

Exposure to waterborne copper and high temperature induces the formation of reactive oxygen species and causes mortality in the Amazonian fish *Hoplosternum littorale*

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Received: 23 December 2015 / Revised: 24 May 2016 / Accepted: 28 May 2016 / Published online: 9 June 2016
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Abstract *Hoplosternum littorale* is an Amazon fish that lives in urban areas surrounded by polluted *igarapés*, where elevated copper concentrations eventually occur. The central goal of this study was to evaluate the associated effects of high temperature and copper contamination on survival time and biochemical responses of the Amazonian fish species *H. littorale*. We exposed fish to two nominal dissolved copper concentrations (50 and 500 $\mu\text{g l}^{-1}$) and combined temperatures of 28 and 34°C. Our findings showed that the combination of these variables affects the survival time of this species. The activity of the biotransformation enzymes ethoxyresorufin-*O*-deethylase and glutathione-*S*-transferase showed no alterations in fish within all treatments. The increase of reactive oxygen species and the decrease in potential

total antioxidant capacity promoted the imbalance in the antioxidant system. An induction in superoxide dismutase activity occurred in fish exposed to copper concentrations of 50 and 500 $\mu\text{g l}^{-1}$ at both temperatures, suggesting liver impairments. Thus, we suggest that *H. littorale* is sensitive to copper, and this sensitivity is increased further with exposure to high temperatures, particularly in the survival time and reactive oxygen species formation of this fish species.

Keywords Antioxidant potential · Catfish · Copper binding · Oxidative stress · Climate change

Introduction

Current global climate challenges and their consequences are considered important threats to aquatic ecosystems' health and chemical safety (Daufresne et al., 2009; Noyes et al., 2009). According to IPCC projections (2013), the global mean surface temperature is expected to increase 6°C by the end of this century, and freshwater environments will be most affected (Straile et al., 2013). Moreover, the human release of contaminants into the aquatic environment results in increased metal concentrations. When water temperature rises, metal solubility and bioavailability also increase (Schiedek et al., 2007). These facts all together can lead to severe impairments to aquatic organisms (Gomiero & Viarengo, 2014; Lee et al., 2014).

Guest editors: Adalberto L. Val, Gudrun De Boeck & Sidinei M. Thomaz / Adaptation of Aquatic Biota of the Amazon

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Copper (Cu) is a trace metal naturally found in aquatic environments (Yu-Ting Lim et al., 2015). It is an essential micronutrient for living organisms, acting as the cofactor for many enzymes involved in vital biological processes (Jiang et al., 2014). However, high levels of waterborne Cu can trigger a range of adverse effects in fish, such as ion regulation impairments, reduced growth, behavioral changes, biotransformation enzyme disturbances, and oxidative stress (Matsuo et al., 2004, 2005; Lushchak, 2011; Duarte et al., 2009; Grosell, 2012; Wang et al., 2014).

The effect of copper toxicity in freshwater aquatic organisms is dependent on a variety of environmental parameters such as pH (Cusimano et al., 1986; Erickson et al., 1996), dissolved organic carbon (DOC) (Welsh et al., 1993; Erickson et al., 1996), and environmental temperature (Carvalho & Fernandes, 2006). In acute exposure experiments, complexation of Cu by dissolved organic carbon (DOC) reduces Cu binding to the gills (Playle et al., 1993) and, therefore, reduces acute toxicity in fish via reductions in free ionic waterborne Cu^{2+} (Erickson et al., 1996).

The exposure to excessive Cu ions has been described as a promoter of oxidative stress by catalyzing the formation of reactive oxygen species (ROS) and generating enzyme inactivation, protein degradation, DNA damage, and peroxidation of the lipid membranes (Van der Oost et al., 2003; Monteiro et al., 2009). These parameters are widely used as biomarkers of oxidative stress in resident species, allowing for early detection of environmental contamination, before the wellness of aquatic organisms is threatened (Frenzilli et al., 2009; Osman et al., 2010).

