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Exposure to aluminum, aluminum + manganese and acid pH triggers different antioxidant responses in gills and liver of *Astyanax altiparanae* (Teleostei: Characiformes: Characidae) males

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ABSTRACT

Exposure to aluminum (Al) and aluminum + manganese (Mn) can trigger an increase in reactive oxygen species (ROS) and modify the activity of oxidative defense enzymes. This study investigated whether exposure to Al and Al + Mn at acid pH for 24 and 96 h causes oxidative stress evidenced by antioxidants and oxidative damage in the gills and liver of sexually mature *Astyanax altiparanae* males. The fish were subsequently immersed in metal-free water for 24 and 96 h to see whether they recovered from the effects of these metals. Exposure to an acid pH boosted the activity of gill superoxide dismutase (SOD) at 96 h and the fish did not recover when immersed for the same period in water at neutral pH. Exposure to Al increased glutathione (GSH) levels (24 h) in the gills, returning to control levels during the recovery period, showing the efficiency of the antioxidant system in preventing lipid peroxidation of the gills and liver. Mn did not modify the activity of the enzymes studied, but did trigger late hepatic lipid peroxidation during the recovery period. The group exposed to Al + Mn exhibited several alterations, including increased concentration of GSH, as well as higher GPx and GR activity in the gills. Despite the defensive responses triggered by acute exposure, during the recovery period there were alterations in catalase (96 h) and an increase in hepatic metallothionein (24 h), but this did not prevent hepatic lipid peroxidation. Al and Al + Mn produced different effects, and the timing of enzymatic and non-enzymatic antioxidant defenses also differed.

1. Introduction

In recent years, the levels of various metals, such as aluminum (Al) and manganese (Mn), have increased in the ecosystem, affecting the health of aquatic organisms (Aravind and Prasad, 2003; Basha and Rani, 2003; Brumbaugh et al., 2005; Atli et al., 2006; Al-Ansari et al., 2010; Correia et al., 2010; Narcizo et al., 2010). Al and Mn are abundant in the earth's crust and both have applications in the daily life of humans (Emsley, 1991). While Mn is an essential metal and accumulates predominantly in the mitochondria, mainly as Mn^{2+} through the Ca^{2+} channel (Gunter and Pfeiffer, 1990), Al has no biological function for aquatic organisms, and is absorbed into the cells by cellular and active transport, essential metal channels and receptor-mediated endocsytosis, leading mainly to lipid peroxidation (Exley et al., 1996; Exley and Mold, 2015). To understand the effect of Al in fish, most studies have been restricted to analyzing toxicity under acidic conditions, since Al is insoluble at pH 6 to 8 due to hydrolysis and formation of $Al(OH)_3$ (Driscoll and Schecher, 1990; Gensemer and Playle, 1999). These studies have shown that Al causes brain and gill lesions, as well as hematological, cardiovascular, metabolic and behavioral changes (Exley et al., 1996; Vuorinen et al., 2003; Walker et al., 2001; Al-Alstad et al., 2005; Barcarolli and Martinez, 2004; Vieira et al., 2013).

LC_{50} values for Al vary widely in fish, 0.70 mg L-1 in *Oryzias latipes* (Ramírez-Duarte et al., 2017), 1.53 mg L^{-1} in *Rasbora sumatrana* (Shuhaimi-Othman et al., 2015), 6.76 mg L^{-1} in *Poecilia reticulata* (Shuhaimi-Othman et al., 2015) and 28.89 mg L^{-1} in *Rutilus kutum* (Zahedi et al., 2014). For Mn, LC_{50} values also varied but they are quite higher, 5.71 mg L^{-1} in *Rasbora sumatrana* (Shuhaimi-Othman et al., 2015), 18.80 mg L^{-1} in *Carassius auratus* (Valbona et al., 2017), 23.91 mg L^{-1} in *Poecilia reticulata* (Shuhaimi-Othman et al., 2015) and 54.39 mg L^{-1} for *Rutilus kutum* (Zahedi et al., 2014).

At high concentrations, Al and Mn are considered pro-oxidant agents, i.e., they are capable of inducing oxidative stress by stimulating the production of reactive oxygen species (ROS) (Lee et al., 2006),

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leading to lipid peroxidation, DNA damage, impaired DNA repair and increased susceptibility to apoptosis, factors that may lead to cytotoxic events and impaired species reproduction (Almroth et al., 2005; García-Medina et al., 2009; Correia et al., 2010; Vieira et al., 2013; Kida et al., 2016; Ramírez-Duarte et al., 2017). Al is known to form complexes with sulfhydryl (SH) groups, damaging several biomolecules, including lipids, proteins and nucleic acids (Goyer, 1997; Wu et al., 2005). Conversely, the ability of Mn to cause oxidative stress is due to the transition from the oxidative state Mn^{+2} to Mn^{+3}, increasing its pro-oxidant capacity (HaMai et al., 2001; Reaney and Smith, 2005), and impairing the mitochondrial function, reducing ATP production, and increasing electron leakage and ROS production (Scholte et al., 1988).

To neutralize the adverse effects on the gills, liver and other organs, the organisms have a complex and effective antioxidant defense system with both enzymatic and non-enzymatic mechanisms, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH), etc. (Ahmad and Mehmood, 2006). These antioxidant defenses are sensitive indicators of oxidative stress, and have therefore been widely used as biomarkers in fish (Lushchak, 2011). SOD is the first line of defense against oxidative stress, eliminating anion superoxide, whereas CAT and GPx transform hydrogen peroxide into water and oxygen (Atli et al., 2006; Elia et al., 2003; Ates et al., 2008). SOD and CAT deal directly with free radicals in cells (Lloyd and Phillips, 1999; Dorval et al., 2003), while other enzymes and proteins, such as GR and GSH, help reinstate the reductive capacity of the cell (Bagnyukova et al., 2005). The effect of oxidative stress on the activity of oxidative stress enzymes has been observed in several freshwater teleosts exposed to metals (Livingstone, 2001; Pandey et al., 2003; Fernandes et al., 2008). This is a common adaptive response mechanism for combating oxidative stress in fish, which can affect different tissues in different ways (Stephensen et al., 2002). An effective antioxidant defense system is essential if the organism is to protect against the damaging effects of metals such as Al and Mn, and studies related to this topic are relevant to different aquatic species (Fernandes et al., 2008). However, the mobilization of energetic substrates for effective antioxidant action can be detrimental to other functions, such as reproduction (Vieira et al., 2013). Exposure of *Astyanax altiparanae* mature males to Al and Al + Mn increased the levels of plasma androgens, showing that these metals act as endocrine disruptors (Kida et al., 2016), as already documented for Al in mature females of *Oreochromis niloticus* (Correia et al., 2010).

