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The combined effect of copper and low pH on antioxidant defenses and biochemical parameters in neotropical fish pacu, *Piaractus mesopotamicus* (Holmberg, 1887)

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Abstract Copper sulfate is widely used in aquaculture. Exposure to this compound can be harmful to fish, resulting in oxidative metabolism alterations and gill tissue damage. Pacu, *Piaractus mesopotamicus*, (wt = 43.4 ± 3.35 g) were distributed in experimental tanks (n = 10; 180 l) and exposed for 48 h to control (without copper addition), 0.4Cu (0.4 mg l^{-1}), 0CupH (without copper addition, pH = 5.0) and 0.4CupH (0.4 mg l^{-1}, pH = 5.0). In liver and red muscle, the superoxide dismutase (SOD) was responsive to the increases in the aquatic copper. The plasmatic intermediary metabolites and hematological variables in the fish of group 0.4Cu were similar to those of the control group. Conversely, the exposure to 0.4CupH caused an increase in the plasmatic lactate, number of red blood cells (RBC) and hemoglobin (Hb). Plasmatic copper concentration [Cu_p] increased in group 0.4Cu and 0.4CupH, which is higher in group 0.4CupH, suggests an effect of water pH on the absorbed copper. Exposure to 0.4Cu and 0.4CupH resulted in a reduction in the Na^+/K^+-ATPase activity and an increase in metallothionein (MT) in the gills. Exposure to 0CupH caused a decrease in glucose and pyruvate concentrations and an increase in RBC, Hb, and the branchial Na^+/K^+-ATPase activity. These responses suggest that the fish triggered mechanisms to revert the blood acidosis, save energy and increase the oxygen uptake. MT was an effective biomarker, responding to copper in different pHs and dissolved oxygen. Combined-factors caused more significant disturbance in the biomarkers than single-factors.

Keywords Biomarkers · Freshwater fish · Oxidative stress · Pollution · Water quality

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Introduction

The global increase in freshwater contamination with innumerous natural and industrial chemical compounds is, to date, one of the main environmental problems in the world (Schwarzenbach et al. 2006). The aquatic environment is frequently exposed to polluting processes caused by a massive input of various chemical substances. As a rule, studies analyzing the behavior and the effects of single compounds on the aquatic organisms are based on observations of the isolated factors. This approach, however, has been changed over the last years due to the increase in investigations on the effects of the combined factors (Ferreira et al. 2008).

Copper sulfate ($CuSO_4$) is one of the most used compounds, such as algaecide and herbicide applied in lakes

and reservoirs (Effler et al. 1980; Carbonell and Tarazona 1993), and has been widely used to control algae bloom and growth of undesired aquatic organisms in aquaculture ponds (Thornton and Rast 1997). Copper toxicity has been studied in many fish species, and is influenced not only by the concentration of this metal in the water, but also by various factors influencing its biodisponibility to the exposed organism. Physicochemical characteristics of water, such as alkalinity, hardness and pH strongly influence copper speciation in water and, therefore, their bioavailability for absorption by fish (Mazon and Fernandez 1999; Tao et al. 1999). Carvalho and Fernandes (2006) have concluded that the use of copper sulfate to control algae and fish parasite should take into consideration the sensitivity of the species to copper and mainly the water pH of aquaculture ponds.

Various factors, such as acid pollution and acid rain, are an increasing environmental problem in the world and affect water pH (Heath 1991). The fish tanks face a daily pH fluctuation due to respiration and photosynthesis (Wurts and Durborow 1992). The ideal pH range for the survival of most fish species is from 6.5 to 9.0. Below or above this range, biochemical and physiological mechanisms are triggered to prevent death. Hydrogen ion concentration (pH) has a marked effect on the chemical form of a number of potentially toxic substances and, consequently, the pH can have major influences on toxicity. Many abiotic and biotic factors influence the bioavailability and toxicity of metals for aquatic organisms. Furthermore, the relationships between factors that affect toxicity are not always linear. For example, a low pH can either increase or decrease toxicity of metals in freshwater ecosystems (Campbell and Stokes 1985). The effects of water pH on the metal activity are complex as the pH affects either the solubility and/or the speciation of many metals (McDonald et al. 1989).

Copper and low pH seem to be similar in the toxicity mechanism as both are characterized by causing excessive mucus production and precipitation on the gills, which may lead to death by asphyxia. Nevertheless, it was also demonstrated that copper absorption and toxicity increased with a decrease in hydrogen ions concentration (Cusimano et al. 1986; Laurén and McDonald 1986; Starodub et al. 1987). Under such circumstances, it is believed that there is no direct relationship between cupric ions concentration and the absorption and toxicity of this metal in aquatic organisms (Blust et al. 1991). Blust et al. (1991) exposed the euryhaline crustacean *Arthemia franciscana* to increasing copper concentrations and observed that this metal absorption increased linearly in relation to its concentration in the solution and this was more evident in alkaline and neutral water than in acid medium. According to these authors, the effect of ions hydrogen on the metal absorption is expressed as ionization of the metal transport system, emphasizing that pH alterations change the copper absorption process. The neotropical fish curimbatá, *Prochilodus lineatus*, showed higher sensibility to copper at pH = 8 (15 μg l^{-1}) if compared with the CL50 values at pH = 4 (200 μg l^{-1}) (Takasusuki et al. 2004). Carvalho and Fernandes (2006) evaluated the copper toxicity in curimbatá at 20 and 30°C. These authors recorded CL50 (96 h) values of 98 and 88 μg l^{-1} at pH = 4.5 and 16 and 14 μg l^{-1} at pH = 8.0, respectively, showing that the species was more sensitive to copper in alkaline pH than in acid pH. They concluded that copper toxicity in the studied species is pH-dependent, corroborating the findings of Takasusuki et al. (2004). The results of Cusimano et al. (1986) also confirm that the effects of copper in rainbow trout decreased in acid pH. Carvalho and Fernandes (2006) suggested that a decrease in copper toxicity at acid pH may result from the competition between H^+ and Cu^{2+} for the binding site in the gill epithelium (Laurén and McDonald 1985), which is the main body surface for water–blood diffusion (Mazon et al. 2002).

The use of copper products in aquatic environments is, to date, a serious problem for environmental chemistry. However, the toxic effects of these compounds on the aquatic organisms have been studied recently and research is usually conducted in highly controlled conditions, factors that may change copper toxicity. Tropical ecosystems are more susceptible to human activities and environmental degradation. However, little has been done to investigate the impact of environmental contaminants (Lacher and Goldstein 1997) and few tropical fish species have been studied in relation to environmental toxicants. The pacu, *Piaractus mesopotamicus*, fed with commercial food presents good food conversion, reaching a marketable size in 1 year. Due to these characteristics, the species has been cultivated in various Brazilian regions (Borghetti and Canzi 1993). According to Tavares-Dias et al. (2001) and Schalch and Moraes (2005), pacu is very susceptible to *Icthyophitirius multifillis* and *Anacanthorus penilabiatus* infections. These contaminations require therapeutic baths of $CuSO_4$ when in aquaculture systems.