Environmental temperature increases may promote the ROS generation, cellular oxidation, and responses of antioxidant enzyme systems. For much freshwater fish, oxidative stress may have serious deleterious effects modifying many cellular functions and even causing death (Lushchak & Bagnyukova, 2006). Recent climate changes can increase the temperature in Amazon region (Hoorn et al., 2010) and can strengthen the effects of Cu ions on fish. Additionally, *Hoplosternum littorale* (Hancock, 1828), a native South American fish that inhabits Rio Negro blackwaters, are commonly found in urbanized and industrialized areas, being subjected to Cu contamination.

In the present study, we aimed to investigate the influence of temperature increase on Cu toxicity in the

biochemical responses of *H. littorale*. We hypothesized that different acute Cu exposure would negatively affect the fish physiology by induction of oxidative stress and that these effects are stronger when associated to high temperature, particularly for the survival time and ROS production.

Materials and methods

Fish sampling and experimental setup

Experiments were conducted during an expedition of ADAPTA Project (Adaptations of Aquatic Biota of the Amazon) to Anavilhanas National Park ($2^{\circ}71'97.10''\text{S}$; $60^{\circ}75'52.99''\text{W}$), Amazon State, Brazil, in December 2014. Adult specimens of *H. littorale* were obtained from research catches carried out in the Rio Negro (Lago Catalão, $3^{\circ}10'04''\text{S}$ e $59^{\circ}54'45''\text{W}$) and brought on board the vessel Ana Clara in aerated containers. Individuals of *H. littorale* (105.8 ± 5.2 g and 14.5 ± 1.5 cm) were caught and maintained in tanks of 150 l with Rio Negro water for 24 h for handling recovery. Then, the fish were divided into two groups in 150 l polystyrene clean tanks and exposed to two different temperatures: 28°C (similar conditions of Rio Negro water) and 34°C (representing 6°C increase of temperature as devised by severe IPCC emission scenario). Water temperature was gradually increased by $1.0 \pm 0.1^{\circ}\text{C}/\text{h}$, using water thermal regulators (Aquarium Heater H-606, Hopar[®]) until the appropriate temperature was reached as pointed out by Mora & Moya (2006). Then, each group was subdivided into three subgroups and distributed simultaneously into six containers filled with 120 l Rio Negro aerated water and exposed to CuSO_4 (Sigma) solution at the following nominal concentrations of copper: 0, 50, and $500 \mu\text{g l}^{-1}$. The real concentrations of copper in the treatment were 47 ± 0.9 and $417 \pm 1.7 \mu\text{g l}^{-1}$, corresponding, respectively, to nominal concentrations of $50 \mu\text{g l}^{-1}$ and 500. Herein we will use nominal concentrations to easy text reading.

The treatments were as follows: (A) $28^{\circ}\text{C} \rightarrow 0 \mu\text{g l}^{-1}$; (B) $28^{\circ}\text{C} \rightarrow 50 \mu\text{g l}^{-1}$; (C) $28^{\circ}\text{C} \rightarrow 500 \mu\text{g l}^{-1}$; (D) $34^{\circ}\text{C} \rightarrow 0 \mu\text{g l}^{-1}$; (E) $34^{\circ}\text{C} \rightarrow 50 \mu\text{g l}^{-1}$; and (F) $34^{\circ}\text{C} \rightarrow 500 \mu\text{g l}^{-1}$. Daily, a fourth of aquarium's water was gently siphoned and replaced with an equal volume of water of the same

water temperature and Cu concentration. Physicochemical parameters were also monitored daily using a YSI multiparameter probe (model 85, Yellow Springs Instruments).

Aquaria water conditions were as follows: temperature (28.1 ± 0.3 and $34 \pm 0.3^\circ\text{C}$), dissolved oxygen (5.3 ± 0.5 and $4.2 \pm 0.4 \text{ mg l}^{-1}$), and pH 5.2. Total calcium ($0.4 \pm 0.02 \text{ }\mu\text{M}$), potassium ($12.4 \pm 0.06 \text{ }\mu\text{M}$), magnesium ($0.2 \pm 0.1 \text{ }\mu\text{M}$), and sodium ($28.5 \pm 1.8 \text{ }\mu\text{M}$) ion concentrations were read on a Perkin-Elmer model 3100 atomic absorption spectrophotometer (AAS) and total chloride ($0.10 \pm 0.2 \text{ }\mu\text{M}$) was measured by the colorimetric method described by Clarke (1950). Dissolved organic carbon (DOC) ($7.7 \pm 0.16 \text{ mg C l}^{-1}$) was read on a total carbon analyzer (Apollo 9000 combustion TOC analyzer, Teledine Tekmar).