The aim of this study was to evaluate the responses of the gill and hepatic antioxidant enzymes of adult males of *Astyanax altiparanae* when exposed to Al and Al + Mn by examining the effects of these metals on oxidative stress responses. This species lives in basins in the State of São Paulo, where, according to reports issued by *Companhia Ambiental do Estado de São Paulo* (CETESB, 2017), the values are higher than 0.1 mg L^{-1} the maximum concentration allowed by Brazilian regulations (CONAMA, 2005). It is based on the hypothesis that the effects of Al and Mn differ, depending on whether Al is present on its own, or combined with Mn. It was also assumed that the fish would be able to recover from the effects of these metals by immersing them in metal-free water for the same period.

2. Materials and methods

2.1. Subjects

The genus Astyanax encompasses over 100 species, widely distributed throughout most of Brazil (Orsi et al., 2004). Some of the characteristics of Astyanax species make them model organisms for biomonitoring the effects of pollutants in watersheds (Uieda and Barreto, 1999). Studies on Astyanax have shown that environmental contamination by industrial waste can alter oxidative stress biomarker responses in these fish (Carrión and López, 2010; Sakuragui et al., 2013; Costa-Silva et al., 2015), and can impair reproduction (Vieira et al., 2013; Kida et al., 2016; Tolussi et al., 2018).

Sexually mature males of *A. altiparanae* with a mean body mass of 24.29 ± 1.17 g and measuring 11.78 ± 0.18 cm long (n = 120) were collected at CESP (*Companhia Energética de São Paulo)* and kept for seven days in 10 glass aquariums (40 × 50 × 70 cm, 132 L of water/tank, 2.20 g L^{-1} fish), with 90% water renewal every 24 h. The fish were fed daily on extruded commercial feed containing 32% crude protein. Feeding was suspended 24 h before beginning the experiment.

2.2. Experimental conditions

The experiment was conducted on five experimental groups (in duplicates; 12 animals in each aquarium, 24 animals per group): 1) control group at neutral pH (ctr - n); 2) acid pH group (acid pH); 3) Al group (Al); 4) Mn group (Mn) and 5) group with both metals (Al + Mn). Neutral pH was set at 7.2 and acid pH at 5.7. Al is insoluble at pH 6 to 8 (Driscoll and Schecher, 1990; Gensemer and Playle, 1999), therefore an Al group in neutral pH was not considered.

The physical and chemical parameters of the water supply were monitored to guarantee the necessary conditions for keeping the fish alive and were previously published by Kida et al. (2016) (Table 1, Supplementary material).

This was a two-stage experiment. The first stage consisted of acute exposure to the metals, further split into two groups for evaluation at 24 h and 96 h. The second consisted of a recovery period. The fish were placed in metal-free water at neutral pH and fed normally to provide the ideal conditions needed for a possible recovery. Samples were taken after 24 and 96 h, with 90% water renewal on a daily basis.

The solution of Al was obtained using aluminum sulfate [Al2(SO4)3; Sigma-Aldrich] from a stock solution prepared with Milli-Q water, acidified to pH 2.5 by adding 65% nitric acid (HNO3) (Suprapur; Merck). MnSO4 (Sigma-Aldrich) was used to prepare the Mn stock solution, together with Milli-Q water acidified to pH 2.5 by adding 65% HNO3. For groups 3, 4 and 5, both metals were kept at a nominal concentration of 0.5 mg L^{-1}, which was chosen as representative of the actual mean concentration of the metals found in the rivers of São Paulo, Brazil (CETESB, 2017). The concentration of both metals are sublethal, below the LC_{50} for *A. altiparanae*, that is 1.0 mg L^{-1} for Al and 4.85 mg L^{-1} (unpublished data, calculated by Probit software). Considering that higher concentrations can be found in the environment (CETESB, 2017), the concentrations used are environmentally realistic.

Total Al and Mn concentration in the water were measured using an atomic absorption spectrophotometer (GBC, AAS 932 Avanta-Plus, IL, USA, following method EPA 6020A). Other water chemical variables were measured according to Standard Methods (2005).

2.3. Biochemical analysis

The fish were anesthetized (1 g of benzocaine, previously diluted in 10 mL of ethanol, per 10 L of water). Stringhetta et al. (2017) studying gills, liver and brain in *Colossoma macropomum* described that benzocaine did not cause oxidative damage. Fish liver and gill samples were homogenized (10 mL/g tissue) in a phosphate buffer solution (0.1 M, pH 7.2) and centrifuged (15,000 × *g*, 30 min, 4 °C). The supernatants were collected to analyze biotransformation enzyme activity, antioxidant parameters, lipid peroxidation and metallothionein concentration, as described below. For all biochemical biomarkers, the protein concentration of the samples was determined using the Bradford method (Bradford, 1976) and the standard was bovine serum albumin.

