Comparative Biochemistry and Physiology, Part C 156 (2012) 178–186

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Antioxidant defenses and biochemical changes in the neotropical fish pacu, *Piaractus mesopotamicus*: Responses to single and combined copper and hypercarbia exposure

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ARTICLE INFO

Article history:
Received 2 February 2012
Received in revised form 30 June 2012
Accepted 3 July 2012
Available online 13 July 2012

Keywords:
Antioxidant defenses
Hematology
Hypercarbia
Metallothionein
Na^+/K^+-ATPase

ABSTRACT

This study investigated the potentially detrimental effects of copper and elevated aquatic CO_2 (hypercarbia), alone or in combination, on pacu, *Piaractus mesopotamicus*. Fish were exposed for 48 h to control (no copper addition in normocarbia), to 400 μg $Cu^{2+}L^{-1}$, to hypercarbic (1% CO_2; $PCO_2 = 6.9$ mm Hg) water and to 400 μg $Cu^{2+}L^{-1}$ + hypercarbia. In liver the single factors caused an increase in lipid hydroperoxide concentration that was not observed when the factors were combined. Copper exposure elicited increased hepatic superoxide dismutase activity, irrespective of aquatic CO_2 level. On the other hand, the effects of copper on hepatic glutathione peroxidase activity were dependent on water CO_2 levels. The two stressors combined did not affect hepatic catalase activity. Hypercarbic water caused a decline in plasma glucose concentration, but this was not observed when hypercarbia was combined with copper exposure. Copper caused a decrease in branchial Na^+/K^+-ATPase activity that was independent of water CO_2 level. Copper caused an increase in branchial metallothionein concentration that was independent of water CO_2 level. Thus, branchial metallothionein and Na^+/K^+-ATPase were effective biomarkers of copper exposure that were not affected by water CO_2 level.

1. Introduction

A range of compounds and environmental factors can influence fish welfare in aquaculture. In recent decades implications of various abiotic factors, e.g., dissolved oxygen and carbon dioxide, and the use of chemicals in aquaculture, have been extensively studied in aquaculture. However, limited data are available on the effects of carbon dioxide (CO_2) on the biology, physiology and biochemistry of cultivated neotropical fish. In aquaculture systems CO_2 may become a limiting factor and an important health issue, mainly in intensified production systems (Tort et al., 2011).

The control of dissolved gas is a key component in preventing poor water quality, especially in recirculating aquaculture systems—RAS (Blancheton, 2000). Fish produce CO_2, which is excreted across the gills, as a normal outcome of aerobic metabolism. High CO_2 concentrations (hypercarbia) frequently occur in aquaculture systems as a result of high fish densities, low water exchange rates per fish biomass and/or high dissolved oxygen concentration (Summerfelt et al., 2000). Long-term hypercarbic exposures affect many fish species, resulting in reduction of food intake and conversion (Smart, 1981), weight gain and growth (Fivelstad et al., 2007), condition factor (Fivelstad et al., 1998, 2003a, 2003b) and in nephrocalcinosis (Hosfeld et al., 2008). Even short-term exposures can be stressful for a number of fish species (Perry and Gilmour, 1996; Crocker et al., 2000). Previous studies have demonstrated that hypercarbia can also be associated with a transient increase in plasma cortisol and hematocrit (Fivelstad, 1999).

In intensive aquaculture systems, constant monitoring of dissolved CO_2 and pH is highly recommended (EFSA, 2008). The upper CO_2 limit for salmonids in aquaculture systems ranges from 10 to 20 mg L^{-1} (Boyd, 1979; MacIntyre et al., 2008). According to Blancheton (2000), the CO_2 concentration in aquaculture tanks with juvenile and adult sea

Abbreviations: HP, lipid hydroperoxide; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; PP, plasma protein; [Cu_P], copper plasma concentration; Ht, hematocrit; RBC, red blood cell count; Hb, hemoglobin; MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; MT, metallothionein; NKA, Na^+/K^+-ATPase.

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1532-0456
doi:10.1016/j.cbpc.2012.07.002

bream, *Sparus aurata*, should not exceed 40 mg L^{-1}. However, some fish species are tolerant of much higher CO_2 levels (Crocker and Cech, 1998; McKenzie et al., 2002, 2003). According to Good et al. (2010) the determination of species-specific CO_2 limits for aquaculture is complicated by various factors influencing the toxicity of this gas. These factors include some metals that could change their chemical speciation, becoming more available to exert toxic effects at high water CO_2 levels (Fivelstad et al., 2003a).

Another important environmental concern in aquaculture is the regular use of copper sulfate ($CuSO_4$). Copper is one of the most widely used compounds as an algaecide and herbicide (Carbonell and Tarazona, 1993), to control algal blooms and growth of undesired organisms in aquaculture ponds. It is also used as a therapeutic agent to control gill diseases caused by bacteria and a variety of parasites (Straus and Tucker, 1993). Copper toxicity has been studied in many fish species, and is influenced not only by the concentration of the metal in the water, but also by various factors influencing its bioavailability. Water physicochemical characteristics have a profound influence on copper speciation and, therefore, bioavailability for absorption by fish (Mazon and Fernandez, 1999; Sampaio et al., 2008, 2010). According to a recent report of the São Paulo State Company of Technology on Environmental Sanitation, in most rivers the dissolved copper concentration exceeds the limit established by the Brazilian legislation. Ponds where copper sulfate was used as an algaecide showed the highest percentage of non-compliance for water copper concentration (CETESB, 2010).

The CO_2 toxicity appears to depend on various factors. Thorarensen and Farrell (2011) concluded that, in Atlantic salmon, *Salmo salar*, the CO_2 toxicity increases when O_2 saturation is low. Fivelstad et al. (2007) also concluded that this species is more sensitive to CO_2 at lower temperatures. There is also evidence that pH affects the CO_2 toxicity either directly or indirectly through effects on the toxicity of metals (Fivelstad et al., 2003b; Portz et al., 2006). Knowing that water physical and chemical characteristics play a key role in copper speciation (Smith et al., 2002) and copper toxicity (Mazon et al., 2002) it is useful to know how increases in water CO_2 concentration can affect copper sulfate toxicity. Larsen et al. (1997) showed that Atlantic cod, *Gadus morhua*, exposed to copper responded differently when also exposed to hypercarbia. However, little is known on the influence of hypercarbia on the toxicity of copper to tropical fishes.