The aim of this study was to examine how the exposure of copper, acid medium and copper/acid medium combinations influence some biomarkers on the neotropical fish pacu, *Piaractus mesopotamicus*. To examine this interaction, we exposed pacu to copper, acid medium (pH = 5.0) and those conditions combined and evaluated the effects of this combination on: (a) Antioxidant status (hydroperoxide levels, activity of superoxide dismutase, glutathione peroxidase and catalase in liver, red muscle and white muscle). (b) Hematological parameters (red blood cells, hematocrit, hemoglobin concentration, mean corpuscular cell volume, mean corpuscular hemoglobin

concentration). (c) Blood pH and copper plasma concentration. (d) Metallothionein concentration in the gills and gill Na^+/K^+-ATPase activity. (e) Plasmatic concentration of glucose, lactate, pyruvate, ammonia and protein.

Materials and methods

Fish and sample preparation

Juvenile specimens of pacu were obtained from Aquapeixe Aquaculture (Conchal, SP, Brazil) and maintained for 2 months in holding tanks (1000 l) with flow-through normoxic water, at a constant temperature (25 ± 1°C), photoperiod of 12-h light: 12-h dark cycle, and constant pH (7.2 ± 0.06). Fish were fed ad libitum daily on commercial dry pellets (38% crude protein). Food was withheld 48 h prior to experimentation. Groups of fish (n = 10; wt = 43.40 ± 3.35 g) were randomly taken from the acclimation tanks and distributed in the experimental glass aquaria (180 l; static system, 2–6 replicates for each group). Fish were exposed for 48 h to control (without copper supply; pH = 7.0) (0Cu), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), to low pH-acid medium (pH = 5.0) (0CupH) and to 0.4 mg Cu^{2+} l^{-1} + pH (pH = 5.0) (0.4CupH). The LC_{50}-48 h 2.37 mg Cu^{2+} l^{-1} was previously determined (unpublished data). The copper agent used was $CuSO_4{\cdot}5H_2O$ (Labsynth Ltd.). A stock solution was prepared by dissolving 5.0 g of $CuSO_4{\cdot}5H_2O$ in 1 l of distilled water and was used to prepare the test solution by diluting it in the water of the experimental aquaria at a desired concentration.

In the acid water aquaria, the pH was adjusted using ultra pure H_2SO_4 and/or NaOH and was continuously monitored during the toxicity test with a pH microelectrode (Quimis Scientific Apparatus, Mod. 400A) and adjustments were made as required. The pH levels of the acid system were maintained near 5.04 ± 0.04 and the neutral system about 7.20 ± 0.06. The measured concentration of copper in the water at the beginning of the experiment was within ±3% of the nominal concentration and was reduced by 48% over the 48 h of exposure. The water quality parameters in the aquariums were measured at the beginning (n = 5) and after the experimental period (n = 5) and the following were maintained constant: dissolved oxygen 138 ± 2 mmHg, hardness 334.59 ± 3.17 mg l^{-1} ($CaCO_3$), alkalinity 10.48 ± 0.06 mg l^{-1} ($CaCO_3$), ammonia 0.03 ± 0.01 mg l^{-1}, Na^+ 1.79 ± 0.29 mEq l^{-1}, K^+ 3.66 ± 0.18 mEq l^{-1}. The water copper concentration was measured at the beginning of the trials and after 48 h in both aquaria. The water copper concentration was determined by atomic absorption spectrophotometry (standard methods AA 6800) and was presented as μg copper l^{-1}.

At the end of the experimental period, fish were taken from the aquaria and anesthetized with benzocaine 0.01% (Synth). Blood was collected from the caudal vein by puncturing it with a heparinized needle and syringe (1 ml). Just afterwards, fish were sacrificed by spinal cord transaction. Tissues (liver, red muscle, white muscle and gills) were collected and washed with saline (0.9% NaCl), dried in filter paper, identified and stocked in a freezer at −80°C. Spectrophotometric readings were carried out in a Spectronic Genesys 5 spectrophotometer. Microplate readings were taken using a Dynex MRXTC 250 (Dynex Technologies Inc., UK). Centrifugations were done with a Hernle-Z323K refrigerated centrifuge.

Oxidative metabolism

The SOD (EC 1.15.1.1), GSH-Px (EC 1.11.1.9) and CAT (EC 1.11.1.6) activities were measured according to the method previously described by Sampaio et al. (2008). The biological samples analysed ranged from 5 to 20 samples and the exact numbers for each group are presented in Tables 2, 3 and 4.

Hematological procedure

Blood and tissue samples were taken from fish of each experimental group to proceed with the hematological and biochemical analysis. The blood was sampled by a caudal puncture with a heparinized needle and syringe (1 ml). Blood pH (BpH) levels were determined with a pH microelectrode (Quimis Scientific Apparatus, Mod. 400A). Plasma protein (PP) was determined by Bradford (1976) and adapted to a microplate reader Dynex MRXTC 250 (Dynex Technologies Inc., UK) as described by Kruger (1994). The copper plasma concentration (Cu_P) was determined by an atomic absorption spectrophotometry (standard methods AA 6800), and was presented as μg copper l^{-1}. The hematocrit (Ht) was determined by the microhematocrit centrifugation technique. The red blood cell count (RBC) was determined optically by a Neubauer chamber. Hemoglobin (Hb) was determined by Drabkin's reagent as an absorbance at 540 nm. The mean cell volume (MCV) and cell hemoglobin concentration (MCHC) were computed from Ht, Hb and RBC. The parameters were evaluated according to the method by Jain (1986). The biological samples analysed ranged from 9 to 30 samples and the exact numbers for each group are presented in Table 6.

Metallothionein concentration in gills and gill Na^+/K^+-ATPase activity

Metallothionein (MT) concentration and Na^+/K^+-ATPase (EC 3.6.1.3) activity were measured according to the

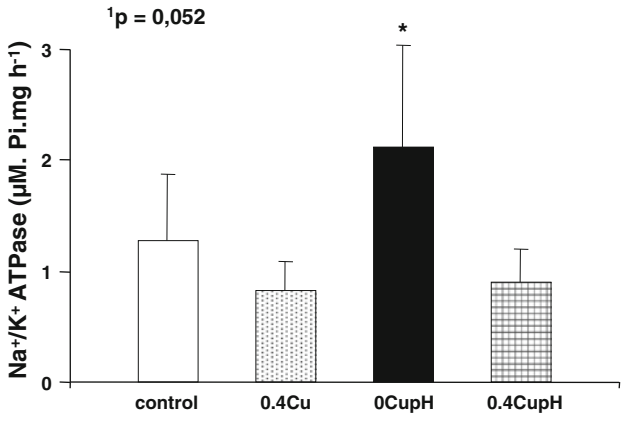

Fig. 1 Na^+/K^+-ATPase (µM Pi mg $prot^{-1}$ h^{-1}) of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control) (n = 10), 0.4 mg Cu^{2+} l^{-1} (0.4Cu) (n = 10), acid medium (pH = 5.0) (0CupH) (n = 13) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) (n = 10) for 48 h. Values are mean ± SD. * Experimental groups differ from the control group (Dunnet test; $p < 0.05$). [1] p value for pH and copper interaction

