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Effects of short-term exposure to copper on biochemical biomarkers in juvenile freshwater fish

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Abstract. Copper is frequently used to control disease and parasites in fish farms. In order to evaluate the $CuSO_4$ effects on biochemical biomarkers of fish, juvenile freshwater *Rhamdia quelen* and *Oreochromis niloticus* were exposed to: 0, 2, 20 and 200 µg.L^{-1} copper (as $CuSO_4$) for 96 h. After exposure, fish were euthanized and brain, muscle, liver and gills removed for acetylcholinesterase, antioxidant enzymes, and carbonic anhydrase activities, and determination of lipoperoxidation levels. Results revealed acetylcholinesterase inhibition, both in brain and muscle, of *R. quelen* exposed to 200 µg Cu L^{-1}. Acute exposure to all concentrations evaluated in *O. niloticus* did not change acetylcholinesterase activity. Althought some differences, the antioxidant system of both species was disturbed by copper exposure, mainly in the 200 µg Cu L^{-1} group. A copper dose-dependent inhibition of carbonic anhydrase activity was detected, in both species. Results suggest potential damaging impacts of copper, with distinct effects in juveniles of two fish species of economic and environmental relevance.

Keywords: *Rhamdia quelen*, *Oreochromis niloticus*, neurotoxicity, carbonic anhydrase, oxidative stress

Resumo. Efeitos da exposição de curto prazo ao Sulfato de Cobre em biomarcadores bioquímicos em peixes juvenis de água doce. O cobre é freqüentemente usado para controlar doenças e parasitas nas fazendas de peixes. Para avaliar os efeitos do $CuSO_4$ em biomarcadores bioquímicos em peixes de água doce, juvenis de *Rhamdia quelen* e *Oreochromis niloticus* foram expostos a três concentrações de cobre: 0, 2, 20 e 200 µg.L^{-1} durante 96 h. Após a exposição, os peixes foram eutanasiados e cérebro, músculo, fígado e brânquias removidas para medida das atividades de acetilcolinesterase, anidrase carbônica e enzimas antioxidantes, e ainda determinação dos níveis de lipoperoxidação. Os resultados mostraram inibição da acetilcolinesterase, tanto no cérebro quanto no músculo, de *R. quelen* exposto a 200 µg de Cu L^{-1}. A exposição aguda a todas as concentrações avaliadas em *O. niloticus* não alterou a atividade da acetilcolinesterase. Apesar de algumas diferenças, o sistema antioxidante de ambas as espécies foi afetado pela exposição ao cobre, principalmente no grupo 200 µg Cu L^{-1}. Foi detectada uma inibição dose-dependente de cobre da atividade de anidrase carbônica, em ambas as espécies. Os resultados sugerem possíveis impactos prejudiciais do cobre, de forma não idêntica, em juvenis de duas espécies de peixes de importância econômica e ambiental.

Palavras-chave: *Rhamdia quelen*, *Oreochromis niloticus*, neurotoxicidade, anidrase carbônica, estresse oxidativo

Introduction

In recent years, environmental contamination due to heavy metals has been intensively investigated in freshwater ecosystems due to their bioaccumulation and potential toxicity (Wang *et al.* 2014). Copper (Cu), which is released into the aquatic environment through agriculture and industrial activities, is considered one of the major polluting metals (Frías-Espericueta *et al.* 2011).

The inadequate management of aquaculture often promotes the proliferation of diseases in aquatic organisms (Burridge *et al.* 2010). Another problem related to aquaculture is the excessive growth of phytoplankton, particularly blue-green algae (Wang *et al.* 2009). Copper, as copper sulfate, has been used as an algaecide for decades, and most algaecides are copper-based (Lin *et al.* 2008). Copper sulfate, when dissolved in water, dissociates into copper ions and sulfate ions, and results in high levels of bioavailable copper (Closson and Paul 2014). Copper contamination is related to the inhibition of sodium absorption in juvenile freshwater fish, possibly acting on carriers such as sodium channels and Na-K-ATPase (Grosell and Wood 2002). The copper added can bioaccumulate and become toxic to the cultivated organisms, and can even cause ecosystem contamination from culture runoffs and effluents (Feldlite *et al.* 2008). Copper is of particular concern because it is considerably toxic to aquatic animals at ecologically relevant concentrations (Vieira *et al.* 2009). Elevated aquatic copper concentrations induced the over-production of reactive oxygen species (ROS), which cause oxidative damage to several fish species (Lushchak 2011, Sevcikova 2011).