During acute exposure tests, the survival times were recorded at the exact moment that fish exhibited total loss of equilibrium or at the end of the experiment (96 h). Then, animals were weighed, measured, and immediately euthanized by cervical sectioning. Hepatic tissue was carefully excised, and part of the liver was directly snap-frozen in liquid nitrogen until analyses of biotransformation and antioxidant enzymes and oxidative parameters were performed.

Copper speciation modeling

Visual MINTEQ ver. 3.1 (Gustafsson, 2010) with the NICA-Donnan model was used to determine the speciation of Cu in the 50 and 500 $\mu\text{g l}^{-1}$ treatment waters at 28 and 34°C respectively. Copper speciation was expressed as percent distribution for all treatments.

Biochemical analyses

Activities of hepatic ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase (GST), and superoxide dismutase (SOD), concentration of the lipid peroxidation (LPO), total antioxidant capacity, ROS, and antioxidant capacity against peroxy radicals (ACAP) were measured. For GST, SOD, and LPO measurements, frozen liver samples were weighed and homogenized (1:4 w/v) in 20 mM Tris buffer (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 500 mM sucrose, and 150 mM KCl. For EROD activity, samples were homogenized (1:4 w/v) in a buffer containing 20 mM HEPES and 15 mM KCl, pH 7.5.

To measure the total antioxidant capacity, ROS assays, fresh liver were homogenized (1:20 w/v) in a buffer solution (pH 7.75) containing 100 mM Tris-HCl, 2 mM EDTA, and 5 mM MgCl_2 . Homogenates were then centrifuged at $10,000\times g$ for 20 min at 4°C. All assays were run on either a SpectraMax Plus 384 spectrophotometer (Molecular Devices) (GST, SOD, and LPO) or a SpectraMax M2 fluorometer (Molecular Devices) (EROD and ROS/ACAP).

EROD activity was performed by the fluorimetric method described by Hodson et al. (1991). Microsomes were isolated from homogenized hepatic samples and added to a reaction mixture containing 100 mM HEPES, 1.28 mM MgSO_4 , 40 mg ml^{-1} bovine serum albumin (BSA), and 0.5 mM NADPH. The reaction was started by the addition of 0.12 mM ethoxyresorufin and was stopped by addition of HPLC-grade methanol (Tedia Brazil). The change in fluorescence was recorded (excitation wavelength 530 nm, emission wavelength 585 nm), and the enzyme activity is expressed in $\text{pmol min}^{-1} \text{ mg}^{-1}$ protein with Resorufin used as the standard.

GST activity was determined according to Keen et al. (1976) by measuring the increase in absorbance at 340 nm, incubating reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as the substrates. GST activity is expressed as $\text{U min}^{-1} \text{ mg protein}^{-1}$.

SOD activity was measured by the indirect method involving the reduction of cytochrome c in the presence of the xanthine oxidase/hypoxanthine system at 550 nm (Flohé & Ötting, 1984). SOD activity is expressed as $\text{U min}^{-1} \text{ mg protein}^{-1}$.

LPO was assessed by Fe^{2+} oxidation in the presence of xylenol orange (FOX, ferrous oxidation-xylenol orange assay) as described by Jiang et al. (1991). For this assay, homogenized samples were treated with 10% TCA (trichloroacetic acid) and centrifuged at $2,236\times g$ for 10 min at 4°C. The supernatants were added to a reaction mixture containing 100 μM xylenol orange, 4 mM $\text{C}_{15}\text{H}_{24}\text{O}$, 25 mM H_2SO_4 , and 250 μM FeSO_4 dissolved in 90% methanol. Samples plus reaction mixture were incubated for 30 min at room temperature for color development before colorimetric measurement at 560 nm. LPO concentration was expressed as $\mu\text{mol cumene hydroperoxide mg protein}^{-1}$.

