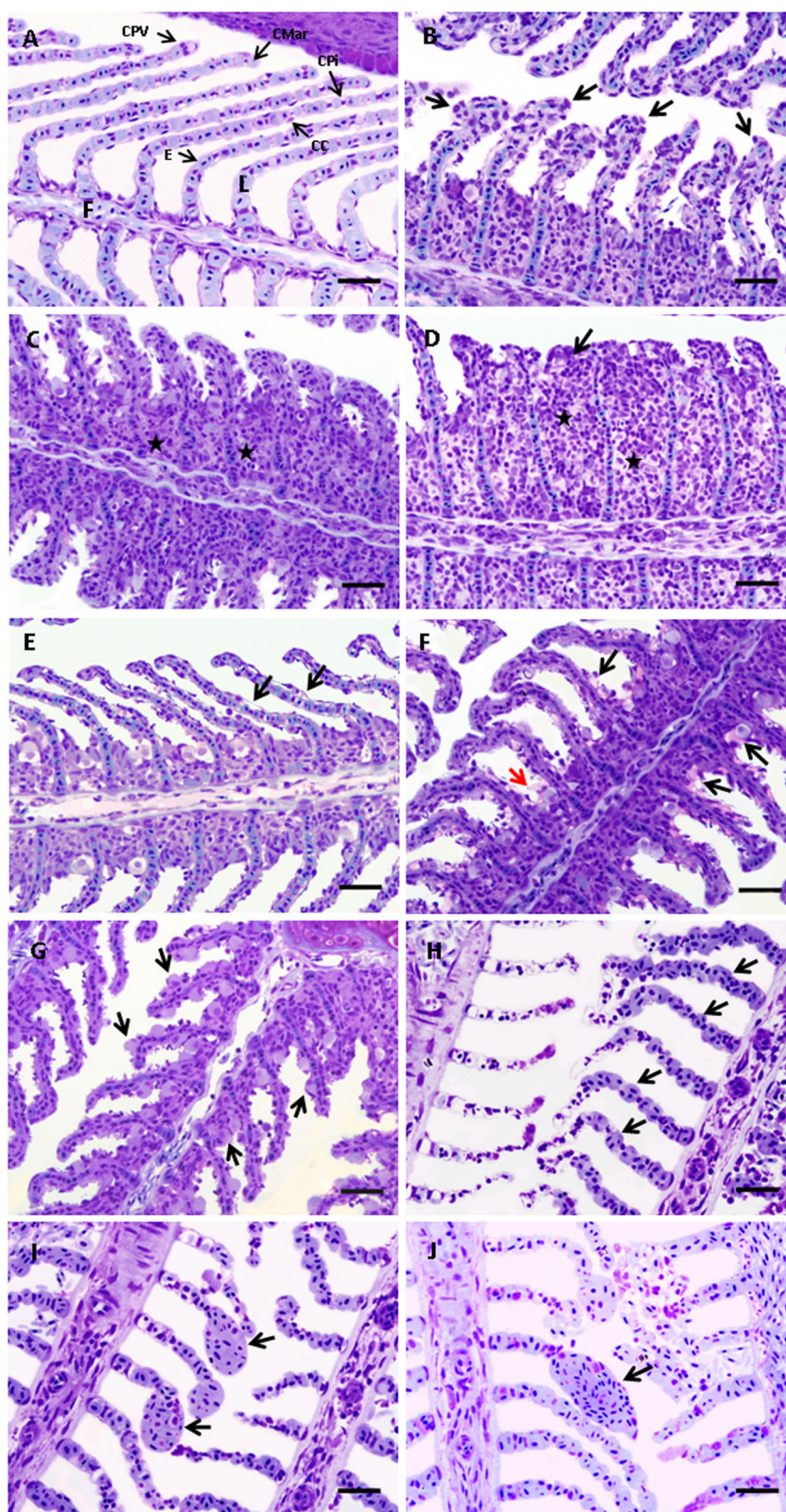


Table 2 Histopathology of gills of *Brycon amazonicus* exposed to sub-lethal concentration ($7.2 \mu\text{g L}^{-1}$) of cypermethrin-based Insecticide (CBI) Galgotrin[®] for 96 h ($n = 4$)