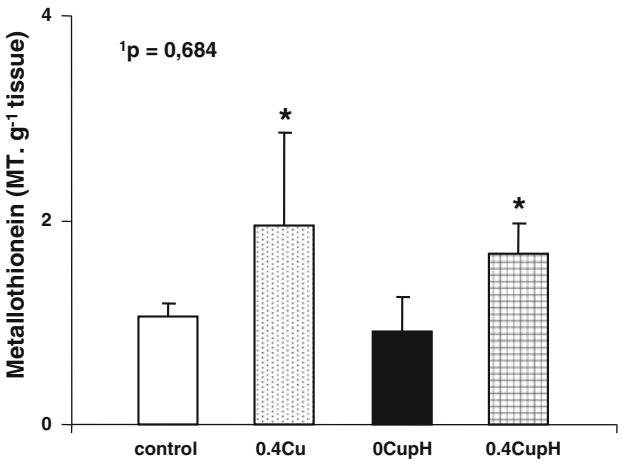

Fig. 2 Metallothionein (MT g^{-1} tissue) of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control) (n = 6), 0.4 mg Cu^{2+} l^{-1} (0.4Cu) (n = 9), acid medium (pH = 5.0) (0CupH) (n = 17) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) (n = 10) for 48 h. Values are mean ± SD. * Experimental groups differ from the control group (Dunnet test; $p < 0.05$). [1]p value for pH and copper interaction

method previously described by Sampaio et al. (2008). The biological samples analysed ranged from 9 to 30 samples and the exact numbers for each group are presented in Figs. 1 and 2.

Plasma intermediary metabolics

To determine the intermediate metabolics, samples of plasma were previously deproteinised using TCA 20%, and centrifuged at 10,000*g*, for 3 min at 4°C. Aliquots of supernatant were taken for protein determination (Bradford 1976) and concentration of glucose (Dubois et al. 1956), pyruvate (Lu 1939), lactate (Harrower and Brown 1972) and ammonia (Gentzkon and Masen 1942). The data were shown as µmol ml of $plasma^{-1}$. The biological samples analysed ranged from 9 to 30 samples and the exact numbers for each group are presented in Table 5.

Statistical analysis

To observe the studied parameter distribution curve, the normality Kolmogorov-Smirnov test was used following the concepts of sample size. The results are presented as a mean ± standard deviation (SD). All statistical calculations were performed using GraphPad InStat (3.00, GraphPad Software), SigmaStat (2.0 software, Jandel Corporation) and Minitab 15 (15.1.30.0, Minitab Brasil). The significance of any approach difference was tested by analysis of variance in a general linear model (GLM). If differences were found ($p < 0.05$), paired Tukey's tests were carried out to determine which individual groups were significantly different from each other. The Dunnet test was performed to determine which individual groups differ from the control ($p < 0.05$).

Results

Aquatic parameters

Table 1 presents the water copper concentration of each aquarium. The water copper concentration (µg l^{-1}) varied, in relation to the nominal concentration, at approximately 2% in the beginning and there was a decrease at the end of the experimental period. Mortality was not observed in any experimental group. Activity values of all enzymes and other parameters were analyzed and are presented in Tables 2, 3, 4, 5 and 6 and Figs. 1, 2, 3 and 4.

Copper and acid pH effects in isolated and associated conditions

Oxidative metabolism

Table 2 presents the results of hepatic oxidative metabolism between copper and pH levels analyzed by Anova GLM, and the Dunnet test comparing the experimental groups with the control. A significant interaction between the copper and pH levels in the hepatic concentration of lipid hydroperoxide HP ($p = 0.014$) and in the activities of superoxide dismutase (SOD) ($p < 0.001$) and catalase (CAT) ($p < 0.013$) was observed. On the other hand, the glutathione peroxidase

Table 1 Initial (0 h) and final (48 h) copper concentration (μg l^{-1}) in the water of different experiments at which pacu, *Piaractus mesopotamicus*, were exposed to copper free and neutral pH (control), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), acid medium (pH = 5.0) (0CupH) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) for 48 h

Copper (μg l^{-1})	0Cu	0.4Cu	0CupH	0.4CupH
0 h	5.70 ± 0.06	460.10 ± 5.60	5.41 ± 0.70	361.33 ± 37.17
48 h	1.04 ± 0.01	5.83 ± 0.07	5.00 ± 1.35	7.30 ± 1.35

Values are mean ± SD, n = 3

Table 2 HP (nmol g $tissue^{-1}$), SOD (USOD mg $protein^{-1}$), GSH-Px (nmol mg $protein^{-1}$) and CAT (BU mg $protein^{-1}$) in pacu liver, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), acid medium (pH = 5.0) (0CupH) and 0.4 mg Cu^{2+} L^{-1} + pH (0.4CupH) for 48 h

	0Cu	n	0.4Cu	n	0CupH	n	0.4CupH	n	Cu × pH[1]
HP (nmol g $tissue^{-1}$)	353.69 ± 23.88^{aA}	6	396.37 ± 27.68^{bA}	9	383.72 ± 52.62^{aA}	15	359.13 ± 17.09^{aA}	8	$p = 0.014$
SOD (USOD $mgPT^{-1}$)	139.30 ± 37.03^{aA}	5	194.91 ± 18.83^{bA}*	9	269.07 ± 63.15^{bB}*	8	172.35 ± 22.52^{aA}	8	$p < 0.001$
GSH-Px (nmol $mgPT^{-1}$)	16.50 ± 3.19	7	13.67 ± 2.29	9	16.19 ± 5.47	10	11.79 ± 3.22	6	$p = 0.482$
CAT (nmol $mgPT^{-1}$)	1.678 ± 0.19^{bB}	4	1.177 ± 0.13^{aA}*	8	1.213 ± 0.22^{aA}*	10	1.114 ± 0.24^{aA}*	8	$p = 0.013$

Values are mean ± SD

Lower case letters show the difference between the groups with the same pH condition, at different copper levels

Capital letters show the difference between the groups with the same copper levels, at different pH conditions

* Experimental groups differ from the control group (Dunnet test; $p < 0.05$)

[1] p value for pH and copper interaction

Table 3 HP (nmol g $tissue^{-1}$), SOD (USOD mg $protein^{-1}$), GSH-Px (nmol mg $protein^{-1}$) and CAT (BU mg $protein^{-1}$) of red muscle of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), acid medium (pH = 5.0) (0CupH) and 0.4 mg Cu^{2+} L^{-1} + pH (0.4CupH) for 48 h