2.4. Antioxidant enzymes

CAT activity was determined according to Beutler (1975),

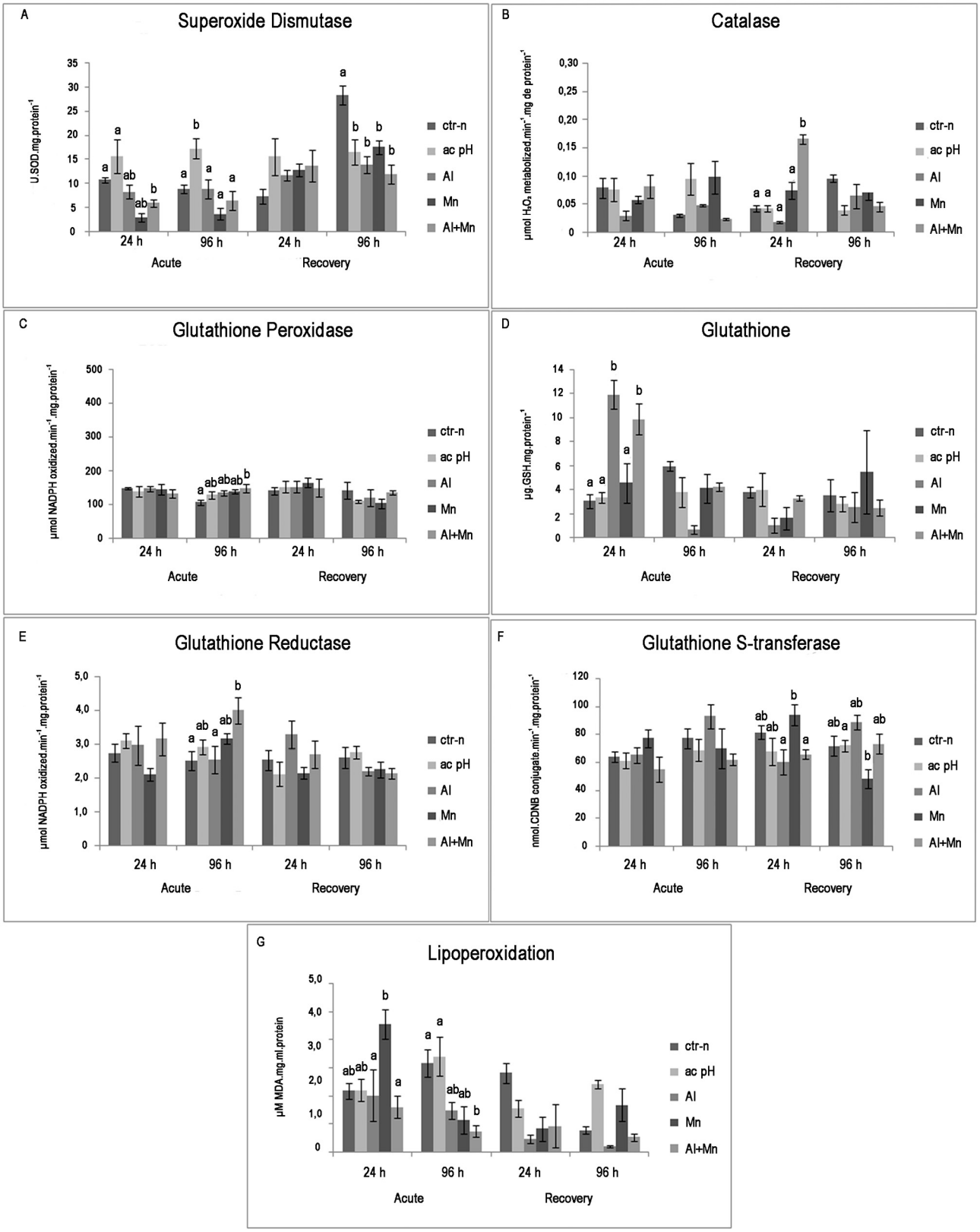

Fig. 1. *Astyanax altiparanae*. Gill oxidative stress biomarkers after exposure to neutral pH, acid pH, Al, Mn and Al + Mn (24 h and 96 h) and recovery (24 h and 96 h). Control group in neutral pH (ctr-n), acid pH group (pH ac), Al (Aluminum), Mn (Manganese), Al + Mn (Aluminum and Manganese). [ab]Different letters indicate statistically significant differences between experimental groups for each period (exposure and recovery individually).

monitoring H_2O_2 decomposition based on the drop in absorbance at 240 nm. CAT activity was expressed as mol of H_2O_2 min^{-1}/mg of protein. SOD activity was determined by measuring the reduction rate of cytochrome *c* by the superoxide radical at 550 nm (25 °C), according to McCord and Fridovich (1969), and expressed in SOD U mg of $protein^{-1}$, where an SOD unit is the amount of enzyme required to cause a 50% drop in the cytochrome *c* reduction rate. GPx activity was determined based on the method proposed by Hopkins and Tudhope (1973), in which the oxidation of NADPH in the presence of H_2O_2 is measured at 340 nm and expressed in nmol of oxidized NADPH min^{-1}/mg $protein^{-1}$. GR activity was determined according to Carlberg and Mannervik (1975), based on the reduction of NADPH in the presence of oxidized GSH at 340 nm, and expressed in nmol of oxidized NADPH min^{-1}/mg $protein^{-1}$. GST activity was determined as described by Keen et al. (1976), with a substrate of 1-chloro-2,4-dinitrobenzene (CDNB). Absorbance activity was measured at 340 nm and expressed as nmol CDNB/min^{-1} mg^{-1} of protein conjugate using a molar extinction coefficient of 9.6 mM cm^{-1}.

2.5. Non-enzymatic antioxidants

GSH concentration was estimated according to Beutler et al. (1963) by reacting glutathione with the 5,5-dithiobis-2-nitrobenzoic acid (DTNB) substrate to form the thiolate (TNB), which was quantified in a microplate spectrofluorometer at 412 nm, using L-glutathione reduced (Sigma-Aldrich, Saint Louis, MO, USA) to yield a standard curve, and expressed in μg GSH/mg of protein-1.

2.6. Metallothionein-type proteins

The concentration of metallothioneins (MT) was estimated based on the concentration of sulfhydryl groups (Viarengo et al., 1997) and quantified by spectrophotometer at 412 nm using Ellman's reagent (2 M NaCl, 0.43 M DTNB in 0.2 M phosphate buffer at pH 8.0).

2.7. Lipid peroxidation

Malondialdehyde (MDA) is one of the end products of lipid peroxidation. It was determined by the TBARS assay (thiobarbituric acid reactive substances) for MDA, and quantified in a microplate spectrofluorometer at 530 nm, using 1,1,3,3-tetramethoxypropane to yield a standard curve, according to Camejo et al. (1998).

2.8. Histological analysis

Histological analysis of the testes was carried out to confirm that all males were at the same gonadal maturation stage. Fractions of the testes were placed in a solution of methacrylate resin and absolute ethanol (1:1) for 2 h, and infiltrated into pure resin solution for a further seven days in the refrigerator. Slices 3 μm thick were obtained using a Leica RM2255 microtome with glass knife. The testes were stained with hematoxylin-eosin, and periodic acid-Schiff (PAS) + hematoxylin + Metanil Yellow (Quintero-Hunter et al., 1991). The prepared sections were analyzed and documented using a computerized image capture system (Leica DM 1000, Leica DFC 295 and Leica Application Suite Professional, LAS V3. 6). The analysis showed that all testes presented the spermatogenic lumen filled with spermatozoa, classified as sexually mature (Schulz et al., 2010) (data not presented).

This experiment was approved by the Ethics Committee on the Use of Animals (CEUA) of the Institute of Biosciences of the University of São Paulo (163/2012).

2.9. Statistical analysis

After verifying sample data normality and homogeneity of variance, one-way ANOVA was run on the parametric data, followed by the Holm-Sidak test. For non-parametric data, Kruskal-Wallis one-way ANOVA on Ranks was followed by Dunn's test. For all tests, each experiment was analyzed separately (24 and 96 h acute exposure; 24 and 96 h recovery period). Data were presented as means ± standard error of the mean (SEM) and the results treated as significant if $P < 0.05$. The fish were deemed to have recovered when the treatment variable showed no significant difference compared to the fish in the control group over the same period.

3. Results

3.1. Gill oxidative stress

Exposure to an acid pH for 96 h increased SOD enzymatic activity compared to the control ($P = 0.002$) and 96 h was not sufficient to reinstate the activity values found at neutral pH ($P = 0.001$) (Fig. 1A). Exposure to both metals and an acid pH did not alter CAT enzymatic activity. However, during the 24-hour recovery period, there was an increase in CAT activity in the gills of fish exposed to Al + Mn ($P = 0.0000293$), which normalized after 96 h ($P = 0.273$) (Fig. 1B).