Reactive oxygen species (ROS) and total oxyradical scavenging capacity (ACAP) were measured

according to the method of Amado et al. (2009). In the homogenates were added the reaction buffer (30 mM HEPES, 200 mM KCl, and 1 mM MgCl₂, pH 7.2) and the fluorescent dye 2',7' dichlorofluorescein diacetate (H2DCF-DA). The acetate groups of H2DCF-DA are cleaved by intracellular esterases present in sample supernatants. After that, the non-fluorescent compound H2DCF was oxidized by ROS to the fluorescent compound, DCF, which was detected in the wavelengths of 488 (excitation) and 525 (emission) nm. Total fluorescence production was calculated by integrating the fluorescence units (FU) over the measurement time and expressed as the area of fluorescence. The results were expressed as area difference of FU × min in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The relative difference between ROS area with and without ABAP was considered as a measure of total antioxidant capacity (ACAP).

For all assays, total protein content was determined previously using the Bradford (1976) method adapted to the microplate reader.

Statistical analysis

The values are presented as mean ± SEM (Standard Errors of Means; $n = 6$). Prior to the comparative statistical tests, distribution and homogeneity of data were checked. All data were analyzed by two-way ANOVA test, with temperature and copper concentration as the factors, followed by Tukey's post hoc test for comparisons. Statistical significance was accepted at the level of $P < 0.05$ (Zar, 1984). All statistical tests were run using SigmaStat 3.5 and the graphs were plotted using SigmaPlot 11.0 software.

Results

Survival time assays

In our findings, the highest temperature (34°C) caused decreases in survival time of animals exposed to both copper concentrations (50 and 500 µg l⁻¹). Fish in the lowest temperature (28°C) presented low survival time (less than 12 h) only when exposed to 500 µg l⁻¹ Cu (Fig. 1).

Copper speciation modeling

Metal speciation modeling (Table 1) showed that the binding of Cu with DOC molecules was dependent upon temperature and concentration. In the present experiments, the free ionic waterborne Cu²⁺ represents less than 1% for both Cu concentrations (50 and 500 µg l⁻¹) and water temperatures (28 and 34°C). Over 50% was weakly bound to DOC for Cu + 2D at both temperatures for the nominal concentration of 50 µg l⁻¹ of Cu, and over 40% was organically complexed with FAI 1 (carboxylic functional groups) and less than 1% with FAI 2 (phenolic functional groups). Additionally, more than 70% was weakly bound to DOC for Cu + 2D for the highest nominal Cu concentration (500 µg l⁻¹), while the Cu organically complexed for FAI 1 was less than 30% and FAI 2 less than 0.5% at both temperatures. We observed that the highest metal concentration makes it difficult the Cu organic complexation with carboxylic and phenolic functional groups of the DOC while facilitating a weak binding to DOC for Cu + 2D. Temperature increase has a similar but weaker effect.

Biotransformation enzyme activity

The results showed that biotransformation pathways were not activated in any treatments, as suggested by no alteration of the activities EROD ($F = 0.973$; $P = 0.391$) and GST ($F = 0.203$; $P = 0.818$) (Fig. 3).

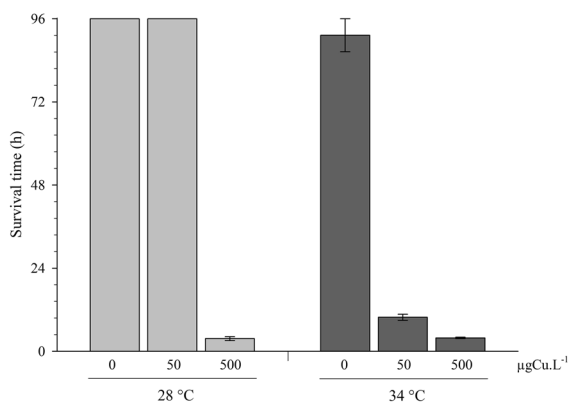


Fig. 1 Survival time of *Hoplosternum littorale* under control conditions (no copper addition) and exposed to two different copper concentrations (50 and 500 µg Cu l⁻¹) at 28 and 34°C, respectively. $N = 6$