This study investigated the effects of CO_2 combined with copper sulfate on pacu, *Piaractus mesopotamicus*, which is one of the main farmed species in Brazilian aquaculture (MPA, 2010). The ability to cope with environmental hypercarbia, alone or in combination with copper exposure, was assessed in pacu over a period of 48 h through the analysis of antioxidant defenses status, intermediary metabolites, metallothionein concentration, Na^+/K^+–ATPase activity in the gills, and a number of hematological variables.

2. Materials and methods

2.1. Fish and sample preparation

Juvenile specimens of *Piaractus mesopotamicus* were obtained from Aquapeixe Aquaculture (Conchal, SP, Brazil) and maintained for two months in holding tanks (1000 L) with flow-through normoxic and normocarbic water, at constant temperature (25 ± 1 °C) and photoperiod (12:12 h light/dark). Fish were fed daily *ad libitum* with commercial dry pellets (38% crude protein). Food was withheld 48 h prior to experimentation.

Groups of fish ($n = 10$; $m = 40.2 \pm 1.1$ g) were randomly taken from the acclimation tanks and distributed in the experimental glass aquaria (180 L; static system, 5 replicates for group control and 3 replicates for groups Hyp, Cu and CuHyp). Fish were exposed for 48 h to control (without copper supply; normocarbic medium = 0.03% CO_2≅0.21 mm Hg) (C), 400 μg Cu^{2+} L^{-1} (Cu), to hypercarbic water (Hyp = 1% CO_2≅6.9 mm Hg) (Hyp) and to 400 μg Cu^{2+} L^{-1} + hypercarbic medium (Hyp = 1% CO_2≅6.9 mm Hg) (CuHyp). The 48 h LC_{50} for copper is 2.37 mg Cu^{2+} L^{-1} in *P. mesopotamicus* (Sampaio, 2008).

The copper agent used was $CuSO_4 \cdot 5H_2O$ (Labsynth®, Diadema, SP, Brazil). A stock solution was prepared by dissolving 5.0 g of $CuSO_4 \cdot 5H_2O$ in 1 L of distilled water and used to prepare the test solution by diluting it in the water of the experimental aquaria to the desired concentration. Hypercarbia was maintained with a CO_2/air certified gas mixture (1% CO_2/99% air balance) prepared and delivered by White Martins Praxair Inc, with adjustments when required. Within 2 h after the onset of equilibration, water PCO_2 had increased to a constant level of about 6.9 mm Hg (local BP = 690 mm Hg). The water CO_2 content from percentage (%) to partial pressure (mm Hg) was converted according to Florindo (2002) and Florindo et al. (2004), based on the water pH, which was continuously monitored, and the PCO_2 calculated using the Åstrup method. The water pH and the oxygen levels were continuously monitored during the toxicity tests with a pH microelectrode connected to a pH-meter Quimis Mod. 400A (Quimis Scientific Apparatus, São Paulo, SP, Brazil) and with a FAC 001 O_2 electrode housed in a thermostatted jacket and connected to a FAC 204A O_2 analyzer (FAC Electronics, São Carlos, SP, Brazil) respectively. The water quality parameters in the aquariums were measured at the beginning ($n = 5$) and after the experimental period ($n = 5$). These variables were maintained constant and are presented in Table 1. The water copper concentration was measured at the beginning of the trials and after 48 h in both aquaria (Table 2). The title of the experimental copper groups refers just to the nominal copper concentration. The water copper concentrations were determined by atomic absorption spectrophotometry (standard methods AA 6800) and presented as μg copper L^{-1}. The quantification limit was around 0.05 μg L^{-1} and the detection limit around 0.017 μg L^{-1}, both obtained from the analytical curve.

At the end of the experimental period, fish were taken from the aquaria and anesthetized with benzocaine at 0.01% (Labsynth, Diadema, SP, Brazil). Blood was collected from caudal puncture with a heparinized needle and syringe (1 ml). Just after, fish were sacrificed by spinal cord transection. Tissues (liver, red muscle, white muscle and gills) were collected and washed with saline (0.9% NaCl), dried in filter paper, identified and stored in a freezer at −80 °C. Spectrophotometric readings were carried out in a Spectronic Genesys 5 (Milton Roy Company, PA, USA) spectrophotometer. Microplate readings were performed with a Dynex MRXTC 250 (Dynex Technologies Inc., UK). Centrifugations were done with a Hermle-Z323K (Hermle LaborTechnik, Germany) refrigerated centrifuge.

2.2. Oxidative metabolism

A ferrous oxidation-xylenol orange, FOX assay was used to determine lipid hydroperoxide (HP) as described by Jiang et al. (1991). HP levels were detected spectrophotometrically at 560 nm, and shown as nmol g^{-1} of tissue. The SOD (EC 1.15.1.1) activity measurement was based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide radicals (Crouch et al., 1981), which was generated by hydroxylamine 37.5 mM in an alkaline solution (Otero et al., 1983). One unit of SOD was defined as the amount of protein needed to decrease the reference rate to 50 % of maximum inhibition. The data were expressed in units of USOD mg^{-1} of protein. GSH-Px (EC 1.11.1.9) activity was assayed by the method from Mills (1959) and modified by Hafeman et al. (1974). One unit of GSH-Px activity was defined as 1 μg of GSH min^{-1}. GSH-Px was shown in nmol mg of $protein^{-1}$. CAT (EC 1.11.1.6) activity was measured by the decrease in the H_2O_2 concentration for 15 s, reading the absorbance at 240 nm according to Aebi (1974). The data were shown as nmol mg of $protein^{-1}$. All the analytical details were previously described by Sampaio et al. (2008). The biological samples

Table 1
Initial (0 h), intermediary (24 h) and final (48 h) water parameters of pH, dissolved oxygen (DO; mm Hg), temperature (*T*; °C), alkalinity (CaCO3; mg L^{-1}), hardness (CaCO3; mg L^{-1}), Na^+ (mEq L^{-1}), K^+ (mEq L^{-1}) and ammonia (mEq L^{-1}) in the water of different experiments at which pacus, *Piaractus mesopotamicus*, were exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h.