At 24 h, the concentration of GSH increased in the gills of fish exposed to Al ($P = 0.003$) and Al + Mn ($P = 0.005$) (Fig. 1D), but after 96 h in metal-free water, GSH normalized, and remained similar to that of the control group. Exposure for 96 h to Al + Mn increased GPx and GR enzymatic activities (Fig. 1C, E) but did not alter GST activity (1F). Exposure for 24 h did not significantly alter MDA levels, but there was a significant increase in MDA in fish exposed to Mn (Fig. 1G) compared to Al and Al + Mn groups ($P = 0.020$). After exposure for 96 h, MDA levels dropped in fish in the Al + Mn group ($P = 0.00239$), and returned to control levels after a 24-h recovery period ($P = 0.083$) (Fig. 1G).

3.2. Hepatic oxidative stress

Exposure for 96 h to acid pH and Al or Al + Mn did not affect SOD activity, nor concentrations of GR and GSH (Fig. 2A). Exposure for 24 h to an acid pH caused a drop in CAT enzymatic activity compared to the control group ($P = 0.000645$). In the recovery period of 96 h CAT enzymatic activity was also lower in acid pH ($P = 0.028$) (Fig. 2B). Exposure to Al + Mn for 24 h triggered a drop in GPx enzymatic activity ($P = 0.000137$), but after 96 h in metal-free water, GPx levels were normalized ($P = 0.154$) (Fig. 2C). Exposure for 96 h to the metals and acid pH did not alter MTL concentrations. However, after a 24-h recovery period, MTL levels rose in the Al + Mn group ($P = 0.000230$) (Fig. 2G). Exposure for 24 h and 96 h to acidic pH and metals did not alter the individuals MDA concentration ($P = 0.101$ and $P = 0.198$ in 24-h and 96-h, respectively), but during the recovery period there was an increase in MDA levels at 96 h in the liver of animals exposed to Mn and Al + Mn ($P = 0.015$).

4. Discussion

Exposure to some metals boosts the production of ROS by inducing oxidative stress (Van der Oost et al., 2003). The present study shows that exposure to metals and an acid pH for 24 h can trigger oxidative stress in sexually mature *A. altiparanae* males. In some cases, a period of 96 h in metal-free water (recovery) was not sufficient for the fish to recover from the effects of the metals and/or pH. Furthermore, the effects of Al and Mn together differed from the effects of the exposure of these metals individually. The simultaneous exposure to two or more metals results in different patterns of ionic distribution in tissues due to factors intrinsic to the fish physiology and the competitive relationship of metal ions in the sites of biological association (Jaben et al., 2012; Oberholster et al., 2012).

The gills are the first point of contact with environmental pollutants in the water. They are multifunctional organs, handling vital functions such as respiration, osmoregulation, acid-base balance and excretion of

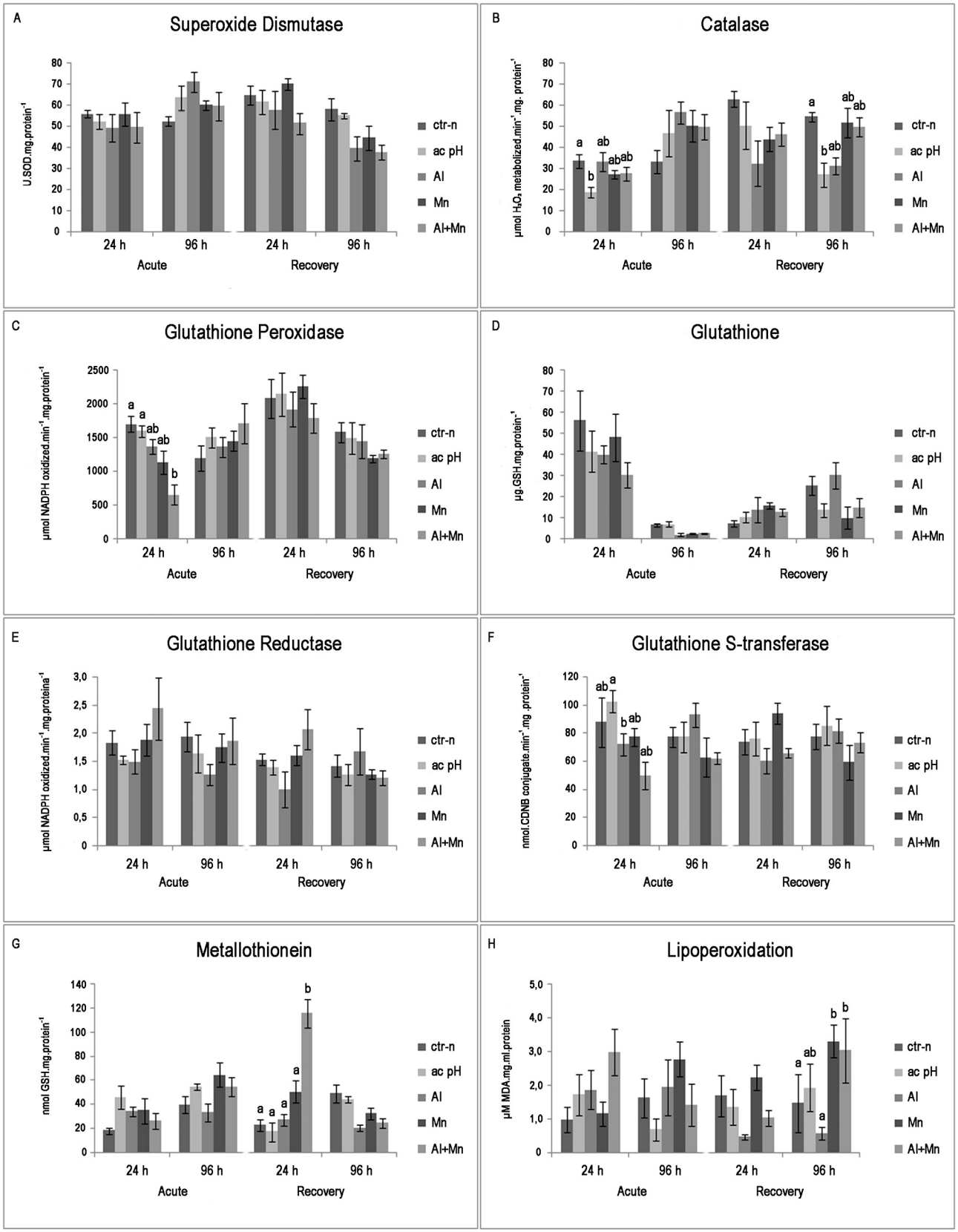

Fig. 2. *Astyanax altiparanae*. Hepatic oxidative stress biomarkers after exposure to neutral pH, acid pH, Al, Mn and Al + Mn (24 h and 96 h) and recovery (24 h and 96 h). Control group in neutral pH (ctr-n), acid pH group (pH ac), Al (Aluminum), Mn (Manganese), Al + Mn (Aluminum and Manganese). [ab]Different letters indicate statistically significant differences between experimental groups for each period (exposure and recovery individually).