Table 1 Copper speciation expressed as percent distribution in experimental waters at the concentrations of 50 and 500 $\mu\text{g l}^{-1}$ Cu and the temperatures of 28 and 34°C

| Copper speciation | % Distribution | | | |
|-------------------|----------------------------|-------|-----------------------------|-------|
| | 50 $\mu\text{g l}^{-1}$ Cu | | 500 $\mu\text{g l}^{-1}$ Cu | |
| | 28°C | 34°C | 28°C | 34°C |
| Cu + 2 | 0.06 | 0.04 | 0.92 | 0.66 |
| (6)Cu + 2D(aq) | 53.88 | 58.51 | 70.98 | 73.3 |
| FA1–Cu(6)(aq) | 45.25 | 40.77 | 27.60 | 25.51 |
| FA2–Cu(6)(aq) | 0.79 | 0.66 | 0.48 | 0.42 |

(6) $\text{Cu}^{2+}\text{D}(\text{aq})$ (weakly bound Cu to DOC), FA1–Cu(6)(aq), FA2–Cu(6)(aq) (organically complexed Cu to dissolved organic acid). Sites 1 and 2 refer to carboxylic and phenolic functional groups, respectively. The program Visual Minteq 3.1 generated these data. $N = 6$

ROS and ACAP analyses, SOD activity, and LPO concentration

ROS levels showed no changes when the animals were exposed to 50 $\mu\text{g l}^{-1}$ Cu at 28°C. Any other condition induced ROS elevation. Moreover, we observed that the ROS increase was dependent of the interaction with the temperature and the concentration in 50 $\mu\text{g l}^{-1}$ Cu at 34°C ($F = 7.745$; $P = 0.002$) (Fig. 2A).

As regards ACAP, the exposure in 50 $\mu\text{g l}^{-1}$ Cu in 28 and 34°C promoted, respectively, an increase of 1.7- and 1.6-fold in the liver ACAP relative area ($F = 10.535$; $P = 0.001$). Antioxidant alterations also were observed in 500 $\mu\text{g l}^{-1}$ Cu by the increase of 1.4-fold at 34°C (Fig. 2B). The temperature did not change scavenging capacity in any situation.

SOD activity showed a concentration-dependent response for *H. littorale*, where the concentrations of 50 and 500 $\mu\text{g l}^{-1}$ induced the increase of SOD by 2.7- and 4.4-fold, respectively, compared to 0 $\mu\text{g l}^{-1}$ at 28°C ($F = 51.726$; $P = 0.001$). At the highest temperature, the increase was 3- and 4-fold after exposure to 50 and 500 $\mu\text{g l}^{-1}$, respectively (Fig. 4A).

Hepatic LPO levels did not change after the exposure to different Cu treatments at both temperatures (Fig. 4B), revealing no membrane damage in the analyzed fish ($F = 0.0854$; $P = 0.918$).

Discussion

In the present work, alterations in oxidative liver parameters were observed in *H. littorale* at both