	Histopathological alterations	Stage	Control	CBI
Hypertrophy and hyperplasia of gill epithelia	Hypertrophy of the lamellar epithelia	I	+	+
	Hyperplasia of the gill filament epithelium	I	+	+
	Hyperplasia of the gill lamellar epithelia	I	+	+
	Decrease of inter-lamellar space	I	+	+
	Epithelial lifting of the lamellae	I	0+	0+
	Epithelial lifting of gill filament epithelium	I	0	0
	Incomplete fusion of several lamellae	I	0+	0+
	Complete fusion of several lamellae	I	0+	0+
	Complete fusion of all the lamellae	II	0	0
	Rupture and peeling of gill filament epithelium	II	0	0
	Rupture of the lamellar epithelium	II	0	0
Changes in mucous and chloride cells	Hypertrophy and hyperplasia of mucous cells	I	+	0+
	Empty mucous cells or their disappearance	I	+	0
	Hypertrophy and hyperplasia of chloride cells	I	+	++
Blood vessel changes	Filament blood vessel enlargement	I	0+	++
	Apical aneurysms	I	0+	+
	Hemorrhages with rupture of epithelium	II	0	0+
	Aneurysms	II	0	0+
Terminal stages	Fibrosis	III	0	0
	Focal necrosis	III	0	0
	Total necrosis	III	0	0

0 = absent; 0+ = rarely present; + = frequent; ++ = very frequent; +++ = extremely frequent

Table 3 Hematological and plasma variables of *Brycon amazonicus* exposed to sub-lethal concentration ($7.2 \mu\text{g L}^{-1}$) of the CBI (cypermethrin-based Insecticide) Galgotrin[®] for 96 h

Variable	Control	CBI
Blood		
Ht (%)	26.0 ± 0.9	29.4 ± 0.5 *
Hb (g 100 mL^{-1})	9.00 ± 0.07	9.80 ± 0.05 *
RBC (10^6 of cells mm^{-3})	2.20 ± 0.07	2.70 ± 0.07 *
MCV (μmm^3)	119.57 ± 6.45	114.08 ± 6.15
MHC (pg cell^{-1})	39.02 ± 1.62	38.90 ± 2.68
MCHC (g dL^{-1})	33.65 ± 1.88	34.33 ± 1.67
Plasma		
Glucose ($\mu\text{mol mL}^{-1}$)	3.30 ± 0.05	3.70 ± 0.05 *
Protein (mg mL^{-1})	20.62 ± 0.69	21.07 ± 0.66
Sodium (mEq L^{-1})	156.5 ± 3.3	172.1 ± 4.8 *
Potassium (mEq L^{-1})	1.61 ± 0.08	1.70 ± 0.09
Chloride ($\mu\text{mol mL}^{-1}$)	173.70 ± 3.15	184.30 ± 3.54 *

Data are presented as mean \pm SEM for $n = 15$. Ht hematocrit, Hb total hemoglobin, RBC red blood cells counting, MCV mean corpuscular volume, MHC mean hemoglobin concentration, MCHC mean corpuscular hemoglobin concentration: (*) indicates significant difference between control and treatment ($P < 0.05$)

matrinxa, likely worsening the LPO effects. That enzyme is responsible for decreasing the levels of H_2O_2 and lipid membrane-not-bound hydro peroxides (Di Giulio and Meyer 2008).

The redox pair glutathione disulfide-glutathione (GSSG-GSH) is a crucial coupling reaction to reduce the oxidative stress level (Di Giulio and Meyer 2008). Concentrations of these co-substrates can be changed depending on factors such as the level of ROS or the direct inhibitory effect of pesticides (Lushchak 2011). The decrease of hepatic GSH in *B. amazonicus* must be evaluated in consonance with the enhancement of G6PDH activity. This enzyme activity is directly linked to $\text{NADPH} + \text{H}^+$ production which is fundamental to GSH regeneration from GSSG. Hepatic GSH and G6PDH profile of *B. amazonicus* is a clear signal of the detrimental effects of the exposure to CBI. In spite of the increased G6PDH activity, this was not enough to supply the hepatic GSH demand. Nevertheless, an increase of GSH has been observed in the liver of *C. punctatus* and *O. niloticus* exposed to deltamethrin and lambda-cyhalothrin, respectively (Sayeed et al. 2003; Piner and Üner 2012). The GSH increase in the gill might be understood when taking into account the role of this molecule in several metabolic

paths, along with the high degree of LPO observed in that tissue.