The concentration required to control algae or pathogens must be below the toxicity threshold for aquatic animals in general, and fishes in particular, but it is well known that copper is extremely toxic to fish, mainly in low alkalinity waters (Perschbache and Wurts 1999). Copper concentrations reported in aquaculture facilities are dramatically variable, ranging from 4 μg Cu L^{-1} used against bacteria to 6000 μg Cu L^{-1} against fungal infections. However, US EPA (1984) copper concentration limits in water for the protection of aquatic life is 20 μg Cu L^{-1}. In Brazil, the maximum level allowed for dissolved copper in aquaculture water is 9 μg CuL^{-1} (CONAMA 2005). According to Lydersen *et al.* (2002) and McGeer *et al.* (2002), copper is acutely toxic to freshwater teleosts at concentrations between 10 and 20 μg Cu L^{-1}. The problem with the use of copper as biocide is the thin line that separates effective treatment levels from overdoses, which can harm fish. Despite many studies on the effect of copper on fish, its effect is still not completely elucidated. Furthermore, toxicity thresholds can be rather variable in different fish species (Mazon *et al.* 2004, Carvalho and Fernandes 2008).

The lack of data about Brazilian fish species upon exposure to copper (Cerqueira and Fernandes 2002, Carvalho and Fernandes 2008) and the extensive use of this metal in aquaculture activities strengthen the importance to investigate copper toxicity in a native commercially relevant species, such as *Rhamdia quelen*, a native species in Brazilian Southern Region. On the other hand, *Oreochromis niloticus* is an exotic invasive species of widespread use in aquaculture in Brazil, commonly used as a biomonitor of water pollution due to its metal tolerance and availability in many polluted sites (Wang *et al.* 2009, Kwok *et al.* 2010). Both are proper species for fish production in the southern part of South America; the two species were chosen due to their economic and ecological relevance, for their different origins, and also because they are widely consumed by humans (Baldisserotto 2009).

All fishes species contain antioxidant enzymes such as superoxide dismutase (SOD), catalases (CAT), glutathione S-transferase (GST), that can be used as biomarkers contamination, and carbonic anhydrase (CA), that are important on physiology response when fish are exposed to metals. Besides, the stress caused by these metals could defeat enzymes activity of antioxidant defense, causing oxidative damage (Zhang *et al.* 2003). Acetylcholinesterase (AChE) has a important physiological role in the degradation of acetylcholine and its inhibiton can affect locomotion and equilibrium of exposed organisms, related to neurotoxicity (Ren *et al.* 2017).

In this context, the present study aimed to investigate the effects of acute exposure to waterborne copper in juvenile *R. quelen* and *O. niloticus* through biochemical analysis related to neurotoxicity by evaluation of AChE activity, potential oxidative stress by antioxidant enzymes and lipid peroxidation levels, and osmoregulation/acid-base regulation by carbonic anhydrase activity. The results can aid in the elaboration of protocols of safer use of copper in

fish culturing and to avoid impacts of this activity to the surrounding natural water bodies.

Material and Methods

R. quelen (silver catfish, 4.5 ± 1.4 g; 8.3± 0.9 cm of total length) and *O. niloticus* (Nile tilapia, 2.7 ± 0.6 g; 5.6 ± 0.4 cm of total length), with one month of age, were obtained from an aquaculture (Peixe&Peixes; Curitiba, Paraná, Brazil), and then transported to the laboratory and maintained in glass aquaria (100 L), following standard procedures (APHA 1998), for 20 days for acclimation. Aquaria water was saturated with oxygen through continuous aeration; in a controlled room, temperature was maintained at 25±1°C under a photoperiod of 12 h light: 12 h dark. Fish were fed with balanced fish food suitable for these species (Primor, Brazil, 32% protein). All procedures using these fish were approved by the Committee on Ethics in Animal Experimentation of the Federal University of Paraná (CEUA/BIO 814/14).

After acclimation to laboratory conditions, each species of fish was randomly divided into 4 groups (12 fish per group), and each group was transferred to a static-test aquarium (25 L), keeping fish at a density not exceeding 1 g fish L^{-1}. The experiment was carried out by exposing the fish for 96 hours to one of the following conditions: control (filtered dechlorinated fresh water without copper), or copper at concentrations of 2, 20, or 200 µg Cu L^{-1} (copper sulfate; Quimidrol, Lot No. 4299). A stock solution of 20 mg Cu/10 ml (78.586 mg $CuSO4.5H_2O$ in 10 ml) was prepared, from which appropriate volumes were further diluted in the experimental 20 liter aquaria. All materials used for the experiment, including aquariums, were previously prepared for removal of possible copper contamination.