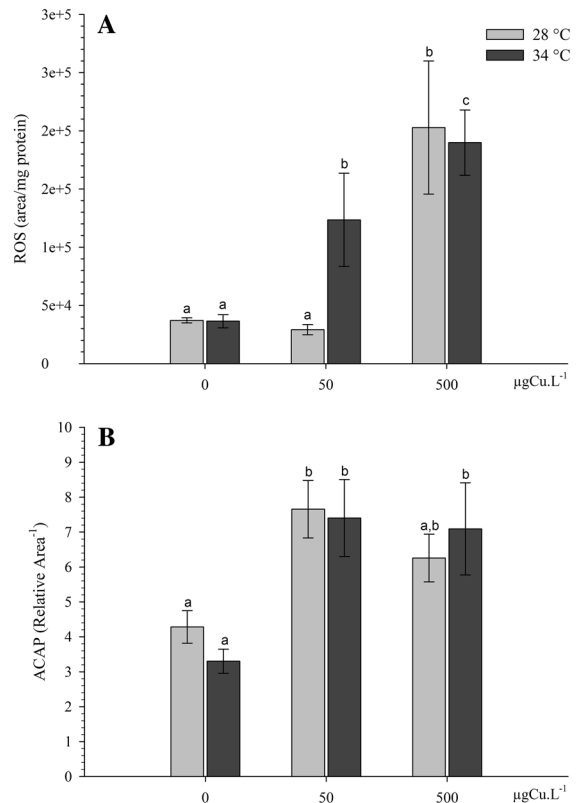


Fig. 2 Reactive oxygen species (ROS) (A) and antioxidant capacity against peroxyl radicals (ACAP) (B) in fresh liver samples from *H. littorale* under control conditions (no copper addition) and exposed to two different copper concentrations (50 and 500 $\mu\text{g Cu l}^{-1}$) at 28 and 34°C, respectively. $N = 6$. Different letters indicate significant differences between treatments ($P < 0.05$)

copper concentrations (50 and 500 $\mu\text{g l}^{-1}$ Cu) and temperatures (28 and 34°C). Richards et al. (1999) observed that the DOC from natural organic matter sources and commercially available DOC (Sigma humic acid) were both effective in reducing physiological impacts of metals in *Oncorhynchus mykiss*. It is also known that high temperatures can lead to higher metal solubility and free metal ions increasing metal bioavailability and uptake rates (Worms et al., 2006), thus increasing metal toxicity. In our findings, the copper speciation model generated for the present experimental conditions (Table 1) showed that complexation of Cu^{2+} with DOC is dependent upon copper concentrations and temperature. Thus, the lower complexation of Cu^{2+} with DOC at higher concentration and temperature caused an increase in bioavailability of the metal, increasing its toxicity to the fish,

as observed for survival time (Fig. 1) and ROS production of the animals (Fig. 2A). Our findings showed that over 90% of Cu^{2+} was somehow bound to DOC (weakly or complexed) resulting in less than 10% of total copper at 34°C available to fish. Thus, the amount of copper uptake by fish may not have been enough to activate biotransformation pathways, as observed by the EROD and GST enzymes (Fig. 3).

Since environmental factors, such as water chemistry and temperature, influence the toxicity of copper, these factors can also indirectly modulate physiological responses such as alterations involved in oxidative metabolism in the organism (Pottinger et al., 2002). For example, Sanchez et al. (2005), evaluating the effect of copper in water with very low DOC concentration, found a decrease in the EROD activity and a consequent increase in GST activity in