Group	Time (h)	pH	DO (mm Hg)	*T* (°C)	Alkalinity CaCO3 (mg L^{-1})	Hardness CaCO3 (mg L^{-1})	Na^+ (mEq L^{-1})	K^+ (mEq L^{-1})	Ammonia (mEq L^{-1})
C	0	7.2	138	21.6	9.0	39.0	1.6	3.7	0.34
	24	7.2	135	21.4	10.4	40.0	1.8	3.8	0.63
	48	7.1	130	21.0	11.0	38.0	1.9	4.0	0.66
Cu	0	7.2	136	21.5	10.3	36.0	1.5	3.7	0.24
	24	7.1	140	21.2	10.3	36.0	1.8	3.5	0.60
	48	7.2	138	21.0	10.7	35.0	1.9	3.5	0.61
Hyp	0	6.5	128	21.0	10.1	36.0	1.4	3.2	0.26
	24	6.6	127	21.0	10.5	38.0	1.8	3.6	0.48
	48	6.5	130	21.3	10.9	37.0	1.7	3.7	0.77
CuHyp	0	6.5	130	21.0	10.3	37.0	1.6	3.4	0.24
	24	6.5	132	21.5	11.3	33.0	1.7	3.6	0.46
	48	6.5	128	21.2	11.1	33.0	1.8	3.7	0.60

analyzed ranged from 5 to 20 samples and the exact sample size (*n*) for each group is presented in Tables 3–5.

2.3. Hematological analyses

Blood and tissue samples were taken from six fish from each experimental group. Plasma protein (PP) was determined by the method of Bradford (1976) adapted to the microplate reader Dynex MRXTC 250 as described by Kruger (1994), using bovine serum albumin (BSA) as reference. The copper plasma concentration ($[Cu_P]$) was determined by atomic absorption spectrophotometry (standard methods AA 6800), and was presented as μg copper L^{-1}. All the hematological variables were evaluated according to the method of Jain (1986). The hematocrit (Ht) was determined by the microhematocrit centrifugation technique. The red blood cell count (RBC) was determined optically using a Neubauer chamber. Hemoglobin (Hb) was determined by Drabkin's reagent at an absorbance at 540 nm. The mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were computed from Ht, Hb and RBC.

2.4. Metallothionein concentration and Na^+/K^+-ATPase activity in the gills

Metallothionein (MT) in the gills was determined by the concentration of SH - groups according to Viarengo et al. (1997), using a reduced glutathione standard (GSH). The concentration of MT was quantified using Ellman's reagent containing NaCl 2 M, DTNB 0.43 in a phosphate buffer 0.2 M (pH 8.0), measured spectrophotometrically, at 412 nm and presented as MT g $tissue^{-1}$. The Na^+/K^+-ATPase (NKA) (EC 3.6.1.3) activity was determined as described by Quabius et al. (1997), and previously adapted to the studied species. In short, the specific NKA activity was defined as the difference between the inorganic phosphate (Pi) release in a medium containing KCl (5 mM) and a medium containing ouabain (2.5 mM). The Pi released was quantified and the activity of the enzyme was expressed in μM Pi mg $protein^{-1}$ h^{-1}. The absorbance was read at 595 nm on a microplate reader Dynex MRXTC 250. All the analytical details were previously described by Sampaio et al. (2008, 2010).

Table 2
Initial (0 h) and final (48 h) copper concentration (μg L^{-1}) in the water of different experiments at which pacus, *Piaractus mesopotamicus*, were exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD, $n = 3$.

Copper (μg L^{-1})	C	Cu	Hyp	CuHyp
0 h	5.70 ± 0.06	460.10 ± 5.60	5.40 ± 0.05	510.10 ± 4.40
48 h	1.04 ± 0.01	5.83 ± 0.07	3.30 ± 0.70	6.30 ± 0.09

2.5. Plasma intermediary metabolites

Samples of plasma were deproteinized with 20% TCA and centrifuged at 4 °C at 10,000*g* for 3 min. 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 (Gentzkow and Masen, 1942). The data were shown as μmol ml^{-1} plasma. All the analytical details were previously described by Sampaio et al. (2010).

2.6. Statistical analysis

The influence of factors and its interaction on response variables were evaluated via two-way ANOVA for unbalance data, based on general linear model (GLM) theory. For each variable, if interaction was significant ($p<0.05$), *F* tests for contrasts were applied to compare responses between levels within each factor. Also, *t*-tests were performed to determine which particular factor combination was different from control ($p<0.05$). Results are presented as mean responses and respective standard errors for each group (factor combination). All statistics were performed with SAS/STAT software, Version 9.01, SAS System for Windows.

3. Results and discussion

3.1. Aquatic parameters

The water quality parameters were kept constant and were within the acceptable levels for fish culture (Boyd, 1990) and with the characteristics of the water in the region where the experiments were performed (Table 1). The water copper concentrations (μg L^{-1}) in the beginning and at the end of experimental period are presented in Table 2. A possible explanation for the decrease in the dissolved copper concentration at the end of the experimental period is the absorption of this ion by the fish and by the low residence time of $CuSO_4$ in the water column (Button et al., 1977; Mastin and Rodgers, 2000). In preliminary studies, the correlation between nominal and measured copper concentrations was $r^2 = 0.8955$ (Sampaio, 2008). The general health conditions of fish were normal throughout the experiments, no mortality was observed in any experimental group.

3.2. Effects of copper and hypercarbia, alone and in combination

3.2.1. Oxidative metabolism

In recent decades, ecotoxicological research has revealed links between reactive oxygen species production and environmental contamination, suggesting that biomarkers of oxidative stress may be used in environmental monitoring programs. Physiological biomarkers have

Table 3
The hydroperoxide (HP; nmol.g tissue^{-1}), superoxide dismutase (SOD; USOD mg protein^{-1}), glutathione peroxidase (GSH-Px; nmol mg protein^{-1}) and catalase (CAT; BU mg protein^{-1}) in the liver of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD.