After 96 h of exposure, fish were anesthetized with benzocaine 80 $mg.L^{-1}$ (diluted in ethanol) and euthanized by spinal cord section. Gills, liver, brain and muscle samples were then obtained and kept at -80ºC until assayed.

Liver, an important tissue for biotransformation and antioxidant system, was used for the assays of the enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST), and for the determination of lipid peroxidation levels (LPO). The liver slices were homogenized in phosphate buffer (0.1 M) at pH 7.0, and centrifuged at 15,000 *x g* for 30 min, at 4°C. SOD activity was assayed at 440 nm based in the method proposed by Gao *et al.* (1998). CAT activity was evaluated at 240 nm according to the method proposed by Aebi (1984). GST activity was measured at 340 nm using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Keen *et al.* 1976). LPO analysis was carried out using the ferrous oxidation–xylenol assay at 570 nm (Jiang *et al*. 1992). Muscle and brain samples, both important tissues to analyses cholinesterases activities, were homogenized in phosphate buffer (0.1 M, pH 7.5), and centrifuged at 10,000 x g for 20 min, at 4 °C, and used for the assay of the acetylcholinesterase (AChE). AChE activity was measured spectrophotometrically at 405 nm, according to Ellman *et al*. (1961), modified by Silva de Assis, (1998). Gills samples were used for the assay of carbonic anhydrase (CA) activity. The gills samples were homogenized in a reaction medium containing mannitol (225 mM), sucrose (75 mM), and Tris-phosphate (10 mM), at pH 7.4. The branchial homogenates were centrifuged (~1,200 xg for 5 min at room temperature), and the supernatant was aliquoted for the protein and enzyme activity assays. CA activity was calculated as CAA= [(CR/NCR) −1]/mg total protein in the sample (Burnett *et al.* 1981, Vitale *et al.* 1999). Protein concentration of all tissue homogenates was determined using Bradford's method (1976), with bovine serum albumin as the standard at 595 nm.

The data analysis of all those biochemical biomarkers were preceded by the Kolmogorov–Smirnov normality test. Data were analyzed using the One-way Analysis of Variance (ANOVA), followed by the Bonferroni's test, using the *GraphPad Prism* 5.00 Software. All tests were regarded as statistically significant when $p < 0.05$.

Results

No mortality was observed during the experiments. The water quality values (mean ± standard error of mean) were as follows: pH (7.2 ± 0.2 units), alkalinity (34 ± 2 mg/L $CaCO_3$), ammonia levels (< 0.01 mg/L), dissolved oxygen (7.0 ± 0.2 mg/L), and temperature (26 ± 2ºC). There was a reduction in the AChE activity both in brain and muscle of *R. quelen* in the group exposed to 200 µg Cu L^{-1} (Fig. 1). However, copper exposure did not affect brain or muscle AChE activity in juvenile *O. niloticus* (Fig. 2).

Hepatic SOD and GST activities in *R. quelen* increased after acute exposure to higher concentration (200 µg Cu L^{-1}) when compared to the control group (Fig. 3a and c), whereas for CAT