Histological changes observed in gills of *B. amazonicus* exposed to CBI can be due to defense/adaptive mechanisms or tissue damages. The HI (histopathological index) from histological ranking indicates that morphological alterations of exposed fish were moderate to severe. This level of alterations is reported to *O. niloticus* and *Cyprinus carpio* exposed to cypermethrin (Korkmaz et al. 2009; Arslan et al. 2017). Exposure of matrinxá to CBI in the present experimental condition led fish to a stressful condition inferred from the increase of plasma glucose, a classical stress response. Blood vessel enlargement observed in the *B. amazonicus* can be resultant of catecholamine release, commonly observed in stressful situations (Pickering and Pottinger 1995). High levels of catecholamines increase lamellar perfusion in order to amplify the oxygen uptake; however, this fact can also enhance ions permeability through the gills (Pickering and Pottinger 1995; Perry 1997) altering the plasma ion concentration, as observed. Although this histological alteration seems to be adaptive, they can result in histological injuries, such as aneurysms and hemorrhages (stage II), observed in the exposed fish to CBI. It is expected that chronic exposure could be more deleterious to fish, leading to irreversible alterations, since a short-term exposure (96 h) already provoked severe alterations. Aneurysms have been also reported in *Aphanius dispar* exposed to deltamethrin (Al-Ghanbousi et al. 2012) and in *O. niloticus* exposed to cypermethrin (Korkmaz et al. 2009). Hypertrophy and hyperplasia of chloride cells (CC) likely occurred in order to compensate an increase of gills permeability from exposure to CBI. Proliferation of CC is commonly observed in stressful situations and could reestablish the ionic balance by increasing ion uptake on the gill epithelia (Perry 1997). Moreover, CC hyperplasia is a defense mechanism to prevent xenobiotic intake, although augmented water-blood barrier can impair gas exchange (Perry 1997). Therefore, enlargement of blood vessels observed in *B. amazonicus* previously discussed can be a physiological response to compensate any impairment of gas exchange. The absence of the empty mucous cells observed in the gills of exposed fish would facilitate the CBI intake, whereas the mucus is often secreted by fish to minimize the irritant toxic effect of pollutant (Verma et al. 1980; Pickering and Pottinger 1995; Kan et al. 2012). High frequency of mucous cells in the gills has been reported in *O. niloticus* and *A. dispar* exposed to deltamethrin (Al-Ghanbousi et al. 2012; Kan et al. 2012).

The proliferation of CC in the gills was likely responsible for increased NKA activity and consequently increased Na^+ intake in *B. amazonicus* exposed to CBI. In order to achieve the electrolytic balance, the Cl^- intake through CC was increased. Thus, the sub-lethal concentration of CBI

provokes an osmoregulatory disorder in matrinxá. An increase of plasma Na^+ concentration is also observed in jundiá *Rhamdia quelen* exposed to cypermethrin (Borges et al. 2007). Otherwise, *C. carpio* exposed to cypermethrin depicts a decrease of NKA activity and Na^+ and Cl^- concentrations due to osmoregulatory alterations and direct action of pyrethroids on gill ATPase (Suvetha et al. 2010).

The hematological changes in *B. amazonicus* exposed to CBI was an adaptive response, indicating increased availability of red blood cells from either a spleen contraction or augmented erythropoiesis, commonly observed in stressed fish (Pickering and Pottinger 1995). This hematological response would compensate a potential decrease of oxygenation in consequence of gill morphological changes able to impair the oxygen uptake. *H. fossilis* exposed to deltamethrin depicts erythropoiesis (Kumar et al. 1999), *R. quelen* exposed to cypermethrin (Cypergold[®]) shows an increase of hemoglobin concentration (Borges et al. 2007), and *A. multispinis* exposed to deltamethrin increases RBC and hemoglobin concentration (Pimpão et al. 2007).

These are the first insights about biochemical, physiological and morphological aspects of *B. amazonicus* exposed to CBI at concentration lower than those observed in aquatic environments (Marino and Ronco 2005; Belluta et al. 2010) but that are used in fish farming (SEPA 1998; EMEA 2003; Haya et al. 2005). These sub-individual level effects can be related to putative effects at upper levels of biological organization because the energetic cost to cope with oxidative damage and the shift on energy demand from regular life cycle activities (mating, searching for food and shelter, etc) to detoxification processes could lead loss of fitness, and consequently to population impairments. Also, oxidative stress induced by cypermethrin are related to DNA damage in fish (Ansari et al. 2011; Kan et al. 2012), and the genotoxic effects can reflect to carcinogenic diseases and morphological abnormalities, which affect the fitness, adaptability, and survivorship of animals (Jha 2008).

In conclusion, this study presents some inferences on tissular poisoning mechanisms of the fish exposed to CBI, bringing attention to abuse or indiscriminate use of pyrethroids in fish farming. In spite of the low and sub-lethal concentration of CBI and the short exposure period, this xenobiotic provokes oxidative stress, gill morphological impairments, osmoregulation disorders, and hematological adjustments in *B. amazonicus*. *B. amazonicus* is not able to cope with CBI, even at low concentration. The impairments reported can be reflected to several ecological parameters related to reproduction, disease resistance, and growth rates, which need to be investigated in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures and experimental conditions involving animals were in accordance with the ethical standards and was approved by the Ethics Committee for Animal Research of the Federal University of Sao Carlos, under the license number CEUA 056/2011.

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