	C	*n*	Cu	*n*	Hyp	*n*	CuHyp	*n*	Cu x Hyp [1]
HP (nmol g tissue^{-1})	353.69 ± 23.88^{aA}	6	396.37 ± 27.68^{bA}*	9	381.09 ± 24.57^{aB}*	8	370.69 ± 18.90^{aA}	8	$p = 0.005$
SOD (USOD mg PT^{-1})	139.30 ± 37.03	5	194.91 ± 18.83*	9	154.77 ± 23.60	8	217.61 ± 34.58*	8	$p = 0.734$
GSH-Px (nmol mg PT^{-1})	16.50 ± 3.19 ^{bB}	7	13.67 ± 2.29 ^{aA}*	9	12.29 ± 1.79 ^{aA}*	7	18.51 ± 1.47 ^{bB}	7	$p < 0.001$
CAT (nmol mg PT^{-1})	1.678 ± 0.19^{bB}	4	1.177 ± 0.13^{aA}*	8	1.034 ± 0.14^{aA}*	7	1.516 ± 0.13^{bB}	8	$p < 0.001$

Lower case letters show the difference between the groups with the same Hyp condition, at different copper levels.
Capital letters show the difference between the groups with the same copper levels, at different Hyp conditions.
* Experimental groups differ from the control group (*t* test; $p < 0.05$).
[1] *p* Value for Hyp and copper interaction.

been used to understand the effects on aquatic organisms of exposure to numerous pollutants. However, such studies have typically focused on single factors or chemical substances. Ferreira et al. (2008) and Sampaio et al. (2008, 2010) recently analyzed the effects on some biomarkers in fish exposed to combinations of factors such as water quality and environmental contaminants.

Many pollutants are strong oxidants (Avci et al., 2005). Freshwater cyprinids from polluted areas showed signs of oxidative stress when compared to fish from unpolluted areas (Gül et al., 2004). The AD mechanisms against stressful conditions are well documented and can be exemplified as enzymes such as SOD, GSH-Px and CAT (Nascimento et al., 2006). Pedrajas et al. (1995) argued that the induction of CAT and GSH-Px after exposure to copper indicates that this metal induces HP species in the liver. Recently, Sampaio et al. (2008, 2010) investigated the effect of copper sulfate exposures on the AD of *P. mesopotamicus*, including in combination with hypoxia or acidic water. These authors showed that exposures to copper in association with hypoxia or acid medium caused significant increase in hydroperoxide production in hepatic tissue.

Table 3 presents the results of the oxidative metabolism in the hepatic tissue. A significant interaction between copper and hypercarbia was observed for the hepatic concentration of HP ($p = 0.005$). Compared to the control group, fish exposed to Cu and to Hyp increased the hepatic HP concentration. However, fish exposed to CuHyp did not change the hepatic HP concentration. The isolated effects of copper sulfate and hypercarbia indicate that single exposure significantly increased the hepatic HP concentration. Nevertheless, in associated exposure the hepatic HP did not differ from the control group. The effect of copper on hepatic SOD activity did not depend on the aquatic CO_2 level ($p = 0.734$). Compared to the control group, hepatic SOD activity increased in fish exposed to Cu and CuHyp. However, there was no significant difference in the response to copper exposure between normocarbic and hypercarbic groups. Petochi et al. (2011) exposed the sea bass, *Dicentrarchus labrax*, at different levels of hypercarbia ($PCO_2 = 2.0$, 13.9, 24.7 and 37.8 mm Hg) and for different periods (8, 22 and 45 days) and concluded that CO_2 treatment did not affect the reactive oxygen metabolites and total antioxidant capacity. However, they suggested a transient increase that can be interpreted as a consequence of an initial blood acidification, which favors the release of metal ions, increasing reactive metabolite formation. In the present study, the hepatic SOD activity was responsive only to copper exposure, characterizing that increases in hepatic SOD activity in response to copper were independent of the aquatic CO_2 concentration. Some authors characterize the increase in SOD activity as an indicator of polluted areas (Rodriguez-Ariza et al., 1991, 1992). When the effects of copper in hypercarbic water were compared to fish from control group, the hepatic SOD activity was intensified. These results showed that hepatic SOD activity is very effective to comprehend the effect of copper sulfate in fish, even in hypercarbic water.

The effect of copper on hepatic GSH-Px activity was dependent on the water CO_2 concentration, and differed between normocarbia and hypercarbia ($p < 0.001$). The groups exposed to Cu and Hyp presented a decrease in the hepatic GSH-Px activity when compared to the control group. Conversely, exposure to CuHyp did not change hepatic GSH-Px activity. The effect of copper on the hepatic CAT activity depended on the water CO_2 concentration ($p < 0.001$). Hepatic CAT activity decreased in response to copper in normocarbic water when compared to the hypercarbic groups. Conversely, exposure to CuHyp did not change hepatic CAT activity, indicating that the factors combined do not affect the activity of this enzyme. The hepatic GSH-Px and CAT induction in response to copper exposure in pacu depends on the water CO_2 level.

In red muscle, there were no significant interactions between copper and CO_2 levels in relation to HP concentration ($p = 0.130$) and SOD ($p = 0.435$), GSH-Px ($p = 0.628$) and CAT ($p = 0.112$) activity (Table 4). These red muscle antioxidant defenses (AD) displayed the same response pattern to copper exposure in both normocarbic and hypercarbic situations. When compared to the control group, the red muscle HP concentration was lower in the Cu, Hyp and CuHyp groups. The responses in red muscle oxidative mechanisms were different from those in the liver. Although red muscle is a highly oxidative tissue, copper and hypercarbia significantly decreased the HP concentration. The red muscle SOD activity was lower in the Hyp and CuHyp groups, when compared to fish from the control group. Red muscle SOD activity did not change in fish exposed to Cu. The oxidative red muscle typically exhibits higher AD activities (Mazeaud et al., 1979). The group of pacu exposed to hypercarbia, whether alone or with copper, had reduced SOD activity, which indicates a higher

Table 4
The hydroperoxide (HP; nmol g tissue^{-1}), superoxide dismutase (SOD; USOD mg protein^{-1}), glutathione peroxidase (GSH-Px; nmol mg protein^{-1}) and catalase (CAT; BU mg protein^{-1}) of red muscle of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD.