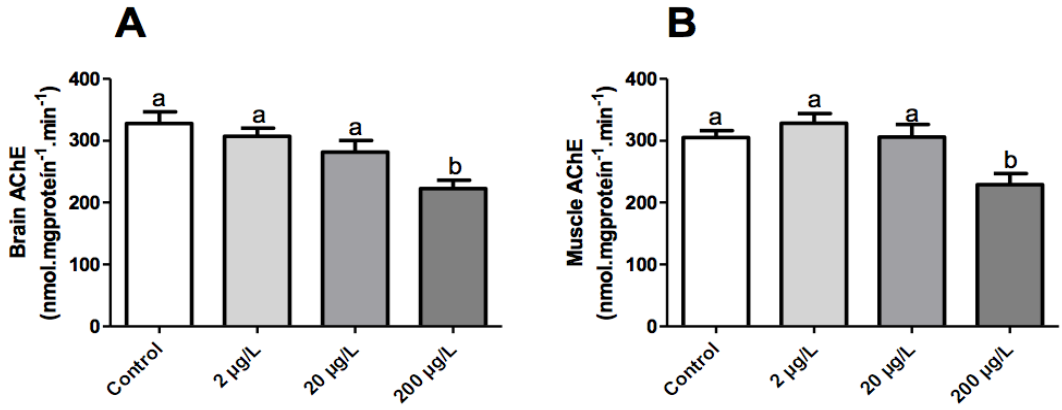

Figure 1. Acetylcholinesterase activity (AChE) in brain (A) and muscle (B) of *Rhamdia quelen* exposed to different copper concentrations (2, 20 or 200 µg L^{-1}) or only water (0 µg L^{-1}) for 96 h. Values are presented as mean ± SEM. Different letters indicate significant difference among groups (ρ<0.05). n=12, ANOVA, Bonferroni.

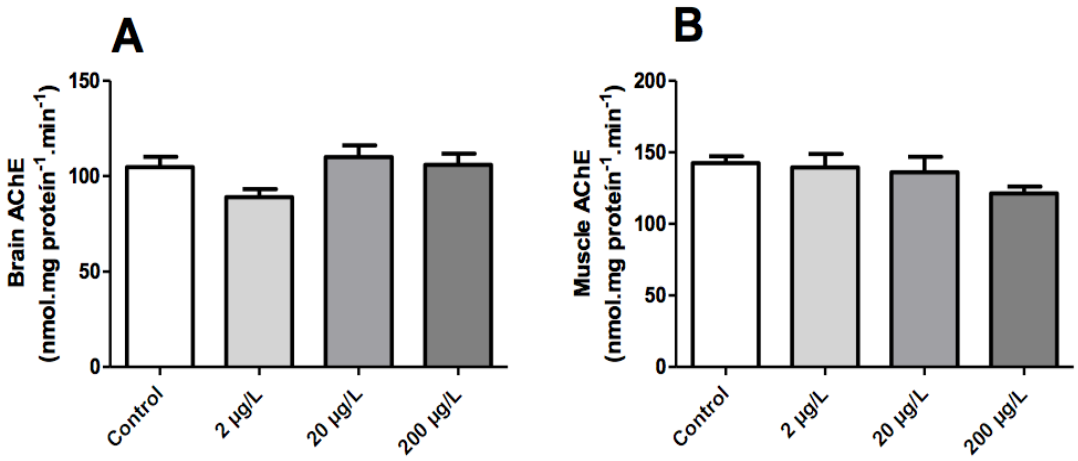

Figure 2. Acetylcholinesterase activity (AChE) in brain (A) and muscle (B) of *Oreochromis niloticus* exposed to different copper concentrations (2, 20 or 200 µg L^{-1}) or only water (0 µg L^{-1}) for 96 h. Values are presented as mean ± SEM. n=12.

activity and LPO level there was no significant difference among groups (Fig. 3b and d).

Hepatic SOD activities in *O. niloticus* had an increase after exposure to higher concentration of copper (200 µg L^{-1}) (Fig. 4a). The CAT activities did not exhibit any significant difference among groups (Fig. 4b). However, there was a significant decrease in GST in all tested copper concentrations (Fig. 4c). Furthermore, it was observed that in the lowest copper concentration used (2 µg Cu L^{-1}) there was an increase in lipid peroxidation (Fif. 4d). Both species, *R. quelen* and *O. niloticus* showed CA inhibition along increased dose of copper (Table I).

Discussion

The potential use of AChE activity as biomarker for monitoring environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during the recent years (Lavado *et al.* 2006). AChE is found mainly at neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission (Jebali *et al.* 2013). AChE activity reduction in the muscle and in the brain of *R. quelen* in the group exposed to 200 µg Cu L^{-1} indicates neurotoxicity induced by copper. AChE is reportedly sensitive to organophosphate and carbamate pesticides, in various species of fish. Furthermore, changes in AChE activity have been related to exposure to some metals (Rabitto *et al.* 2005, Pretto *et al.* 2010). Study published by Mela *et al.* (2013), with *R. quelen* exposed to 2, 7, and 11 µg Cu L^{-1}, for 96 hours, did not result in changes of the AChE enzyme activity, as it happened here with