	0Cu	n	0.4Cu	n	0CupH	n	0.4CupH	n	Cu × pH[1]
HP (nmol g $tissue^{-1}$)	275.57 ± 17.41^{aB}	7	240.59 ± 7.69^{aA}*	9	211.65 ± 31.37^{aA}*	17	234.02 ± 13.91^{bA}*	8	$p = 0.016$
SOD (USOD $mgPT^{-1}$)	459.99 ± 39.06	7	429.50 ± 37.52	8	230.06 ± 38.87*	20	241.12 ± 39.47*	8	$p = 0.102$
GSH-Px (nmol $mgPT^{-1}$)	16.65 ± 7.92	6	13.44 ± 2.72	8	19.29 ± 10.15	17	15.27 ± 6.43	8	$p = 0.887$
CAT (nmol $mgPT^{-1}$)	0.237 ± 0.09^{bB}	7	0.144 ± 0.02^{aA}*	9	0.129 ± 0.02^{aA}*	8	0.152 ± 0.02^{aA}*	8	$p < 0.001$

Values are mean ± SD

Lower case letters show the difference between the groups with the same pH condition, at different copper levels

Capital letters show the difference between the groups with the same copper levels, at different pH conditions

* Experimental groups differ from the control group (Dunnet test; $p < 0.05$)

[1] p value for pH and copper interaction

(GSH-Px) activity was not influenced by the interaction between copper and pH levels ($p = 0.482$). The hepatic HP concentration in response to copper addition to water depends on the medium pH. In neutral pH, fish exposure to 0.4Cu increased the hepatic HP concentration by 12%. However, fish exposed to the same copper concentration in acid medium did not change the hepatic HP concentration. This suggests that the effect of the increase in the hepatic HP concentration in response to the copper increase in neutral pH was abolished in acid pH. The exposure of fish to 0CupH did not change the hepatic HP concentration. There was no difference between the fish of experimental groups exposed to the acid medium in relation to the control, showing that there is no effect of acid medium exposure on the hepatic HP production.

The SOD hepatic activity in response to the copper levels depends on the aquatic pH. The response pattern of the SOD hepatic activity in response to copper exposure in neutral pH differs from that of acid pH. Fish exposed to 0.4Cu presented an increase of 40% in the SOD hepatic activity in relation to those of the control group. Conversely, the exposure to 0.4CupH did not change the SOD

Table 4 HP (nmol g tissue^{-1}), SOD (USOD mg protein^{-1}), GSH-Px (nmol mg protein^{-1}) and CAT (BU mg protein^{-1}) in white muscle of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), acid medium (pH = 5.0) (0CupH) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) for 48 h

	0Cu	n	0.4Cu	n	0CupH	n	0.4CupH	n	Cu × pH[1]
HP (nmol g tissue^{-1})	100.34 ± 4.31^{aA}	7	190.27 ± 12.10^{bA}*	8	171.37 ± 34.95^{aB}*	17	211.38 ± 30.28^{bA}*	7	$p = 0.015$
SOD (USOD mgPT^{-1})	235.27 ± 64.58	7	168.13 ± 35.32	8	195.85 ± 74.59	17	187.83 ± 30.71	8	$p = 0.136$
GSH-Px (nmol mgPT^{-1})	35.00 ± 12.38	7	24.12 ± 6.21*	9	33.77 ± 9.91	16	20.13 ± 3.02*	8	$p = 0.652$
CAT (nmol mgPT^{-1})	0.066 ± 0.03	6	0.033 ± 0.01*	9	0.045 ± 0.02	15	0.024 ± 0.01*	8	$p = 0.297$

Values are mean ± SD

Lower case letters show the difference between the groups with the same pH condition, at different copper levels

Capital letters show the difference between the groups with the same copper levels, at different pH conditions

* Experimental groups differ from the control group (Dunnet test; $p < 0.05$)

[1] p value for pH and copper interaction

Table 5 Plasma concentrations of glucose (μmol ml^{-1}), lactate (μmol ml^{-1}), pyruvate (μmol ml^{-1}), ammonia (μmol ml^{-1}) and protein (mg ml^{-1}) of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), acid medium (pH = 5.0) (0CupH) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) for 48 h

	0Cu	n	0.4Cu	n	0CupH	n	0.4CupH	n	Cu × pH[1]
Glucose (μmol ml^{-1})	1.23 ± 0.04	5	1.17 ± 0.33	7	0.88 ± 0.19*	15	1.15 ± 0.41	6	$p = 0.114$
Lactate (μmol ml^{-1})	1.28 ± 0.10^{aB}	5	1.27 ± 0.44^{aA}	7	0.86 ± 0.19^{aA}	12	1.80 ± 0.42^{bB}	6	$p < 0.001$
Pyruvate (μmol ml^{-1})	0.130 ± 0.01	5	0.140 ± 0.03	7	0.110 ± 0.03	12	0.112 ± 0.02	6	$p = 0.694$
Ammonia (μmol ml^{-1})	0.230 ± 0.01	5	0.214 ± 0.04	7	0.147 ± 0.09	16	0.213 ± 0.06	6	$p = 0.102$
Protein (mg ml^{-1})	9.09 ± 0.32	5	9.02 ± 1.74	6	9.09 ± 1.10	12	7.78 ± 1.10	5	$p = 0.181$

Values are mean ± SD

Lower case letters show the difference between the groups with the same pH condition, at different copper levels

Capital letters show the difference between the groups with the same copper levels, at different pH conditions

* Experimental groups differ from the control group (Dunnet test; $p < 0.05$)

[1] p value for pH and copper interaction

Table 6 RBC ($\times 10^6$ μl^{-1}), Htc (%), Hb (g dl^{-1}), MCHC (%) and MCV (fl) of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control), 0.4 mg Cu^{2+} l^{-1} (0.4Cu), acid medium (pH = 5.0) (0CupH) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) for 48 h

	0Cu	n	0.4Cu	n	0CupH	n	0.4CupH	n	Cu × pH[1]
RBC ($\times 10^6$ μl^{-1})	1.55 ± 0.34	10	2.01 ± 0.32*	10	1.94 ± 0.31*	27	2.23 ± 0.23*	9	$p = 0.442$
Htc (%)	33.60 ± 2.76	10	38.10 ± 8.71	10	31.48 ± 3.64	27	34.20 ± 4.10	10	$p = 0.528$
Hb (g dl^{-1})	7.88 ± 1.27	10	9.21 ± 1.56	10	9.77 ± 1.30*	27	10.41 ± 1.47*	10	$p = 0.380$
MCHC (%)	23.64 ± 4.53	10	25.06 ± 5.82	10	31.30 ± 4.77*	27	30.62 ± 4.33*	10	$p = 0.448$
MCV (fl)	225.64 ± 49.66	10	193.81 ± 55.37	10	166.30 ± 26.49*	27	154.12 ± 36.63*	9	$p = 0.386$

Values are mean ± SD

* Experimental groups differ from the control group (Dunnet test; $p < 0.05$)

[1] p value for pH and copper interaction

hepatic activity. This indicates that the increase in SOD hepatic activity in neutral pH was abolished in the acid medium. The exposure of fish to 0CupH increased the SOD hepatic activity by 93%. The effect of copper on the CAT hepatic activity depends on the water pH. The CAT hepatic activity decrease in response to copper was of 30% for the fish in group 0.4Cu, 28% for the group 0CupH and of 34% for the group 0.4CupH. Then, both isolated and associated exposures had a negative effect on the CAT hepatic activity. The GSH-Px hepatic activity was similar for fish exposure to both isolated and associated copper and pH conditions.