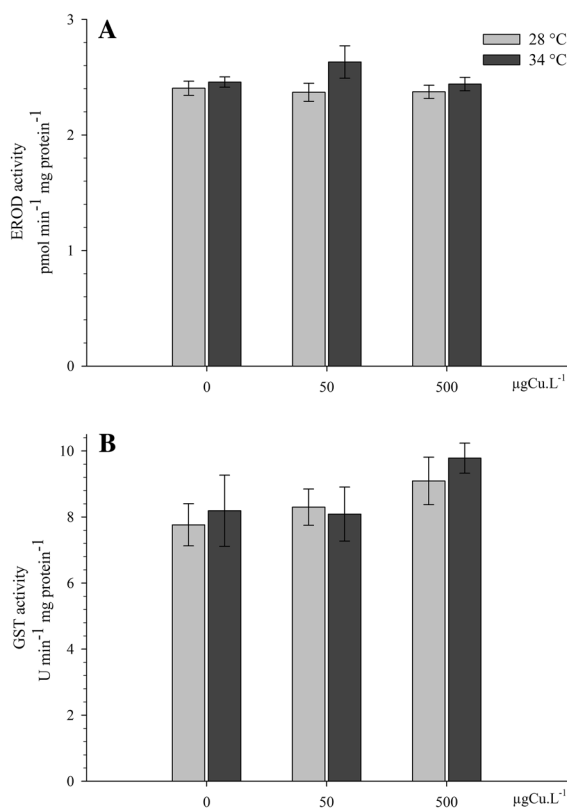


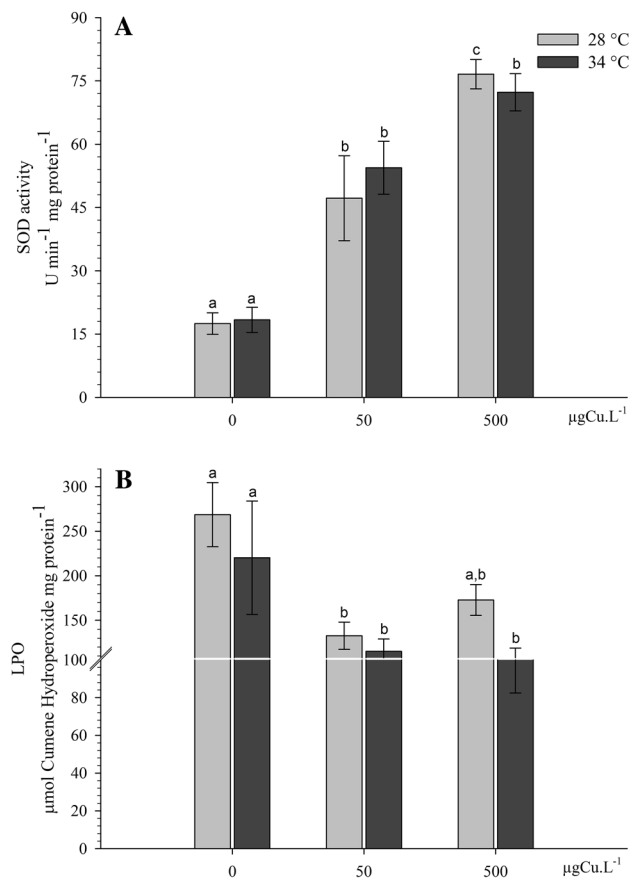
Fig. 3 Ethoxyresorufin-*O*-deethylase (EROD) (A) and glutathione-*S*-transferase (GST) (B) activities in liver samples from *H. littorale* under control conditions (no copper addition) and exposed to two different copper concentrations (50 and $500 \mu\text{g l}^{-1}$) at 28 and 34°C , respectively. $N = 6$. See Fig. 2 for the caption

Gasterosteus aculeatus exposed to copper sulfate at 25, 100, and $200 \mu\text{g l}^{-1}$. Similar results were observed for *Pomatoschistus microps* exposed to identical concentrations of copper in the artificial medium with very low DOC concentrations, revealing the same pattern for these two enzymes (Vieira et al., 2009). However, as mentioned above, in this study no difference was observed between treatments in regards to these two enzymes. Both EROD and GST did not change in any experimental conditions, suggesting that these biotransformation enzymes were not necessary for such conditions. It is important to mention that in both cases the water used in those experiments (Sanchez et al., 2005; Vieira et al., 2009) contained very low concentrations of DOC, different from the conditions of the present work, where the water had high levels of DOC, around 8 mg C l^{-1} . According to Wood et al. (2011), interactions between the DOC and copper may lead to an indirect protective effect in the gills preventing the uptake of the copper by fish. These authors also suggest a direct protective capacity that is associated with the ability of DOC to increase Na^+ uptake, affect Na^+ , K^+ -ATPase activity and transepithelial potential, and reduce paracellular permeability (Wood et al., 2011).

Thus, in the present work, *H. littorale* did not activate any biotransformation pathways, and we speculate that this is due to the protective role of DOC, which results from the complexation of Cu with DOC of the Rio Negro water, as evidenced by copper speciation modeling (Table 1).

Some contaminants, such as copper, may directly interact with biomolecules, leading to oxidative damages to these molecules, which in turn are reflected in part by increased LPO values (Stohs & Bagchi, 1995). Therefore, LPO and ACAP responses (Figs. 4B, 2B) have been pointed as useful biomarkers of the effect and exposure, respectively (Rose et al., 2006). In the present work, the antioxidant capacity against peroxy radicals (ACAP) values following copper exposure at both 28 and 34°C were increased, indicating a larger relative area and consequently lower efficiency to avoid or reduce tissue damage induced by the increased ROS content and associated with it, to the LPO content showed a decrease in *H. littorale*. Based on these results, we suggest that the depletion of LPO levels in these animals might have arisen from the induction of antioxidants, which could have reduced the peroxidative effects.