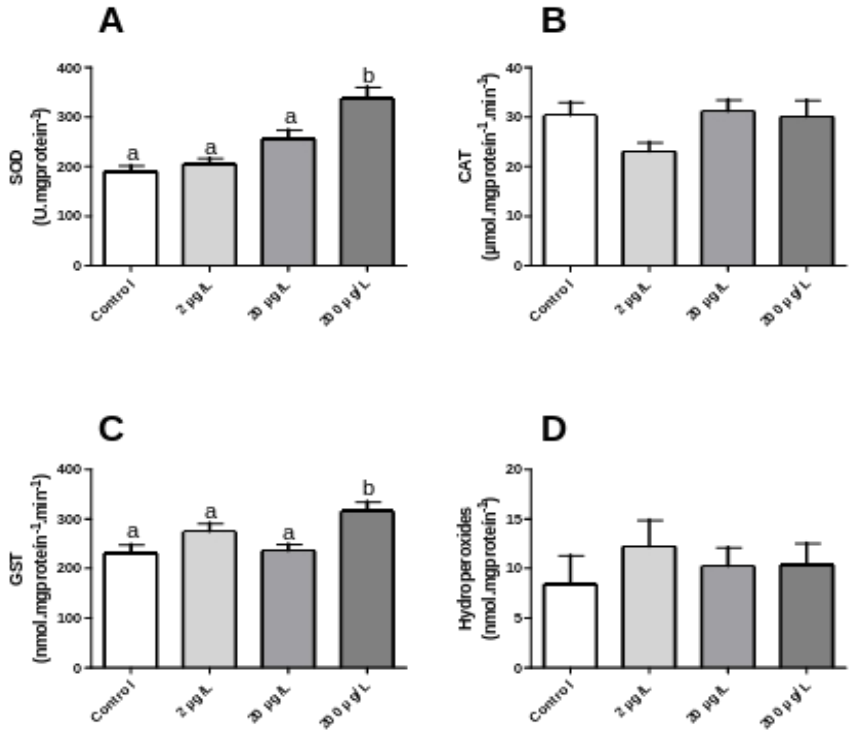

Figure 3. Superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione S-transferase (GST) activities (C) and determination of lipid peroxidation levels (LPO) (D) of *Rhamdia quelen* exposed to different copper concentrations (2, 20 or 200 µg L-1) or only water (0 µg L-1) for 96 h. Values are presented as mean ± SEM. Different letters indicate significant difference among groups ($\rho<0.05$). n=12, ANOVA, Bonferroni.

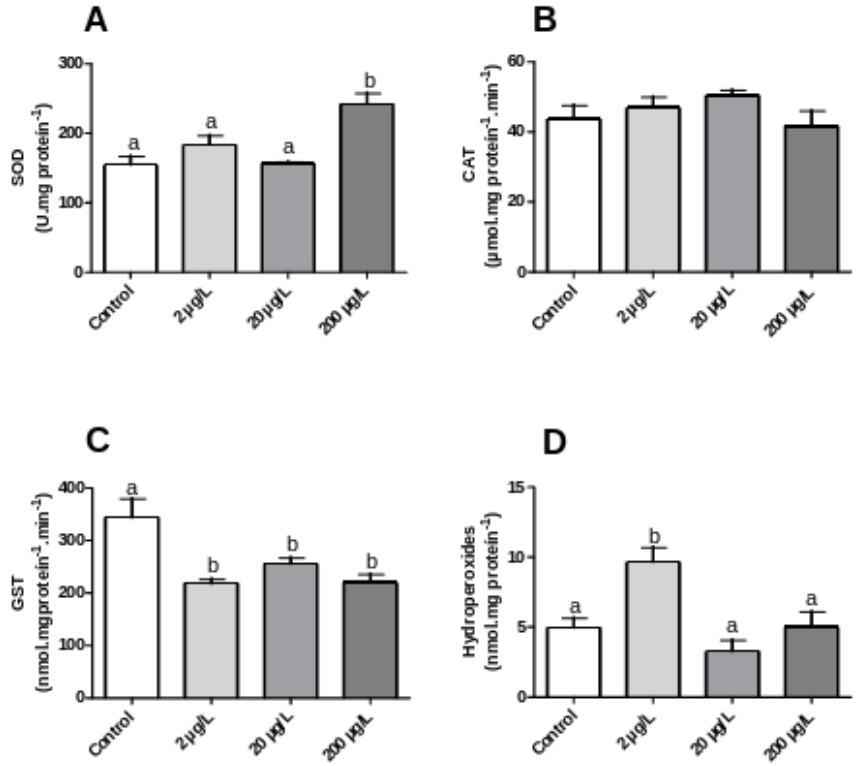

Figure 4 (previous page). Superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione S-transferase (GST) activities (C) and determination of lipid peroxidation levels (LPO) (D) of *Oreochromis niloticus* exposed to different copper concentrations (2, 20 or 200 μg L^{-1}) or only water (0 μg L^{-1}) for 96 h. Values are presented as mean ± SEM. Different letters indicate significant difference among groups (ρ<0.05). n=12, ANOVA, Bonferroni.