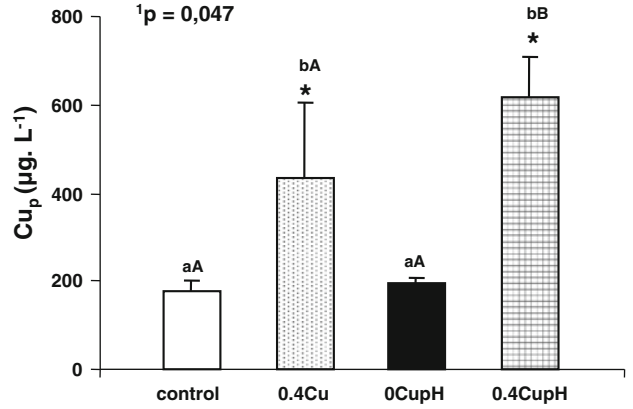

Fig. 3 [Cu_p] (µg l^{-1}) of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control) (n = 7), 0.4 mg Cu^{2+} l^{-1} (0.4Cu) (n = 10), acid medium (pH = 5.0) (0CupH) (n = 8) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) (n = 4) for 48 h. Values are mean ± SD. Lower case letters show the difference between the groups with the same pH condition, at different copper levels. Capital letters show the difference between the groups with the same copper levels, at different pH conditions. * Experimental groups differ from the control group (Dunnet test; $p < 0.05$). [1] p value for pH and copper interaction

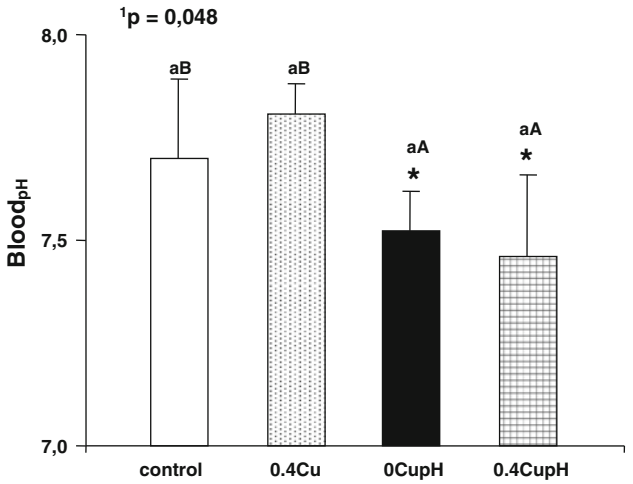

Fig. 4 Blood pH of pacu, *Piaractus mesopotamicus*, exposed to copper free and neutral pH (control) (n = 9), 0.4 mg Cu^{2+} l^{-1} (0.4Cu) (n = 10), acid medium (pH = 5.0) (0CupH) (n = 17) and 0.4 mg Cu^{2+} l^{-1} + pH (0.4CupH) (n = 10) for 48 h. Values are mean ± SD. Lower case letters show the difference between the groups with the same pH condition, at different copper levels. Capital letters show the difference between the groups with the same copper levels, at different pH conditions. * Experimental groups differ from the control group (Dunnet test; $p < 0.05$). [1] p value for pH and copper interaction

A significant effect of the copper and pH interaction on the HP concentration ($p < 0.016$) and in the CAT activity ($p < 0.001$) was observed in red muscle (Table 3). On the other hand, the SOD and GSH-Px activities were not influenced by the interaction between copper and pH levels ($p = 0.102$ and $p = 0.887$, respectively). When compared to the fish in the control group, the HP concentration was 23 and 15% lower in the fish in groups 0CupH and 0.4CupH, respectively. The exposure to acid medium in the isolated group or associated to copper decreased the SOD activity in red muscle by about 50%. The red muscle CAT activity in response to copper exposure depends on the water pH. In relation to the control group, the CAT activity decreased by 39, 46 and 36% in the fish of groups 0.4Cu, 0CupH and 0.4CupH, respectively.

A significant effect of the copper and pH interaction on the HP concentration ($p < 0.015$) was observed in white muscle (Table 4) having an increase in HP concentration by 90, 71 and 111% in the fish of groups 0.4Cu, 0CupH and 0.4CupH, respectively. Interactions between copper and pH levels were not observed for the activities of SOD ($p = 0.136$), GSH-Px ($p = 0.652$) and CAT ($p = 0.297$) of white muscle. Consequently, the antioxidant defense enzyme activities in white muscle, in response to copper, are similar in neutral and acid pH. The white muscle GSH-Px activity decreased by 31 and 42% in the fish in groups 0.4Cu and 0.4CupH when compared to the control. Likewise, the white muscle CAT activity decreased by 50 and 64% in the fish in groups 0.4Cu and 0.4CupH, respectively.

Intermediary metabolics

Table 5 shows the values of the plasmatic intermediary metabolics glucose, lactate, pyruvate, ammonia and protein of fish exposed to isolated and associated copper and pH levels. Plasmatic lactate concentration ($p < 0.001$) in response to copper depends on the water pH. The lactate levels did not change in response to the enhancement of copper in neutral pH and increased by 41% in fish exposed to copper in acid medium (group 0.4CupH). There was no significant interaction between copper and pH levels in the plasmatic concentrations of glucose ($p = 0.114$), pyruvate ($p = 0.694$), ammonia ($p < 0.102$) and protein ($p < 0.239$). These plasmatic variables presented the same response pattern to copper exposure in both neutral and acid water pH. When compared to the control group, the plasmatic glucose concentration was 28% lower in the fish in group 0CupH.

Branchial Na^+/K^+-ATPase activity and metallothionein concentration

The values of branchial Na^+/K^+-ATPase activity and metallothionein concentration of fish exposed to isolated and combined copper and pH levels are presented in Figs. 1 and 2. There was no significant interaction between copper and pH in the branchial Na^+/K^+-ATPase activity ($p < 0.052$). In pacu, regardless of the water pH, the exposure to copper decreased the branchial Na^+/K^+-ATPase activity.

This suggests that the branchial Na^+/K^+-ATPase activity in response to aquatic copper is independent of the water pH. Conversely, when compared to the control group, the isolated exposure of pacu to 0CupH increased the branchial Na^+/K^+-ATPase activity by 66%. There was no significant interaction between copper and pH in the branchial MT concentration ($p = 0.684$). Fish exposed to 0.4Ch and 0.4CupH increased the branchial MT concentration by 84 and 58%, respectively. As a consequence, the increase in branchial MT concentration occurs exclusively in response to water copper enhancement, regardless of the water pH.

Plasmatic copper concentration [Cu_p] and blood pH (pH_b)

Figure 3 presents the values of plasmatic copper concentration [Cu_p]. The data show an interaction between copper and pH ($p = 0.047$). Compared with the control, [Cu_p] was 145 and 251% higher in fish of groups 0.4Cu and 0.4CupH, respectively, confirming that the exposure of pacu to copper and acid pH increased [Cu_p]. The values of pH_b are presented in Fig. 4. There was a decrease of 2 and 3%, respectively, in the pH_b of the fish in groups 0CupH and 0.4CupH, showing a significant interaction between copper and pH levels in the pH_b ($p < 0.048$).