Fig. 4 Superoxide dismutase (SOD) activity (A) and lipid peroxidation (LPO) levels (B) in liver samples from *H. littorale* under control conditions (no copper addition) and exposed to two different copper concentrations (50 and 500 $\mu\text{g L}^{-1}$) at 28 and 34°C, respectively. $N = 6$. See Fig. 2 for the caption



Alterations in ROS content are associated with changes in the activity of several enzymes and concentration of antioxidant molecules to adequately scavenge the distinct types of ROS avoiding subsequent deleterious oxidative damages in tissues (Lushchak, 2011). The enzyme SOD is recognized as free radical scavengers, acting to neutralize superoxide anion (O_2^-) produced by different pathways that involve endogenous compounds and various xenobiotics (Kehrer, 2000). Our findings showed a progressive increase of the SOD activity according to increases of copper concentrations for *H. littorale*, and these changes were not directly related with assay temperature. In general, copper is considered to have a high oxidative potential (Stohs & Bagchi, 1995). Its effectiveness in inducing oxidative stress is, in fact, linked to its ability to participate in Fenton-like reactions and cycle between Cu(II) and Cu(I) oxidation states within the cell (Jomova et al., 2012). This characteristic could explain the increase in SOD

caused by increased ROS production, thereby leading to the toxic effects of this metal. Therefore, our results also provide evidence that the toxic effects of copper in *H. littorale* are dependent on the metal concentration in the water and water temperature, which had a considerable effect on copper toxicity.

Molecules of ROS are generated naturally as products of certain metabolic pathways (Lushchak, 2011), especially, during aerobic metabolism, when animals show mechanisms to scavenge these ROS molecules (Geracitano et al., 2002; Valko et al., 2005). These mechanisms are crucial to providing whole-organism homeostasis since ROS can react with biomolecules causing damages to DNA, proteins, and lipids (Stohs & Bagchi, 1995). For *H. littorale*, the toxicity could be directly associated with the dose-dependent effect of metal on the imbalance between ROS production and antioxidant defenses, as seen with the concomitant ACAP (antioxidant capacity against peroxy radicals) increased values found in the

liver of *H. littorale* under experimental conditions (Fig. 2B), indicating that ROS formation exceeded antioxidant defense capability or disrupted redox signaling (Jones, 2006), once the higher ACAP levels mean lower competence to neutralize peroxy radicals. These results may have contributed to the fish mortality observed in the present work (Fig. 1). As observed, both copper concentrations promoted mortality in this species at both temperatures, when fish exposed to the concentration of $500 \mu\text{g l}^{-1}$. Moreover, both increased copper and temperature levels have been widely recognized as inducers of ionoregulatory disturbances in freshwater fishes (Carvalho & Fernandes, 2006), where the fall in plasma ion levels causes a shift of fluid from the extracellular to intracellular compartments, resulting in a reduced blood volume and elevated blood viscosity and pressure that might lead fish to dead.

Additionally, reduced survival time in the treatment of $50 \mu\text{g l}^{-1}$ at high temperature was also observed suggesting that the higher temperature increases the toxicity of copper even at lower concentration and can be considered an additional stressor for these animals. Thus, the effect of copper as well as temperature, at 34°C , was clearly effective in causing mortality of all fish after 12 h of exposure in the treatment of $50 \mu\text{g l}^{-1}$ and after 6 h of exposure for the concentration of $500 \mu\text{g l}^{-1}$. In the animals exposed to the natural river temperature (28°C), the mortality effects caused by copper were not observed in fish exposed to $50 \mu\text{g l}^{-1}$ of copper. Although the interactive effects of temperature and metal over antioxidants and cellular repair mechanisms have not been extensively studied in ectotherms and require further investigation to determine whether similar limitations of protective response at high temperatures can occur (Sokolova & Lanning, 2008), these are the first results showing that temperature strengthens Cu^{2+} effect decreasing survival time and increasing ROS production for this Amazon fish species, even at high DOC contents.

Acknowledgments We acknowledge the joint grant from the Brazilian National Research Council (CNPq) and Amazonas State Research Foundation (FAPEAM) to ADAPTA Project (Environmental Adaptations of Aquatic Organisms) that supported this work. We thank Maria de Nazaré Paula-Silva for all support during the ADAPTA expedition.

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