	C	*n*	Cu	*n*	Hyp	*n*	CuHyp	*n*	Cu x Hyp [1]
HP (nmol g tissue^{-1})	275.57 ± 17.41	7	240.59 ± 7.69*	9	228.13 ± 16.89*	8	228.89 ± 14.00*	8	$p = 0.130$
SOD (USOD mg PT^{-1})	459.99 ± 39.06	7	429.50 ± 37.52	8	244.76 ± 23.81*	8	232.78 ± 28.09*	8	$p = 0.435$
GSH-Px (nmol mg PT^{-1})	16.65 ± 7.92	6	13.44 ± 2.72	8	16.18 ± 2.51	7	14.75 ± 4.47	6	$p = 0.628$
CAT (nmol mg PT^{-1})	0.237 ± 0.09	7	0.144 ± 0.02 *	9	0.141 ± 0.05 *	8	0.116 ± 0.05*	8	$p = 0.112$

* Experimental groups differ from the control group (*t* test; $p < 0.05$).
[1] *p* Value for Hyp and copper interaction.

Table 5
The hydroperoxide (HP; nmol g tissue^{-1}), superoxide dismutase (SOD; USOD mg protein^{-1}), glutathione peroxidase (GSH-Px; nmol mg protein^{-1}) and catalase (CAT; BU mg protein^{-1}) in white muscle of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD.

	C	*n*	Cu	*n*	Hyp	*n*	CuHyp	*n*	Cu x Hyp [1]
HP (nmol g tissue^{-1})	100.34 ± 4.31	7	190.27 ± 12.10 *	8	217.85 ± 19.65*	6	186.18 ± 14.58*	8	$p = 0.550$
SOD (USOD mg PT^{-1})	235.27 ± 64.58	7	168.13 ± 35.32 *	8	254.82 ± 33.80	7	162.55 ± 42.93*	7	$p = 0.808$
GSH-Px (nmol mg PT^{-1})	35.00 ± 12.38	7	24.12 ± 6.21*	9	20.83 ± 5.32*	7	19.16 ± 2.72*	7	$p = 0.104$
CAT (nmol mg PT^{-1})	0.066 ± 0.03 ^{Bb}	6	0.033 ± 0.01 ^{aB}*	9	0.023 ± 0.01 ^{aA}*	8	0.015 ± 0.01 ^{aA}*	8	$p = 0.027$

Lower case letters show the difference between the groups with the same Hyp condition, at different copper levels.
Capital letters show the difference between the groups with the same copper levels, at different Hyp conditions.
* Experimental groups differ from the control group (*t* test; $p<0.05$).
[1] *p* Value for Hyp and copper interaction.

sensitivity of red muscle SOD to hypercarbia. Unlike the results in the liver, SOD activity in red muscle was not responsive to water copper concentration in hypercarbic conditions.

In relation to the control group, red muscle CAT activity was lower in fish of the Cu, Hyp and CuHyp groups, respectively. The red muscle GSH-Px activity was similar for fish exposed to both isolated and associated copper and CO_2 conditions. In the red muscle the results indicate that, among the peroxidases measured, red muscle CAT activity was more sensitive to hypercarbia than GSH-Px. According to Moore et al. (2008), CAT is more sensitive to oxidation due to the presence of a heme group. The GSH-Px may have a greater role in the detoxification of lipoperoxides (Matés, 2000).

There was no significant interaction between copper and CO_2 levels on the white muscle HP concentration ($p = 0.550$), or in SOD ($p = 0.808$) and GSH-Px activities ($p = 0.104$) (Table 5). These white muscle antioxidant defenses presented the same response pattern to copper exposure in both normocarbic and hypercarbic conditions. In contrast, a significant interaction between copper and CO_2 was observed for CAT activity ($p = 0.027$) in this tissue. The CAT activity following copper exposure was dependent on the presence of hypercarbia.

When compared to the control group, the white muscle HP concentration was higher in the Cu, Hyp and CuHyp groups, respectively. The white muscle SOD activity was lower in the Cu and CuHyp groups, respectively, when compared to control group. White muscle SOD activity decreased in fish exposed to 400 μg Cu L^{-1}, whether alone or in combination with hypercarbia. When compared to the control group, the white muscle GSH-Px activity was lower in the Cu, Hyp and CuHyp groups. In relation to the control group, the CAT activity decreased in the Cu, Hyp and CuHyp groups, respectively. The increase in HP concentration in white muscle can be correlated with the low activities of the AD. White muscle is less oxidative than other tissues and possibly has less effective AD mechanisms. The white muscle AD demonstrated similar responses in pacu exposed to copper in both normocarbia and hypercarbia. The low SOD activity and the decrease in the GSH-Px and CAT activities suggest that the AD mechanisms were not efficient enough to avoid an oxidative stress process in this tissue.

As found in red muscle, the white muscle CAT activity was more sensitive to the stressors than GSH-Px was. This suggests that copper and/or hypercarbia modify the relationship between these peroxidases. Furthermore, the use of antioxidant enzymes and the HP concentration as biomarkers of copper exposure is contingent upon the physical and chemical water conditions. The data also indicate that copper exposure under hypercarbia is an oxidative stress for pacu.

3.2.2. Intermediary metabolites

Table 6 shows the values for plasma glucose, lactate, pyruvate, ammonia and protein in fish exposed to copper and hypercarbia. Significant interactions between copper and hypercarbia were observed in pacu plasma glucose ($p<0.011$) and lactate ($p<0.001$) concentration. In normocarbia, exposure to copper had no effect on plasma glucose concentration whereas exposure to Hyp caused a decrease when compared to the control group. Conversely, the same copper concentration in hypercarbia conditions increased plasma glucose concentration. The effects of copper on plasma lactate concentration depended on the water CO_2 level. Lactate levels did not change in the Cu group, but they decreased in the Hyp group and increased in the CuHyp group. The hypoglycemia and reduced lactate plasma concentration of fish exposed to Hyp was abolished when they were exposed to CuHyp. The fish of group CuHyp exhibited lactate plasma concentration significantly higher than those of control. Metabolic changes can be measured though different indicators in plasma. According to Begum and Vijayaraghavan (1999) an increase in plasma lactate can be an indicator of a metabolic disorder and suggests respiratory stress in fish tissues. Some authors consider plasma lactate concentration the more sensitive indicator of acute stressors (Acerete et al., 2004).