Hematological parameters

Table 6 presents the hematological values of RBC, Htc, Hb, MCHC and MCV of pacu exposed to copper in neutral and acid pH. There was no interaction between the copper and pH for RBC ($p = 0.442$), Htc ($p < 0.528$), Hb ($p = 0.380$), MCHC ($p < 0.448$) and MCV ($p = 0.386$). All the hematological parameters evaluated presented the same pattern and intensity of response to copper exposure in neutral and acid water pH. Compared to the control, the RBC of the fish in groups 0.4Cu, 0CupH and 0.4CupH increased by 30, 25 and 44%, respectively. The Hb and MCHC were 25 and 30% higher in the fish in groups 0CupH and 0.4CupH than those of the control.

Discussion

Aquatic parameters and health conditions

The water quality parameters were kept within the normality of the experimental protocols, according to culture patterns (Boyd 1990) and maintained the characteristics of the region where the experiments were performed. As described by Aragão et al. (2003), the water hardness of most of the Brazilian river basins is low (soft water), which enables toxicity tests to be carried out. According to Mastin and Rodgers (2000) the fast decrease in copper concentration may occur as a consequence of the transference of the cation to other compartments of the system or by the low residence time of $CuSO_4$ in the water column. The general health conditions of fish were normal throughout the experiments. The studied species was resistant to the exposure to both isolated and associated conditions.

Copper and acid pH effects in isolated and associated conditions

Oxidative metabolism

Evaluating the isolated effects of copper on the hepatic oxidative metabolism of fish, we observed that there was an increase in the HP concentration and in the SOD activity compared to the fish of the control group. These data show a possible influence of copper on the hepatic HP increase and the stimulation of this metal on SOD activity (Sanchez et al. 2005). According to Warner (1994), increases in SOD activity can be accompanied by increases in CAT and/or GSH-Px activities. In contrast, we observed that CAT activity decreased significantly in fish exposed to 0.4Cu when compared to the fish in the control group. In this same comparison, there was no difference in the hepatic GSH-Px activity. The CAT and GSH-Px activities were not effective in preventing the excess of H_2O_2 formation resulting from the increase in SOD activity. The decrease or low activity of CAT and GSH-Px can validate the high hepatic HP concentrations as these enzymes did not intermediate the oxidative process, allowing peroxidation and characterizing the oxidative stress in this tissue. According to Kono and Fridovich (1982), excessive production of superoxide resulting from SOD activity, can inhibit CAT activity. Roberts et al. (1987) and Rodriguez-Ariza et al. (1993) characterize the SOD activity increase as an indicator of exposure to polluted waters. Pacu exposed to 0CupH did not show increases in the hepatic HP concentration. Probably, the GSH-Px activity was sufficient to combat the superoxides produced by SOD, preventing oxidative damage.

The hepatic HP concentration decreased in fish exposed to copper in acid water (group 0.4CuHp). SOD activity was also influenced by the interaction between copper and pH. As the SOD activity was intensified when copper was added to water with neutral pH, this response was abolished when copper was added to water at acid pH. The hepatic GSH-Px activity was not different in pacu exposed to copper in the diverse pH levels. This demonstrates that hepatic GSH-Px activity in this species was not sensitive to the medium alterations. The hepatic CAT activity was stopped when the fish were exposed to 0.4Cu, 0CupH and 0.4CupH, showing the interaction between copper and

water pH. Jemec et al. (2008) suggested that chemically stressed animals can increase or decrease the CAT activity according to the chemical nature, time and dose of exposure. According to Scandalios (2005), if CAT activity is inhibited, the H_2O_2 concentration tends to increase in the liver.

The oxidative mechanisms in response to copper in red muscle seem to be different from those in the liver. Pacus of group 0.4Cu increased the red muscle CAT activity. Even being a highly oxidative tissue, this copper concentration did not modify the oxidative status. The decrease in CAT activity seems to be an inhibitory process of copper. According to Taylor et al. (1988), copper inhibited CAT activity in rats. The red muscle GSH-Px activity did not change in fish exposed to both isolated and associated conditions. However, CAT was inhibited when pacu was exposed to these conditions. The GSH-Px activity characterizes this enzyme as an important tissue protector against oxidative peroxidation damage (Fehér et al. 1987). The GSH-Px levels may increase in cells of fish exposed to copper (Freedman et al. 1989). Low levels of H_2O_2 are the preferential substrate of GSH-Px, but in higher H_2O_2 concentrations, this peroxide is combated by CAT (Yu 1994).

Compared to the control group, when pacu was exposed to acid water, a decrease in the HP concentration and in the activity of the enzymes SOD and CAT was observed in the red muscle. The fish exposure to acid water caused a decrease in plasmatic glucose and lactate levels, suggesting a reduction in the metabolism. This response seems to avoid acidosis as observed in Fig. 4. The metabolism depression leads to a reduction in O_2 uptake, decreasing reactive oxygen species (ROS) formation in the respiratory chain. Wilhelm Filho and Boveris (1993) found a positive correlation between antioxidant enzymes and metabolic intensity. Pacu exposed to acid water, isolated or associated to copper, presented SOD activity inhibition and the low H_2O_2 concentration was an appropriate condition to the GSH-Px activity. The antioxidant defense systems (AD) can adapt organisms to new and adverse conditions. Rodriguez-Ariza et al. (1993) also observed a higher GSH-Px activity in mullet (*Mugil* sp.) collected from polluted areas. Steadman et al. (1991) suggest that the increased GSH-Px activity prevents the lipidic peroxidation in fish.

In the red muscle of pacu, the HP concentration and CAT activity were influenced by the interaction between copper and pH. The exposure of pacu to 0CupH and to 0.4CupH elicited a decrease in the HP concentration in red muscle. The red muscle is rich in mitochondria and these organelles are the main site of the ROS production (Graham 1990). The reduction in the red muscle HP concentration can be a consequence of the decrease in the transportation of electrons in the respiratory chain. The red muscle, supposedly more oxidative, also presents higher AD activity (Mazeaud et al. 1979). The SOD activity in the group of pacu exposed to acid water, isolated or associated with copper, was reduced, which indicates the high sensitivity of SOD to acid medium. Likewise, CAT was inhibited in fish exposed to copper in neutral and acid pH. Most of these responses suggest the sensitivity of AD in red muscle to water pH, regardless of the association with copper. Due to the presence of a heme group, CAT is more sensitive to oxidation (Moore et al. 2008). This characteristic may be related to the higher sensitivity shown by the CAT activity in the different tissues studied in pacu exposed to acid medium. The precise regulation of the H^+ concentrations is essential as it affects the activity of most of the enzymes. The water pH affects the enzyme structure and activity, and low pH may cause denaturation of mitochondrial enzymes (Ling 1981). This may explain the reduction in the red muscle enzyme activity in pacu exposed to acid water and the correlation of this inhibition with the decrease in pH_b.