nitrogenous residues (Pandey and Upadhyaya, 2008). Paradoxically, they are highly vulnerable to toxic chemicals primarily because of their large surface area which facilitates increased toxic interaction and absorption, and also because the gill detoxification system is not as robust as that of the liver (Playle and Wood, 1990; Pereira et al., 2010). Thus, the gills are often used in assessing the impact of water pollutants on marine and freshwater habitats, providing important indications of acute exposure (Atli et al., 2006) and reliable results for assessing environmental water contamination (Pereira et al., 2010). The gill responses reported herein show that the gills can advantageously be used as biomarkers for effective oxidative damage. The antioxidative responses differed for each treatment, implying that the gills provide effective protection for the other organs, given that the survival rate was 100%, corroborating the findings of Livingstone (2001) and Zhang et al. (2004). However, note that when the fish were exposed to Al + Mn, catalase increased only during the recovery period. In contrast, GSH increased during exposure (24 h) preventing gills and hepatic LPO during the acute exposure, but not the hepatic LPO during the recovery period (96 h), suggesting that the GPx is the enzyme that is promoting the breakdown of hydrogen peroxide into water and oxygen. GR is responsible for the reduction of glutathione oxidized by GPx, through oxidation of NADPH in NADP+, maintaining adequate concentrations of GSH in its reduced state (Van der Oost et al., 2003; Hermes-Lima, 2004). The exposition to Al + Mn triggered this first line of defense, coordinated by the increase, initially of the no-enzymatic defense (GSH), followed by the enzymatic defenses. The increase in GSH levels occur in two ways: increase in its synthesis or recycling via the GSH/GSSG pathway, which depends on GR (Van der Oost et al., 2003). The increase in GR activity in the gills, after 96 h, suggests that the second mechanism was triggered. The results herein found suggest that enzymatic and non-enzymatic defenses were brought into play at different times, and even when transferred to metal-free water, antioxidant defense is still necessary against Al + Mn.

The liver is also a target organ of great importance for fish, since it is involved in biotransformation processes and the elimination of xenobiotics. Both the liver and gills are highly sensitive to pollutants and good candidates for biomonitoring (Farrel et al., 2011). However, the liver exhibited specific variations in response to the contaminants, and was less sensitive than the gills, corroborating the results of Velma and Tchounwou (2010). It is also possible that the gills provided some initial protection by boosting GSH activity and partially reducing metal levels as part of the detoxification process (Zhang et al., 2004).

pH plays a key role in the bioavailability of many metals (Poléo et al., 1995). pH values between 6 and 9 are recommended for freshwater fish in Brazil (CONAMA 357, 2005). However, episodes of rapid acidification in inland water bodies can occur as a result of incidents and ecological changes. In these situations, Al and Mn in the soil are mobilized, boosting the presence of these metals in their dissolved, more toxic forms and affecting fish populations (Monette and McCormick, 2008). The environmental pH is known to influence antioxidant enzyme activity, modifying enzymatic action and substrate concentration in the organism (Moore et al., 2008). In this study, an acid pH triggered a coordinated action of hepatic CAT and gill SOD and the exposition period was relevant in the response. After 24 h, hepatic CAT decreased the breakdown of hydrogen peroxide, but after 96 h, the increased in gill SOD activity reestablished this process, what can be confirmed by the absence in lipoperoxidation in both organs during the acute exposure. However, 96-h recovery period was not sufficient to reinstate hepatic CAT and gill SOD activity levels. These findings corroborate the experiments conducted by Nilsen et al. (2013) on Atlantic salmon, in which a period of over two weeks was required for complete recovery after exposure to an acid pH.

Similar to an acid pH, the pro-oxidant action of Al can also alter the activity of antioxidant enzymes. Several studies have shown that exposure to high concentrations of Al can induce oxidative stress by stimulating ROS in cells (Sinha et al., 2007), inducing LPO, altering the activity of several antioxidant enzymes including SOD, CAT and GPX, and inducing protein oxidation (Almroth et al., 2005; Vlahogianni et al., 2007) activating CAT and then returning to normal levels. In the liver, GPx activity decreased within 24 h of exposure to Al + Mn, since this enzyme is an active participant, with CAT, in maintaining H_2O_2 levels (Hermes-Lima, 2004). Once it is deactivated, other detoxification systems, such as MT, are necessary to give a significant boost to GPx levels during the 24-h recovery period. However, only a few studies show a correlation between exposure to Al and Mn, individually or combined, and MT production in fish (Giguère et al., 2006; Leonard et al., 2014). When fish were exposed to Mn alone, antioxidant defenses were not activated, triggering late hepatic LPO during the recovery period (96 h). The absence of an antioxidant defense response on exposure to Mn was also observed by Oliveira et al. (2018) in *Prochilodus lineatus*.

The data clearly show that the action of Al + Mn triggers different antioxidant responses, as observed by Oliveira et al. (2018) studying the effects of Mn and Fe in *Prochilodus lineatus*. Giguère et al. (2006) analyzed the bioaccumulation pattern of copper, zinc, nickel and cadmium in *Perca flavescens*, reporting that MT helps protect against oxidizing agents. Similarly, Chowdhury et al. (2005) observed differentiated patterns of cadmium accumulation from water and feed in different tissues of *Oncorhynchus mykiss*, associated with an increase in MT at higher cadmium concentrations. Exposure to Mn alone resulted in high concentrations of MT in the intestine, liver and kidney of *Oncorhynchus mykiss* (Filipovi and Raspor, 2003; Amiard et al., 2006; De Boeck et al., 2010). The simultaneous exposure to two or more metals can trigger different patterns of ionic distribution in tissues due to factors intrinsic to the physiology of each species and the competitive relationship of metal ions to sites of biological association (Jaben et al., 2012; Oberholster et al., 2012).

In addition to the observed effects on oxidative stress, acid pH and metals are also known as endocrine disrupters, acting on hormones in the hypothalamic-pituitary-gonadal axis (Zelennikov et al., 1999; Correia et al., 2010; Vieira et al., 2013; Atli et al., 2015). Exposure to Al + Mn in the fish analyzed herein did not alter the plasma cortisol concentration, suggesting that there was no change in metabolic demand (Kida et al., 2016). However, the production of hormones that modulate the hypothalamic-pituitary-gonadal axis, such as 11-keto-testosterone, was found to impair the reproductive success of *A. altiparanae* (Kida et al., 2016). Since all physiological systems are intrinsically related, changes in the immune and endocrine systems generate adaptive responses that can disrupt processes and even impair survival (Kime and Nash, 1999). In summary, endogenous and extrinsic processes induce variations in oxidative stress biomarkers levels in individuals during the reproductive phase, and can initiate adaptive responses in this process (Aras et al., 2009).