There was no interaction between copper and CO_2 levels for their effects on plasma concentrations of pyruvate ($p = 0.884$), ammonia ($p = 0.097$) and protein ($p = 0.072$). These variables presented the same response to copper exposure in both normocarbic and hypercarbic water. Compared to the control, plasma ammonia concentrations were lower in the Hyp and CuHyp groups. Plasma pyruvate and protein concentrations were similar in all groups.

Hypercarbic water is suggested as a stressor inducing increases in plasma glucose and lactate concentrations. Petochi et al. (2011) observed an increased glycemia in sea bass exposed to hypercarbia for 8 days of exposure and concluded that this occurred as a consequence of stress. However, in the present study the exposure to hypercarbia caused a significant decrease in plasma glucose, lactate and ammonia concentrations, while pyruvate and protein remained unchanged. These results indicate that exposure to hypercarbic water caused hypoglycemia without induction of anaerobic metabolism. Perry et al. (1988) evaluated the interactive effects of catecholamine and hypercapnia on hepatic metabolism of rainbow trout (*Oncorhynchus mykiss*). These authors observed a rapid depletion on liver glycogen and concomitant hyperglycemia when fishes were exposed to hypercapnia. According to Devlin (1997), a decrease in the glycolytic metabolism is related to metabolic acidosis prevention as glycolysis is the main H^+ source in the organism.

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

The branchial metallothionein concentration and NKA activity of fish exposed to copper and hypercarbia are shown in Figs. 1 and 2, respectively. There was no significant interaction between copper and CO_2 for their effects on branchial metallothionein (MT) concentration ($p<0.230$). Fish exposed to Cu and CuHyp had higher branchial MT concentrations, by comparison to the other groups. Thus, the increased branchial MT was exclusively in response to water copper levels. The action of metallothionein in gill tissues of aquatic organisms has been described and characterized by many authors (Mouneyrac et al., 1998). Canli et al. (1997) highlighted these proteins as potential indicators of cellular stress and sublethal heavy metal exposure. Considered as the first defense system against metal exposure (Bragigand and Berthet,

Table 6
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 normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD.

	C	*n*	Cu	*n*	Hyp	*n*	CuHyp	*n*	Cu x Hyp [1]
Glucose (μmol mL^{-1})	1.23 ± 0.04 ^{aB}	5	1.17 ± 0.33^{aA}	7	0.83 ± 0.21^{aA}*	8	1.34 ± 0.27 ^{bA}	5	$p = 0.011$
Lactate (μmol mL^{-1})	1.28 ± 0.10^{aB}	5	1.27 ± 0.44^{aA}	7	0.87 ± 0.30^{aA}*	8	1.99 ± 0.37^{bB}*	5	$p < 0.001$
Pyruvate (μmol mL^{-1})	0.130 ± 0.01	5	0.140 ± 0.03	7	0.107 ± 0.02	8	0.114 ± 0.04	5	$p = 0.884$
Ammonia (μmol mL^{-1})	0.230 ± 0.01	5	0.214 ± 0.04	7	0.146 ± 0.03*	8	0.182 ± 0.05*	5	$p = 0.097$
Protein (mg mL^{-1})	9.09 ± 0.32	5	9.02 ± 1.74	6	8.37 ± 0.74	8	9.47 ± 0.56	5	$p = 0.072$

Lower case letters show the difference between the groups with the same Hyp condition, at different copper levels.
Capital letters show the difference between the groups with the same copper levels, at different Hyp conditions.
* Experimental groups differ from the control group (*t* test; $p < 0.05$).
[1] *p* Value for Hyp and copper interaction.

2003), cellular MT concentrations are generally very low but increase considerably during copper exposure (Roesijadi, 1994). In the present study, the branchial MTs were responsive to aquatic copper, which corroborates the findings of Dang (2000) and Ryu et al. (2003).

Some other factors not related to metal contamination can also induce the synthesis of MT. Variables such as temperatures (Serafim et al., 2002), pH (Carvalho et al., 2004), salinity (Leung et al., 2002), organism size (Leung and Furness, 2001), capture stress, harvest and transport, salinity and reproductive stage (Baer and Thomas, 1990), variation in dissolved oxygen concentration and freezing (English and Storey, 2003) and the presence of antibiotics or herbicides (Mosleh et al., 2004) are the main mechanisms inducing increases in the MT concentration in fish. However, the level of induction in these parameters is usually lower than that caused by metals (Kägi, 1993), therefore the utilization of MT as a biomarker depends on the environmental conditions, not only on the concentration of metals (Amiard et al., 2006). Although the MTs are considered as general biomarkers of pollution, the results of the present study indicate that they are not sensitive to hypercarbia. Sampaio et al. (2008, 2010) found that branchial MT also presented a specific response to increased copper, irrespective of dissolved O_2 and pH.

There was no significant interaction between copper and CO_2 for branchial NKA ($p < 0.875$). Regardless of the water CO_2, exposure to copper decreased branchial NKA activity in the Cu. Conversely, the groups Hyp and CuHyp maintained the branchial NKA at control levels. Copper may cause a decrease in the plasma Na^+ and Cl^- concentration due to a disruption in the branchial ionic regulation system (Nussey et al., 1995; Pelgrom et al., 1995). Under low copper concentrations, the ion uptake can be suppressed by the inhibition of NKA (Li et al., 1998). In the present study, the inhibition of NKA activity in pacu exposed to copper may be linked to the metal's capacity to bind to the active site of the enzyme (Grosell et al., 2002). The results also indicate that the inhibition of NKA by copper occurred only when copper was added in normocarbic water. Baker et al. (2009) suggested that the ability of the white sturgeon, *Acipenser transmontanus*, to tolerate hypercarbic exposures is due to the increase of the branchial NKA, which contributed to maintaining whole animal pH homeostasis. In pacu, the increased NKA activity in hypercarbic water, without copper, may have had a similar role.

3.2.4. Hematological variables and plasma copper concentrations [Cu_p]

Due to the close association between the fish circulatory system and the external environment, water quality is an important factor responsible for variations in hematological variables (Cassillas and Smith, 1977). In recent decades, hematological variables have been used in clinical diagnosis of fish health, helping to assess the effects of xenobiotics and toxic substances (Wendelaar Bonga, 1997). Hematological parameters are considered indicators of stress (Sancho et al., 2000) and exposure to toxic substances as metals (Wepener et al., 1992). Thus, hematology might be considered a key indicator of overall health in fishes (Nussey et al., 1995), and variations in hematological profile indicate disturbances in physiological processes (Ranzani-Paiva et al., 2000).