Pacus of groups 0.4Cu, 0CupH and 0.4CupH showed a significant increase in white muscle HP concentration when compared to the control. The interaction between copper and pH was evident with the increase in white muscle HP concentration in fish exposed to the isolated and associated factors. The increase in HP concentration in this tissue can be correlated to the low activity of the AD enzyme system. White muscle is considered less oxidative than other tissues and possibly presents antioxidant defense mechanisms which are less effective. The white muscle AD presented similar responses in pacus exposed to copper in both neutral and acid pH. Fish exposed to 0.4Cu and 0.4CupH showed inhibition in the activity of GSH-Px and CAT. The low SOD activity and the decrease in the GSH-Px and CAT activity suggest that the AD mechanisms were not effective to avoid the oxidative stress. According to Sant'Ana and Mancini-Filho (2000), the muscle oxidative stress directly affects flesh organoleptic proprieties, such as texture and color.

Singh et al. (2004) determined an optimal pH range (6.5–7.0) for CAT activity. Moore et al. (2008) proposed that environmental pH changes may influence the CAT activity, modifying the relation between enzymatic action and substrate concentration. The sensitivity of CAT to decrease in the water pH was observed in the liver, red and white muscles of pacu exposed to acid water. On the other hand, the GSH-Px was kept constant in these tissues, suggesting that pH changes did not modify this enzyme activity in pacu. The evidence suggests that the use of antioxidant enzymes and the HP concentration as biomarkers of copper exposure have to take into consideration the physical and chemical water conditions. The present data support the idea that there is a significant interaction

between copper and pH water levels in the oxidative metabolism of the analyzed tissues of pacu. The data also conclude that the exposure to copper in acid water was more harmful to oxidative metabolism than the exposure to copper in the neutral pH.

Intermediary metabolics

According to Heath (1995), copper exposure causes hepatic glycogen mobilization, increasing glycemia. This was not observed in the present study. When compared to control, pacus exposed to 0.4Cu did not change the glucose, lactate, pyruvate, ammonia and protein plasma concentration, i.e., the whole intermediary metabolism was not affected by copper exposure in neutral pH. Similar results were obtained by Dethloff et al. (1999) when rainbow trout was exposed to 16 μgCu l^{-1} of copper in neutral pH, and by Griffin et al. (1999) with catfish, *Ictalurus punctatus*, also exposed to copper. Brown et al. (1990) studied the effect of acid water (pH ~ 4.7–5.6) in Atlantic salmon, *Salmo salar*. These authors observed an increase in the plasma glucose concentration, which was attributed to the maintenance of the carbohydrate metabolism. In the present study, the exposure to acid medium caused a decrease in the plasma glucose and lactate concentrations, while the pyruvate, ammonia and protein did not change. These results indicate that exposure to acid water caused hypoglycemia without stimulating the anaerobic metabolism. These responses characterize a metabolic depression, decreasing the energetic cost, avoiding intensifying the plasmatic acidosis caused by the exposure of fish to acid water (pH 5.0). Winkaler et al. (2001) characterized the hypoglycemia of the characid fish *Astyanax* sp. as a response to chronic stress. A decrease in the glycolytic metabolism may be related to metabolic acidosis prevention as glycolysis is the main H^+ source in the organism (Devlin 1997). Our results corroborate this assertion as the pacu exposure to acid water decreases the pH_b.

A significant interaction between copper and the pH was observed in pacu plasma lactate concentration. The reduction of lactate plasma concentration of fish exposed to 0CupH was abolished when they were exposed to 0.4CupH. These alterations indicate the differences of responses to copper exposure to neutral and acid pH. The fish of group 0.4CupH presented lactate plasma concentration significantly higher than in the control group. These findings are compatible with those of Takasusuki et al. (2004) who observed an increase in the lactate plasma concentration of curimbata, *Prochilodus lineatus*, exposed to copper in acid water, suggesting the use of anaerobic glucosis to supply the energetic needs. The pyruvate, ammonia and protein plasma concentrations were not influenced by the exposure to both isolated and associated factors. The association of copper and pH exposure presented a significant interaction in pacu pH_b. This interaction points out different responses to copper exposure in neutral and acid pH. The blood acidosis was observed in fish exposed to 0CupH and 0.4CupH. This pH_b decrease shows that copper exposure in neutral pH did not induce acidosis. However, when associated to the acid medium, the same copper dose was sufficient to cause acidosis in pacu. According to Jobling (1994), decreases in blood pH may decrease the red blood cells pH, interfering in the Hb-O_2 affinity and impairing the O_2 transport to the tissues. The increase in lactate plasma concentration in fish of group 0.4CupH could act as a buffer to prevent plasma acidosis. Variations in plasma lactate levels are frequently correlated to changes in blood pH, and the lactate is considered an important buffer (Brooks 2000).

Branchial Na^+/K^+-ATPase activity, metallothionein concentration and [Cu_p]

A considerable part of the copper diffused through the gills is transported by plasma (Pelgrom et al. 1995). Carbonell and Tarazona (1994) studied the copper toxicity in rainbow trout and concluded that copper plasma concentration reached a limiting situation after a few hours or days of exposure. Pacu of group 0.4Cu showed a significant increase in [Cu_p], clearly indicating that this metal was absorbed by the gills and diffused in the blood. However, the exposure of pacu to acid water alone did not change [Cu_p]. Copper toxicity can result in branchial ionic regulation impairment (Grosell et al. 2002). This metal affects the gill morphology inducing it to necrosis, hypertrophy, epithelium enlargement and vacuolation (Fernandes and Mazon 2003). It also stimulates chloride cell proliferation via cortisol (Wendelaar Bonga 1997) and mucus hypersecretion, which plays an important role in the detoxication process (Handy et al. 2002). However, chloride cell proliferation reduces the respiratory surface area and the mucus covering the secondary lamellae represents an important barrier to oxygen diffusion. Consequently, the respiratory efficiency is seriously impaired.

Considered as the first defense system against metal exposure (Bragigand and Berthet 2003), the MT action in the gill tissue of aquatic organisms was described and characterized by many authors (Mouneyrac et al. 1998). Branchial MTs of pacu were responsive to the aquatic copper enhancement in neutral pH (group 0.4Cu). The same findings were described by Heath (1995), Dang (2000), Bragigand and Berthet (2003) and Ryu et al. (2003). These authors describe the MTs as mechanisms of metabolic defense to copper tolerance. Pelgrom et al. (1995), Li et al. (1998) and Dang et al. (1999) related an increase in branchial MT in tilapia mossambica,

Oreochromis mossambicus, in response to copper exposure. Our data corroborate these authors and show evidence of the importance of branchial MT as a protective mechanism and a biomarker of fish exposed to copper. Compared to the control group, pacus exposed to 0CupH did not change the branchial MT concentration. Although the MTs are considered as environmental pollution biomarkers, in the present study the MTs were not responsive to the acid medium exposure. There was no interaction between the copper and pH concerning the branchial MT concentration. Branchial MT concentration of pacu in response to copper exposure increased in the neutral and acid pH. This response enables us to indicate the branchial MT concentration as an important biomarker of copper exposure regardless of the aquatic pH. Carvalho et al. (2004) exposed *Prochilodus lineatus* to different copper levels, pH and temperature and concluded that the hepatic MT concentration is an effective biomarker of copper exposure regardless of the different conditions.