Table I. Specific activity of the branchial carbonic anhydrase (CA) of *Rhamdia quelen* and *Oreochromis niloticus* exposed to different copper concentrations (2, 20 or 200 μg L^{-1}) or only water (0 μg L^{-1}) for 96 h. Values are presented as mean ± SEM. Different letters indicate significant difference among groups (ρ<0.05). n=12, ANOVA, Bonferroni.

	Copper concentrations (μg L^{-1})			
	Control	2	20	200
Rhamdia quelen	7.5 ± 1.1^{a}	7.5 ± 0.6^{a}	4.1 ± 0.5^{b}	0.01 ± 0.02^{c}
Oreochromis niloticus	7.5 ± 1.2^{a}	5.8 ± 1.1^{ab}	4.1 ± 0.5^{b}	0.28 ± 0.25^{c}

juveniles of this same catfish, and that of the Nile tilapia, upon the lowest copper concentrations tested: 2 and 20 μg Cu L^{-1}. Juveniles of R. *quelen*, in the present experiment, responded similarly to adults of the same species, to acute copper exposure. Machado da Silva (2006) showed that the brain activity of AChE was reduced in *R. quelen* after exposure for 45 days to 40 and 80 μg Cu L^{-1}, while muscle AChE activity was not affected. According to Sturm *et al*. (1999), inhibition of AChE activity occurs when the animals are exposed to high concentrations of metals, corroborating with the findings of this study, that AChE activity was reduced only at the highest concentration. In contrast, some authors showed AChE stimulation due to sub chronic exposure of copper in different species of fish, as Romani *et al.* (2003), with *Sparus auratus* (20 days of exposure) and Gioda *et al.* (2003), with *Leporinus obtusidens* (45 days of exposure). Stimulation of enzyme activity is actually a possibility, as a compensatory response upon chronic exposure, for instance, to a mixture of contaminants in the field, such as was proposed in Freire *et al.* (2015), using small characins as bioindicators (*Astyanax* spp.).

O. niloticus is an useful species to be used as bioindicator, given its widespread occurrence and availability, and reported ability to accumulate metals (Zhou *et al.* 1998, Ay *et al.* 1999, Dang *et al.* 1999). In the tilapia, the induction of the expression of the metalothionein gene has been demonstrated (Cheung *et al.* 2004). This induction may relate to the fact that copper exposure under the protocol employed here did not affect brain or muscle AChE activity in juvenile *O. niloticus*; juveniles of this species were more resistant to copper neurotoxicity than *R. quelen.* According to Lam *et al*. (1998), *O. niloticus* is relatively resistant to copper with a 24h LC_{50} value of 2,820 μg L^{-1} and 96 h LC_{50} value of 1,520 μg L^{-1}, concentrations even higher than used in this experiment.

Many environmental pollutants are capable of inducing oxidative stress in animals by disturbing the antioxidant capacity of the cells, and enhancing intracellular ROS levels, which often result in DNA damage, LPO, and enzyme inhibition (Van der Oost *et al.* 2003). The liver is known to be an important organ for coping with oxidative stress, as it has the highest antioxidant enzyme activities to protect them from oxidative stress caused by metals (Atli *et al.* 2006, Atli and Canli, 2010). Antioxidant enzymes are crucial in the effort to counteract oxidative stress caused by toxicants, once the supply of other antioxidant compounds are depleted (Martinez-Alvares *et al.* 2005). Antioxidant responses have been suggested as biomarkers of exposure to metals in aquatic organisms (Atli and Canli 2010). The roles of antioxidant enzymes can be summarized as follows: superoxide dismutase (SOD) converts superoxide anion radical to hydrogen peroxide and catalase (CAT) reduces hydrogen peroxide to water. Glutathione S-transferase (GST) has multiple biological roles, including cell protection against oxidative stress and several toxic molecules, and is involved in the synthesis and modification of leukotrienes and prostaglandins. As an example, GSTs protect cellular DNA against oxidative damage that can lead to an increase in DNA

mutations or induce DNA damage (Allocati *et al*. 2018). Lipoperoxidation (LPO) quantification, in particular, has been found to have a high predictive value as a biomarker of effect (Abdel-Moneim *et al*. 2012), being involved in pathological processes and in the etiology of many fish diseases. LPO has been reported as a major contributor to the loss of cell function under oxidative stress conditions (Storey 1996). It has been reported that LPO may be induced by a variety of environmental contaminants (Pandey *et al.* 2008, Carvalho *et al.* 2012).