Table 7 presents the RBC, Hct, Hb, MCHC and MCV values in the experimental groups. There was no interaction between copper and CO_2 for effects on RBC ($p = 0.503$), Hct ($p < 0.796$), Hb ($p = 0.964$), MCHC ($p < 0.807$) or MCV ($p = 0.857$). All of these variables showed the same pattern and intensity of response to copper exposure in both normocarbic and hypercarbic conditions. Compared to the control

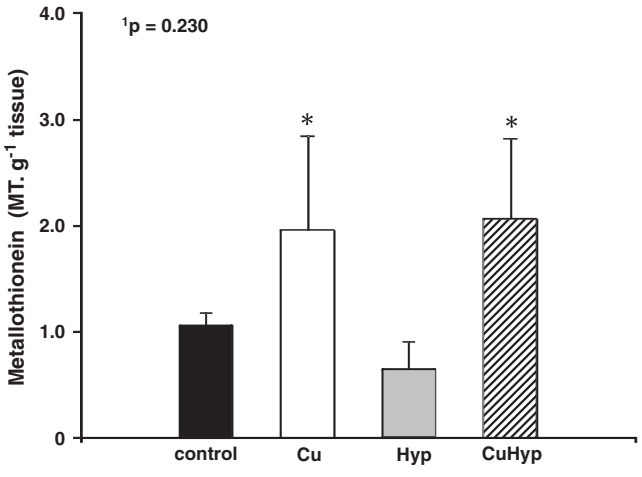

Fig. 1. Metallothionein (MT g^{-1} tissue) of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD. *Experimental groups differ from the control group (*t* test; $p < 0.05$). *p* Value for Hyp and copper interaction.

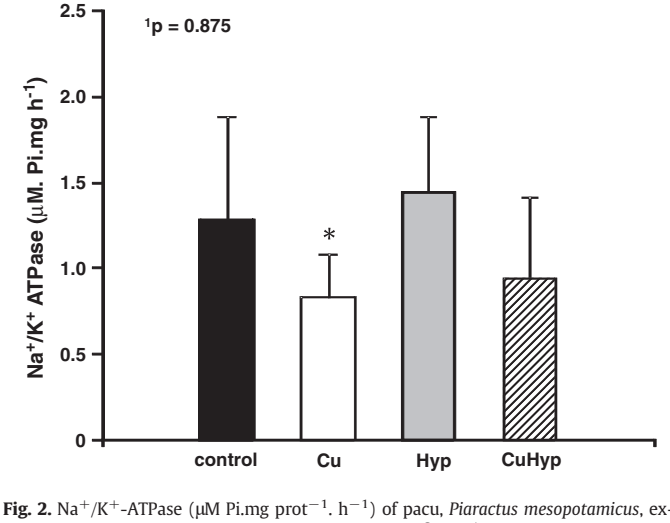

Fig. 2. Na^+/K^+-ATPase (μM Pi.mg $prot^{-1}$. h^{-1}) of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD. *Experimental groups differ from the control group (*t* test; $p < 0.05$). *p* Value for Hyp and copper interaction.

group, the RBC and Hb increased in fish exposed Cu, Hyp and CuHyp. The Hct was higher in the Cu group compared to the control. Also comparing to the control group, the MCHC was higher in the Hyp and CuHyp groups and the MCV decreased in Cu, Hyp and CuHyp groups. Nussey et al. (1995) exposed tilapia mossambica, *Oreochromis mossambicus*, to 0.40 mg L^{-1} copper, and found an increase in RBC levels, similar to the current results on pacu, which may indicate that copper induced erythropoiesis. Hypercarbia also had significant effects on hematological variables in the pacu, so it is perhaps not surprising that the association of copper and hypercarbia stimulated mechanisms involved in O_2 uptake and transport more than either factor alone. Petochi et al. (2011) observed a modest transient increase in Hb and Hct of sea bass exposed to acute hypercarbia. Gilmour (1998) classified these responses as a quantitative strategy controlling blood gas transport and compensating changes in blood O_2 carrying capacity induced by hypercarbia. According to Perry et al. (1999), hypercarbia activates the autonomic nervous systems, stimulating sympathetic pathways inducing spleen contraction in fish. Stressed fish tend to deplete their splenic stores, elevating their Hct (Gallaugher and Farell, 1998). A transient increase of Hct is also attributed by different authors to the erythrocyte swelling, as reported by Fivelstad (1999) and Fivelstad et al. (2003a) for Atlantic salmon exposed to over 19 mg L^{-1} CO_2 and juvenile yellowtail kingfish, *Seriola lalands*, maintained at 50 mg L^{-1} CO_2 (Moran et al., 2008). Nevertheless, the transient increase in Hb and Hct could also be caused by higher amount of RBC, primarily due to spleen contraction (Yamamoto, 1987) activated by adrenergic response to carbon dioxide (Gilmour, 1998) and also by an increase in erythropoietin synthesis by the kidney (Lai et al., 2006). The hematological responses of pacu exposed to copper in normocarbic or hypercarbic water indicate that the presence of copper in the water induced an increase in Hb concentration.

Fig. 3 presents the plasma copper concentrations [Cu_p], there was no interaction between copper and dissolved CO_2 ($p = 0.063$), [Cu_p] was higher in the Cu and CuHyp groups when compared to the control. This confirms that exposure to copper increased [Cu_p], regardless of water CO_2 concentration. A considerable part of the copper diffused across the gills is transported by plasma (Pelgrom et al., 1995), and the current results clearly indicate that the pacu exposed to copper had absorbed it across the gills and into the blood, and that this process was not affected by hypercarbia. This shows that [Cu_p] is an important biomarker to monitor copper toxicity. Sampaio et al. (2010) found that [Cu_p] of fish exposed to copper in acid medium was higher than that presented by fish exposed to copper at neutral pH. Another possibility is that low pH increases the Cu^{2+} species, increasing the bioavailability of copper. Copper toxicity can result in branchial ionic regulation impairments (Wood, 2001; Grosell et al., 2002). This metal affects the gill morphology inducing necrosis, hypertrophy, epithelium enlargement and vacuolation (Beaumont et al., 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. As a result, the gas exchange efficiency is seriously impaired. The results of the present study indicate that, if hypercarbia influenced ventilatory variables, this had no effect on copper uptake at the gills. The present results suggest that the increased branchial MT concentration observed in fish exposed to CuHyp could be a protective mechanism against the copper exposure, avoiding the increase in [Cu_p]. As proposed by Mayer et al. (2003) the MTs scavenge metals, maintaining low free concentration and acting as a protective mechanism.