Under low copper concentrations, the branchial ion uptake can be suppressed by the inhibition of Na^+/K^+-ATPase (Li et al. 1998). The Na^+/K^+-ATPase activity was inhibited in pacu exposed to 0.4Cu. According to Grosell et al. (2002), this inhibition occurs mainly due to the copper's capacity of linking to the active site of Na^+/K^+-ATPase. Unlike mammals, the main blood buffering mechanisms in fish depends on the internal pH regulation, based on the H^+ excretion to the aquatic environment. One of the processes involved in this mechanism is low intracellular Na^+ concentration, maintained by the Na^+/K^+-ATPase (Claiborne et al. 2008). Many fish species quickly adjust their metabolism by excreting H^+ through the gills (Heisler 1989). Claiborne and Evans (1992) observed that spiny dogfish, *Squalus acanthias*, can recover from a respiratory acidosis within 24 h due to H^+ excretion and an increase in plasma HCO_3^-, compensating the blood pH decline. Pacus exposed to acid medium increased the Na^+/K^+-ATPase activity, possibly as an attempt to revert the decreased pH_b. The branchial Na^+/K^+-ATPase activity did not change as a consequence of the interactions between copper levels and different pH at which pacus were subjected. Branchial Na^+/K^+-ATPase activity increased during exposure to the acid medium alone, while copper exposure, in both neutral and acid water, induced a reduction in the activity of this enzyme. The lack of interaction between these factors indicates that the stimulus of the acid pH to Na^+/K^+-ATPase activity was abolished when pacus were exposed to copper in both neutral and acid pH.

The present results point out interaction between the copper levels and the different pHs at which pacus were exposed to in the copper absorption process. The $[Cu_p]$ of fish exposed to copper in acid medium was higher than that presented by fish exposed to copper in neutral pH. The ventilation volume, the gill epithelium thickness and gill perfusion affect the O_2 transport rate and may similarly influence the toxic absorption through the gills (Yang et al. 2000), and the O_2 transference rate can indicate the toxic compounds conveyance (Murphy and Murphy 1971). Yang et al. (2000) found a direct correlation between toxic compound absorption through the gills and the O_2 uptake. The respiratory variables were not measured in the present study, however it is possible that changes in these parameters influenced the copper absorption rate by the gills. Blust et al. (1991) observed that changes in hydrogen ions concentrations had an important effect in the copper absorption in *Artemia franciscana* and the observed absorption variation could not be attributed solely to the variations in the aquatic free ion copper concentration. According to these authors, the transport system binding sites are vulnerable to acid medium. Carvalho and Fernandes (2006) confirmed that copper toxicity is water pH dependent and may affect the metal specificity and its toxicity in a complex manner. Hence, acid medium may affect the copper bioavailability. According to Buchwalter et al. (1996), copper toxicity can be enhanced with a pH decline as the amount of free copper linked to carbon can decrease in response to increases in H^+, which would compete with copper to the carbon molecule binding sites. Due to this competition, a higher amount of free ions copper would be available to be binded by the gill surface. Çoğum and Kargin (2004) evaluated the copper bioconcentration in the liver, gills and muscle of Nile tilapia, *Oreochromis niloticus*, exposed to different pHs (5.5, 7.8 and 9.5) for 7, 15 and 30 day copper concentrations of 0.1, 0.5, 1.0 and 5.0 mg l^{-1}. These authors observed a significant increase in the copper accumulation in the tissues, in every experimental pH, following increases in water copper concentrations and time of exposure. Curimbatá exposed to rising copper concentrations increased this metal plasma accumulation in both high and low pH. However, in pH = 4.5 the plasma copper concentration was higher than in pH = 8.0 (Takasusuki et al. 2004).

Hematological parameters

The hematological characteristics of various cultivated fish species have been studied to characterize their hematological profile. Therefore, variations in this profile indicate disturbances in physiological processes (Ranzani-Paiva et al. 2005). Pacu exposed to 0.4Cu increased the RBC, corroborating the results of Nussey et al. (1995) with tilapia mossambica, *Oreochromis mossambicus*, exposed to 0.40 mg l^{-1} of copper. However, in this copper exposure pacu did not change the other hematological parameters when compared to the control group, indicating that this

copper concentration stimulated erythropoesis. These results, however, diverge from those of Tavares-Dias et al. (2002) who reported a reduction in RBC, Hb and MCHC in pacu exposed to 0.50 and 1.0 mg l^{-1} of copper evaluated after 24 h of second exposure. According to Heath (1995), during chronic exposures to low pH, an enhancement in hematological parameters can be a response to a reduced O_2 flow to the blood. Due to the Bohr Effect, the blood will present a lower O_2 affinity in lower pHs. Further stimulation of RBC and Hb production will increase the O_2 transport capacity and, then, partially compensate these alterations. The present results indicate that mechanisms to improve the O_2 uptake and transport were activated in pacu exposed to acid medium. This is confirmed by the increase in RBC, Hb and MCHC.

According to Heath (1995), proteins are important intracellular buffers and the hemoglobin can operate in the same way. Our data indicate that the increase in Hb concentration may be a mechanism involved in the reversion of the plasma acidosis. In spite of being resistant to acid water (mortality was not recorded), the pacu hematological variations were effective to improve the O_2 uptake complicated by blood acidosis, such as described by Nussey et al. (1995) in tilápia mossambica. RBC and Hb concentrations in pacu exposed to copper were similar in neutral and acid pH, and there was no interaction between the isolated and associated factors in the hematological variables evaluated. However, fish from group 0.4CupH showed a more significant enhancement in RBC and Hb than those of the control group. Although without interaction, the association of factors stimulated the mechanisms of O_2 uptake and transport when compared to the exposure to isolated factors. The hematological responses of pacu exposed to copper in neutral and acid pH indicate that, regardless of this metal absorption, the presence of copper in the water impaired the gas exchange, inducing the fish to activate mechanisms to improve the O_2 uptake and transport. This condition was more significant in fish exposed to acid water as the influence of pH_b can interfere in the Hb-O_2 affinity. Takasusuki et al. (2004) described a decreased copper toxicity in acid medium in rainbow trout, tambaqui and curimbatá, respectively. Carvalho and Fernandes (2006) suggested that this toxicity reduction may result from the competition of H^+ and Cu^{2+} by the same binding site in the gill epithelium which is the main body surface of water–blood diffusion (Mazon et al. 2002). Our data indicate an increase of copper absorption in acid pH. Moreover, the hematological differences in fish exposed to copper in different pHs point out possible gill damage and excessive mucus production, impairing the gas exchange in fish exposed to 0.4CupH.

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