5. Conclusion

The exposure of sexually mature *A. altiparanae* males to Al, regardless of water acidity, triggered enzymatic and non-enzymatic antioxidant responses that were able to protect the liver and gill membranes against oxidative damage. However, the exposure of these animals to Mn, individually or combined with Al, did not trigger the same responses, resulting in lipid peroxidation of hepatocytes during the recovery period. Immersing the fish in metal-free water for 24 h was not always sufficient to induce recovery from the effects of exposure. These findings suggest that this kind of contaminated environment can impair the metabolism and reproductive physiology of this fish species.

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Conflict of interest

We have no conflict of interest.

Acknowledgments

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References

Ahmad, I., Mehmood, Z., 2006. Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk. J. Biol. 30, 177–183.
Almroth, B.C., Sturve, J., Berglund, A., Forlin, L., 2005. Oxidative damage in eelpout (*Zoarces viviparous*), measured as protein carbonyls and TBARS, as biomarkers. Aquat. Toxicol. 73, 171–180.
Al-Alstad, N.E.W., Kjelsberg, B.M., Vollestad, L.A., Lydersen, E., Poléo, A.B.S., 2005. The significance of water ionic strength on aluminium toxicity in brown trout (*Salmo trutta* L.). Environ. Pollut. 133, 333–342.
Al-Ansari, C.A.M., Saleem, A., Kimpe, L.E., Sherry, J.P., Vance, L., Blais, J.M., 2010. Bioaccumulation of the pharmaceutical 17a-ethinylestradiol in shorthead redhorse suckers (*Moxostoma macrolepidotum*) from the St. Clair River, Canada. Environ. Pollut. 158, 2566–2571.
Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202.
Aras, M.A., Hara, H., Hartnett, K.A., Kandler, K., Aizenman, E., 2009. Protein kinase C regulation of neuronal zinc signaling mediates survival during preconditioning. J. Neurochem. 110, 106–117.
Aravind, M.N.V., Prasad, L., 2003. Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* a free floating freshwater macrophyte. Plant Physiol. Biochem. 41, 391–397.
Ates, B., Orun, I., Talas, Z.S., Durmaz, G., Yilmaz, I., 2008. Effects of sodium selenite on some biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss*) Walbaum, exposed to Pb and Cu. Fish Physiol. Biochem. 34, 53–59.
Atli, G., Alptekin, O., Tukel, S., Canli, M., 2006. Response of catalase activity to Ag, Cd, Cr, Cu and Zn in five tissues of freshwater fish *Oreochromis niloticus*. Comp. Biochem. Physiol. C 143, 218–224.
Atli, G., Ariyurek, S.Y., Kanakc, E.G., Canli, M., 2015. Alterations in the serum biomarkers belonging to different metabolic systems of fish (*Oreochromis niloticus*) after Cd and Pb exposure. Environ. Toxicol. Pharmacol. 40, 508–515.
Bagnyukova, T.V., Vasylkiv, O.Y., Storey, K.B., Lushchak, V.I., 2005. Catalase inhibition by amino triazole induces oxidative stress in goldfish brain. Brain Res. 1052, 180–186.
Barcarolli, I.F., Martinez, C.B.R., 2004. Effects of aluminum in acidic water on hematological and physiological parameters of the neotropical fish *Leporinus macrocephalus* (Anostomidae). Bull. Environ. Contam. Toxicol. 72, 639–646.
Basha, P.S., Rani, A.U., 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxicol. Environ. Saf. 56, 218–221.
Beutler, E., 1975. Catalase. In: Beutler, E. (Ed.), Red Cell Metabolism. A Manual of Biochemical Methods. Grune and Straton, New York, pp. 89–90.
Beutler, E., Duron, O., Kelly, B.M., 1963. Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 61, 882–890.
Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Brumbaugh, W.G., Schmitt, C.J., May, T.W., 2005. Concentrations of cadmium, lead, and zinc in fish from mining-influenced waters of Northeastern Oklahoma: sampling of blood, carcass, and liver for aquatic biomonitoring. Arch. Environ. Contam. Toxicol. 49, 76–88.
Camejo, G., Wallin, B., Enojärvi, M., 1998. Analyses of oxidation and antioxidants using microtiter plates. In: Amstrong, D. (Ed.), Free Radical and Antioxidants Protocols. Humana Press, New Jersey, pp. 377–387.
Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.
Carrión, T.E., López, E.L., 2010. Helminths and lipid peroxidation in *Astyanax aeneus* (Pisces: Characidae) from a river in the humid subtropics of southeastern Mexico. Dis. Aquat. Org. 88, 215–224.
CETESB – Companhia De Tecnologia De Saneamento Ambiental, 2017. Relatório de Qualidade das Águas Interiores do Estado de São Paulo. Governo do Estado de São Paulo, Secretaria do Meio Ambiente, São Paulo.
Chowdhury, M.J., Baldisserotto, B., Wood, C.M., 2005. Tissue-specific cadmium and metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. Arch. Environ. Contam. Toxicol. 48, 381–390.
CONAMA - Conselho Nacional do Meio Ambiente, 2005. Resolução n° 357/05. In: Estabelece a classificação das águas doces, salobras e salinas do Território Nacional. SEMA, Brasília.
Correia, T.G., Narcizo, A.M., Bianchini, A., Moreira, R.G., 2010. Aluminum as an endocrine disruptor in female Nile tilapia (*Oreochromis niloticus*). Comp. Biochem. Physiol. Toxicol. Pharm. 151C, 61–66.
Costa-Silva, D.G., Nunes, M.E.M., Wallau, G.L., Martins, I.K., Zemolin, A.P.P., Cruz, L.C., Rodrigues, N.R., Lopes, A.R., Posser, T., Franco, J.L., 2015. Oxidative stress markers in fish (*Astyanax sp.* and *Danio rerio*) exposed to urban and agricultural effluents in the Brazilian Pampa biome. Environ. Sci. Pollut. Res. 7, 37–47.