The SOD-CAT system provides the primary defense against oxygen toxicity. The SOD has great importance to allow the organisms to survive in the presence of O_2 and to tolerate increases in the concentration of ROS (Halliwell and Gutteridge, 1985). According to Sanchez *et al*. (2005), being a metaloenzyme, SOD has a fast response to copper exposure as a result of the metal interaction with the enzyme. CAT is a common enzyme, found in nearly all cells, mainly in the liver and blood of vertebrates. Given that copper toxicity is exerted by a mechanism of enhanced ROS generation, it would be expected that exposed animals modulate their antioxidant defense system, to prevent damage of macromolecules such as proteins, lipids, DNA, carbohydrates (Geracitano *et al.* 2004).

GSTs catalyze the conjugation of GSH — via a sulfhydryl group — to electrophilic centers on a wide variety of substrates in order to make the compounds more water soluble. This activity detoxifies endogenous compounds such as peroxidized lipids and enables the breakdown of xenobiotics (Salinas and Wong, 1999). In addition, GST activities increased after acute exposure to copper (200 μg L^{-1}). The GST can be considered an antioxidant enzyme for facilitating the elimination of ROS. The increase at the exposure to 200 μg Cu L^{-1} may have increased ROS, leading to an increase in the antioxidant enzymes (SOD and GST). However the no change in LPO levels indicating that probably these enzymes prevented oxidative stress in cells of these fish exposed to copper. The result of no increase in CAT - an enzyme that acts in a SOD-subsequent step to reduce oxidative damage - could be explained by an increase in GPx activity, since this peroxidase also operates in hydrogen peroxide conversion to O_2 and water, but in a different way. Unfortunately, due to the small size of the livers of juvenile specimens, GPx was not evaluated here. Craig *et al*. (2007) also observed increased activity of SOD without alterations in CAT activity after acute copper exposure (8 and 15 μg L^{-1} Cu for 48 h) in *Danio rerio*. After copper exposure (0, 25, 100 and 200 μg.L^{-1}) in the stickleback, *Gasterosteus aculeatus aculeatus*, Sanchez *et al*. (2005) observed rapid increase in SOD and CAT activity, these doses are similar to those used in this experiment.

O. niloticus also had an increase in SOD activity after exposure to the highest concentration of copper (200 μg L^{-1}), with no accompanying increase in CAT activity. However, the decrease of GST in all tested copper concentrations may impair the biotransformation and protection against the oxidative stress resulting from the increase of ROS. In zebrafish, *Danio rerio* exposed to 40 and 140 μg L^{-1} of $CuSO_4$, for two weeks, there was inhibition of activity on GST and CAT (Paris-Palacios *et al.* 2000). Likewise, in common carp, CAT and GST were inhibited after 96 h of exposure to copper at concentrations of 100 and 250 μg L^{-1} (Dautremepuits *et al.* 2002).

Concerning oxidative damage, in *O. niloticus*, there was an increase in lipid peroxidation in the lowest copper concentration used (2 μg Cu L^{-1}) demonstrating the occurrence of oxidative stress. LPO was high only at the lowest tested concentration, therefore the effects were possibly reversed, under higher copper concentrations, due the activation of the antioxidant enzymatic systems. Toxic effects of fish exposure to copper have been reported in several studies; however, most of those studies evaluated the effect of copper on adult animals. In fish there are reports that copper can either stimulate or inhibit the antioxidant enzymes, depending on several parameters, as the concentration, species, and route of exposure or experimental conditions. In a study conducted by Dogan *et al*. (2014), adult fish *O. niloticus* were exposed to copper in soft water (SW) (~ 80 mg CaCO3 L^{-1}, conductivity 1.77 mS/cm) or hard water (HW) (~ 320 mg CaCO3 L^{-1}, conductivity 5.80 mS/cm) using 2 exposure protocols (acute: 20 μM for 48 h and subchronic: 10 μM for 144 h) and antioxidant enzyme activities were measured in the liver of fish. In acute and subchronic copper exposures SOD activity increased in both waters and CAT activity increased in SW and decreased in HW.