De Boeck, G., Eyckmans, M., Lardon, I., Bobbaers, R., Sinha, A.K., Blust, R., 2010. Metal accumulation and metallothionein induction in the spotted dog fish *Scyliorhinus canicula*. Comp. Biochem. Physiol. 155A, 503–508.
Dorval, J., Leblond, V.S., Hontela, A., 2003. Oxidative stress and loss of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) exposed in vitro to endosulfan, an organochlorine pesticide. Aquat. Toxicol. 63, 229–241.
Driscoll, C.T., Schecher, W.D., 1990. The chemistry of aluminium in the environment. Environ. Biochem. Health 12, 28–48.
Elia, A.C., Galarini, R., Taticchi, M.I., Dorr, A.J.M., Mantilacci, L., 2003. Antioxidant responses and bioaccumulation in *Ictalurus melas* under mercury exposure. Ecotoxicol. Environ. Saf. 55, 162–167.
Emsley, J., 1991. The Elements, 2nd edition. Oxford University Press, New York.
Exley, C., Mold, M.J., 2015. The binding, transport and fate of aluminium in biological cells. J. Trace Elem. Med. Biol. 14, 1–23.
Exley, C., Burgess, E., Day, J.P., Jeffery, E.H., Melethil, S., Yokel, R.A., 1996. Aluminum toxicokinetics. J. Toxicol. Environ. Health 48, 569–584.
Farrel, A.P., Joseph, C.J., Richards, J.G., Stevens, E.D., 2011. Encyclopedia of Fish Physiology From Genome to Environment. 3. Elsevier, pp. 2061–2083.
Fernandes, C., Fontaínhas-Fernandes, A., Ferreira, M., Salgado, M.A., 2008. Oxidative stress response in gill and liver of *Liza saliens*, from the Esmoriz-Paramos Coastal Lagoon, Portugal. Arch. Environ. Contam. Toxicol. 55, 262–269.
Filipovi, V., Raspor, B., 2003. Metallothionein and metal levels in cytosol of liver, kidney and brain in relation to growth parameters of *Mullus surmuletus* and *Liza aurata* from the Eastern Adriatic Sea. Water Res. 37, 3253–3262.
García-Medina, S., Razo-Estrada, C., Gómez-Oliván, L., Amaya-Chávez, A., Madrigal Bujaidar, E., Galar-Martínez, M., 2009. Al-induced oxidative stress in lymphocytes of common carp (*Cyprinus carpio*). Fish Physiol. Biochem. 19, 459–467.
Gensemer, R.W., Playle, R.C., 1999. The bioavailability and toxicity of aluminum in aquatic environments. Crit. Rev. Environ. Sci. Technol. 29, 315–450.
Giguère, A., Campbell, P.G.C., Hare, L., Couture, P., 2006. Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient. Aquat. Toxicol. 77, 178–189.
Goyer, R.A., 1997. Toxic and essential metal interactions. Annu. Rev. Nutr. 17, 37–50.
Gunter, T., Pfeiffer, D., 1990. Mechanisms by which mitochondria transport calcium. Am. J. Phys. 258, 755–786.
HaMai, D., Campbell, A., Bondy, S.C., 2001. Modulation of oxidative events by multivalent manganese complexes in brain tissue. Free Radic. Biol. Med. 31, 763–768.
Hermes-Lima, M., 2004. Oxygen in biology and biochemistry: role of free radicals. In: Storey, K.B. (Ed.), Functional Metabolism: Regulation and Adaptation. Wiley-Liss, Hoboken, pp. 319–368.
Hopkins, J., Tudhope, G.R., 1973. Glutathione peroxidase in human red cells in health and disease. J. Haematol. 25, 563–575.
Jaben, G., Javed, M., Azmat, H., 2012. Assessment of heavy metals in the fish collected from the river Ravi, Pakistan. Pak. Vet. J. 32, 107–111.
Keen, J.H., Habig, W.H., Jakobi, W.B., 1976. Mechanism for the several activities of the glutathione-S-transferases. J. Biol. Chem. 251, 6183–6188.
Kida, B.M.S., Abdalla, R.P., Moreira, R.G., 2016. Effects of acidic water, aluminum, and manganese on testicular steroidogenesis in *Astyanax altiparanae*. Fish Physiol. Biochem. 16, 222–232.
Kime, D.E., Nash, J.P., 1999. Gamete viability as an indicator of reproductive endocrine disruption in fish. Sci. Total Environ. 233, 123–129.
Lee, B., Pine, M., Johnson, L., Rettori, V., Hiney, J.K., Dees, W.L., 2006. Manganese acts centrally to activate reproductive hormone secretion and pubertal development in male rats. Reprod. Toxicol. 22, 580–585.
Leonard, E.M., Banerjee, U., D'Silva, J.J., Wood, C.M., 2014. Chronic nickel bioaccumulation and sub-cellular fractionation in two freshwater teleosts, the round goby and the rainbow trout, exposed simultaneously to waterborne and diet borne nickel. Aquat. Toxicol. 154, 141–153.
Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.
Lloyd, D.R., Phillips, D.H., 1999. Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutat. Res. Fundam. Mol. Mech. Mutagen. 424, 23–36.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.
McCord, J.E., Fridovich, I., 1969. Superoxide dismutase an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.
Monette, M.Y., McCormick, S.D., 2008. Impacts of short-term acid and aluminum exposure on Atlantic salmon (*Salmo salar*) physiology: a direct comparison of parr and smolts. Aquat. Toxicol. 86, 216–226.
Moore, M., Lawrence, A.J., Arukwe, A., Sayer, M., Thain, J., 2008. Molecular/cellular processes and the physiological response to pollution. In: Lawrence, A.J., Hemingway, K.L. (Eds.), Effects of Pollution on Fish: Molecular Effects and Population Responses. Blackwell Publishing, Oxford, pp. 83–133.
Narcizo, A.M., Correia, T.G., Moreira, R.G., 2010. Evaluation of Subchronic Exposure to Aluminum in the Reproductive Physiology Parameters in *Astyanax fasciatus*. Fish Biology Congress, Barcelona, pp. 177.
Nilsen, T.O., Ebbenson, L.O.E., Handeland, S.O., Kroglund, F., Finstad, B., Angotzi, A.R., Stefansson, S.O., 2013. Atlantic salmon (*Salmo salar* L.) smolts require more than two weeks to recover from acidic water and aluminum exposure. Aquat. Toxicol. 142–143, 33–44.

Oberholster, P.J., Myburgh, J.G., Ashton, P.J., Coetzee, J.J., Botha, A.M., 2012. Bioaccumulation of aluminium and iron in the food chain of Lake Loskop, South Africa. Ecotoxicol. Environ. Saf. 75, 34–141.
Oliveira, L.F., Santos, C., Risso, W.E., Martinez, C.B.R., 2018. Triple-mixture of Zn, Mn and Fe increases bioaccumulation and causes oxidative stress in freshwater neotropical fish. Environ. Toxicol. Chem. 37, 1749–1756.
Orsi, M.L., Carvalho, E.D., Foresti, F., 2004. Biologia populacional de *Astyanax altiparanae* (Teleostei, Characidae) do médio Rio Paranapanema, Paraná, Brasil. Rev. Bras. Zootec. 21, 207–218.
Pandey, S.K., Upadhyaya, A.R., 2008. Heavy metal pollution induced due to coal mining effluent on surrounding aquatic ecosystem and its management through naturally occurring aquatic macrophytes. Bioresour. Technol. 99, 930–936.
Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish Wallago attu (Bl &Schn.). Sci. Total Environ. 309, 105–115.
Pereira, P., Pablo, H.D., Vale, C., Pacheco, M., 2010. Combined use of environmental data and biomakers in fish (*Liza aurata*) inhabiting a eutrophic and metal-contaminated coastal system—gills reflect environmental contamination. Mar. Environ. Res. 69, 53–62.
Playle, R.C., Wood, C.M., 1990. Is precipitation of aluminum fast enough to explain aluminum deposition on fish gills? Can. J. Fish. Aquat. Sci. 47, 1558–1561.
Poléo, A.B.S., Oxvenad, S.A., Ostbye, K., Andersen, R.A., Oughton, D.H., Vsllestad, L.A., 1995. Survival of crucian carp, *Carassius carassius*, exposed to a high low molecular weight inorganic aluminium challenge. Aquat. Toxicol. 45, 78–90.
Quintero-Hunter, I., Grier, H.J., Muscato, M., 1991. Enhancement of histological detail using yellow as countersatin in period acid Schiff's hematoxylin staining of glycol methacrylate tissue sections. Biotech. Histochem. 66, 169–172.
Ramírez-Duarte, W.F., Kurobe, T., Teh, S.J., 2017. Impairment of antioxidant mechanisms in Japanese Medaka (*Oryzias latipes*) by acute exposure to aluminum. Comp. Biochem. Physiol. 198C, 37–44.
Reaney, S.H., Smith, D.R., 2005. Manganese oxidation state mediates toxicity in PC12 cells. Toxicol. Appl. Pharmacol. 205, 271–281.
Sakuragui, M.M., Paulino, M.G., Pereira, C.D.S., Carvalho, C.S., Sadauskas-Henrique, H., 2013. Integrated use of antioxidant enzymes and oxidative damage in two fish species to assess pollution in man-made hydroelectric reservoirs. Environ. Pollut. 178, 41–51.
Scholte, A.M., Roodhooft, C., Ceuterick, L., Luyt-Houwen, E.M., 1988. Defect in succinate oxidation by isolated muscle mitochondria in a patient with symmetrical lesions in the basal ganglia. J. Neurol. Sci. 84, 189–200.
Schulz, R.W., França, L.R., Layere, J.J., Legac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.
Shuhaimi-Othman, M., Yakub, N., Ramle, N.A., Abas, A., 2015. Comparative toxicity of eight metals on freshwater fish. Toxicol. Ind. Health 31, 773–782.
Sinha, S., Mallick, S., Misra, R.K., Singh, S., Basant, A., Gupta, A.K., 2007. Uptake and translocation of metals in *Spinacia oleracea* L. grown on tannery sludgeamended and contaminated soils: effect on lipid peroxidation, morphoanatomical changes and antioxidants. Chemosphere 67, 176–187.
Stephensen, E., Sturve, J., Förlin, L., 2002. Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. Comp. Biochem. Physiol. 133C, 435–442.
Stringhetta, G.R., Barbas, L.A.L., Maltez, L.C., Sampaio, L.A., Monserrat, J.M., Garcia, L.O., 2017. Oxidative stress responses of juvenile tambaqui *Colossoma macropomum* after short-term anesthesia with benzocaine and MS-222. An. Acad. Bras. Cienc. 89, 2209–2218.
Tolussi, C.E., Gomes, A.D., Kumar, A., Ribeiro, C.S., Lo Nostro, F.L., Bain, P., Souza, G.B., Da Cuña, R.H., Honji, R.M., Moreira, R.G., 2018. Environmental pollution affects molecular and biochemical responses during gonadal maturation of *Astyanax fasciatus* (Teleostei: Characiformes: Characidae). Ecotoxicol. Environ. Saf. 147, 926–934.
Uieda, V.S., Barreto, M.G., 1999. Composição da ictiofauna de quatro trechos de diferentes ordens do rio Capivara, bacia do Tietê, Botucatu, São Paulo. Rev. Bras. Zootec. 1, 55–67.
Valbona, A., Mihallaq, Q., Eldores, S., Valon, M., Caterina, F., 2017. Antioxidant defense system, immune response and erythron profile modulation in goldfish, *Carassius auratus*, after acute manganese treatment. Fish Shellfish Immunol. 76, 101–109.
Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.
Velma, V., Tchounwou, P.B., 2010. Chromium-induced biochemical, genotoxic and histopathologic effects in liver and kidney of goldfish, *Carassius auratus*. Mutat. Res. 698, 43–51.
Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: application to Mediterranean and Antarctic molluscs. Mar. Environ. Res. 44, 69–84.
Vieira, V.A.R.O., Correia, T.G., Moreira, R.G., 2013. Effects of aluminum on the energetic substrates in neotropical freshwater *Astyanax bimaculatus* (Teleostei: Characidae) females. Comp. Biochem. Physiol. 157C, 1–8.
Vlahogianni, T., Dassenakis, M., Scoullos, M.J., Valavanidis, A., 2007. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. Mar. Pollut. Bull. 54, 1361–1371.
Vuorinen, P.J., Keinänen, M., Peuranen, S., Tigerstedt, C., 2003. Reproduction, blood and plasma parameters and gill histology of vendace (*Coregonus albula* L.) in long-term exposure to acidity and aluminum. Ecotoxicol. Environ. Saf. 54, 255–276.
Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 2001. Principles of Ecotoxicology, 2nd edition. Taylor and Francis, London (321 p).
Wu, J.H., Tung, Y.T., Wang, S.Y., Shyur, L.F., Kuo, Y.H., Chang, S.T., 2005. Phenolic antioxidants from the heartwood of *Acacia confusa*. J. Agric. Food Chem. 53, 5917–5921.
Zahedi, S., Vaezzade, H., Rafati, M., Dangesaraki, M.Z., 2014. Acute toxicity and accumulation of iron, manganese and, aluminum in Caspian Kutum Fish (*Rutilus kutum*). Iran. J. Toxicol. 8, 1028–1033.
Zelennikov, O.V., Mosyagina, M.V., Fedorov, K.E., 1999. Oogenesis inhibition, plasma steroid levels, and morphometric changes in the hypophysis in Russian sturgeon (*Acipenser gueldenstaedti Brandt*) exposed to low environmental pH. Aquat. Toxicol. 46, 33–42.
Zhang, J., Shen, H., Wang, X., Wu, J., Xue, Y., 2004. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere 55, 167–174.