Copper exposure increased the activity of antioxidant enzymes in erythrocyte of *Dicentrarchus labrax* (Gwozdzinski *et al.* 1992) and increased the CAT activity in the liver and kidney of common carp (Dautremepuits *et al.* 2004). Upon copper intraperitoneal administration, there was inhibition of SOD in carp after 48 h (Varanka *et al.* 2001)

while the enzyme was rapidly induced in *Spaurus aurata* (Pedrajas *et al.* 1995).

The CA is a metalloenzyme crucial for several essential physiological processes, and which catalyzes the reversible hydration of CO_2, yielding H^+ and HCO_3^-. Given its widespread occurrence in bacteria, plants and animals, this enzyme can be used as a general biomarker in toxicology, and environmental monitoring studies. CA activity as a biomarker has been shown to be especially relevant for the detection of the toxic effects of metals and pesticides (Lionetto *et al.* 2012, Mela *et al.* 2013). At the present study the CA activity was affected by the exposure to copper in both species. However, a dose-dependent effect was more evident in *R. quelen* than in *O. niloticus*.

Confirming metal/copper toxicity to the enzyme carbonic anhydrase (Lionetto *et al.* 2012), similar results have been observed before, in Mela *et al.* (2013), in adult specimens of *R. quelen* (30 ± 2g) which were exposed to control, 2, 7, 11 µg Cu/l conditions for 96 hours, with resulting inhibition of CA at the 7 µg condition. Zimmer *et al.* (2012), used *Poecillia vivipara* (0.1 - 1.1 g) with 12 and 96 hours of exposure to 20 µg/l of copper sulphate, both in freshwater and in seawater, and observed gill CA inhibition at 12 hours and recovery at 96 hours. Gill CA activity inhibition was also observed in Souza-Bastos *et al.* (2014) where *R. quelen* were exposed to three ammonia concentrations for 5 and 24 hours, and this inhibition was more evident at 24 hours and at higher concentrations, similar to our results. Our data confirm this trend, showing a clear inhibition of the enzyme CA activity by copper. In summary, gill CA activity is a good biomarker due to its sensitivity to metals (as well as other polluting agents), showing concentration-dependent inhibition upon short-term exposure (Christensen and Tucker, 1976; Lionetto *et al.* 2000; 2012).

Conclusions

Juveniles of the species *O. niloticus* and *R. quelen* were differently affected by copper. While *O. niloticus* AChE activity was unaffected by copper, in *R. quelen* copper exposure (200 µg L^{-1}), under the conditions employed here, reduced AChE activity in both brain and muscle, demonstrating that *R. quelen* is more sensitive to copper-induction neurotoxicity. The antioxidant system of both species responded differently, but both were disturbed by copper. The antioxidant system of *R. quelen* was activated and it was able to metabolize the reactive species avoiding the occurrence of oxidative stress. The same response was not observed in *O. niloticus*, even thought there was an increase in the SOD activity, the GST activity was inhibited by all concentrations of copper and the metal still caused oxidative stess in this species. Both species had also their branchial carbonic anhydrase strongly inhibited by copper, in a distinct dose-dependent manner. Thus, these two cultivated species responded differently, but were both sensitive to aquaculture and environmentally-realistic levels of copper in the water.

Although most of the disturbances observed in the present study occurred under the highest concentration (Copper 200 µg L^{-1}), as reduction of AChE activity and increase of SOD, some disruptions occurred also in the dose below that recommended by the regulatory agencies for aquaculture (Copper 2 µg L^{-1}) - increased LPO and CA. US EPA (1984) copper concentration limits in water for the protection of aquatic life is 20 µg Cu L^{-1} and in Brazil the maximum level allowed for dissolved copper in aquaculture water is 9 µg Cu L^{-1} (CONAMA, 2005). The results obtained in this study demonstrate that levels of copper currently used in aquaculture are not entirely safe for fish juveniles of *R. quelen* and *O. niloticus*. Further studies are needed for improving the elaboration of protocols of safer use of copper and to avoid impacts to the surrounding natual water bodies, and to fish species consumed by humans.

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