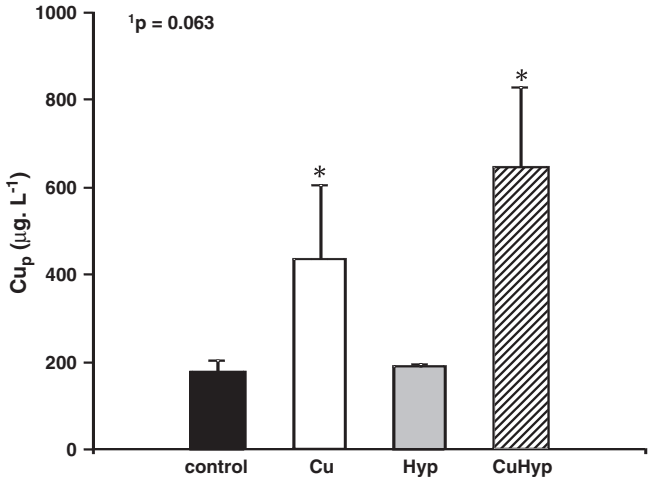

Fig. 3. [Cu_p] (μg L^{-1}) of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD. * experimental groups differ from the control group (*t* test; $p<0.05$). *p* Value for Hyp and copper interaction.

Understanding the effects of hypercarbia in fish is important for the management of intensive aquaculture farms and for fish welfare. The hypercarbic conditions, characteristic of many polluted and non-polluted aquatic environments, may increase the contaminants toxicity in fish. Since most toxicity tests were conducted in normocarbic water, it is important to characterize the toxicity factors affecting environments with increased carbon dioxide. The present study shows that pacu is able to cope well with the hypercarbic experimental level. However, little is known on the tolerance of this species to higher CO_2 tensions and how more hypercarbic levels can affect its welfare in aquaculture tanks. Our data points out that the association of hypercarbia plus copper induced changes in several physiological responses of pacu when compared to the responses to single conditions. This study provides new information on copper-induced effects associated with hypercarbic conditions in freshwater fish and is addressed to comprehend the use of this compound.

Acknowledgements

The authors are thankful to CNPq (F.G. Sampaio doctoral fellowship) and Aquapeixe Aquaculture (Conchal, SP, Brazil) fish farm by providing the fish. We are also grateful to Prof. Dr. Pedro de Magalhães Padilha (Laboratory of Chemistry and Biochemistry, Institute of Bioscience, São Paulo State University, Botucatu — SP, Brazil) for the laboratory analysis,

Table 7
The red blood cell (RBC; $\times 10^6$ μL^{-1}), hematocrit (Hct; %), hemoglobin (Hb; g dL^{-1}), mean corpuscular hemoglobin concentration (MCHC; %) and mean corpuscular volume (MCV; fL) of pacu, *Piaractus mesopotamicus*, exposed to copper-free and normocarbia (C), 400 μg Cu^{2+} L^{-1} (Cu), hypercarbia (Hyp) and 400 μg Cu^{2+} L^{-1} + Hyp (CuHyp) for 48 h. Values are mean ± SD.

	C	*n*	Cu	*n*	Hyp	*n*	CuHyp	*n*	Cu x Hyp [1]
RBC ($\times 10^6$ μL^{-1})	1.55 ± 0.34	10	2.01 ± 0.32 *	10	1.86 ± 0.17 *	8	2.44 ± 0.26 *	10	$p = 0.503$
Hct (%)	33.60 ± 2.76	10	38.10 ± 8.71*	10	33.44 ± 1.54	8	37.10 ± 2.55	10	$p = 0.796$
Hb (g dL^{-1})	7.88 ± 1.27	10	9.21 ± 1.56*	10	9.52 ± 0.58*	8	10.80 ± 1.66*	10	$p = 0.964$
MCHC (%)	23.64 ± 4.53	10	25.06 ± 5.82	10	28.55 ± 2.74*	8	29.21 ± 4.84*	10	$p = 0.807$
MCV (fL)	225.64 ± 49.66	10	193.81 ± 55.37*	10	180.70 ± 15.11*	8	153.67 ± 22.42*	10	$p = 0.857$

* Experimental groups differ from the control group (*t* test; $p<0.05$).
[1] *p* Value for Hyp and copper interaction.

and Dr. David John McKenzie for the text revision. The present project was supported by the São Paulo State Research Foundation — FAPESP (Proc. 03/06105-7).

References

Acerete, L., Balasch, J.C., Espinosa, C., 2004. Physiological responses in Eurasian perch (*Perca fluviatilis* L.) subjected to stress by transport and handling. Aquaculture 237, 167–178.

Aebi, H., 1974. Catalase. In: Bergmeyer, H.-U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 671–684.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202.

Avci, A., Kaçmaz, M., Durak, I., 2005. Peroxidation in muscle and liver tissues from fish in a contaminated river due to a petroleum refinety industry. Ecotoxicol. Environ. Saf. 60, 101–105.

Baer, K.N., Thomas, P., 1990. Influence of capture stress, salinity and reproductive status on zinc associated with metallothionein like-proteins in the liver of three teleost species. Mar. Environ. Res. 29, 277–287.

Baker, D.W., Matey, V., Huynh, K.T., Wilson, J.M., Morgan, J.D., Brauner, C.J., 2009. Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, *Acipenser transmontanus*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296, R1868–R1880.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 2003. Exposure of brown trout, *Salmo trutta*, to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation. J. Exp. Biol. 206, 153–162.

Begum, G., Vijayaraghavan, S., 1999. Effect of acute exposure of the organophosphate insecticide rogor on some biochemical aspects of *Clarias batrachus* (Linnaeus). Environ. Res. A 80, 80–83.

Blancheton, J.P., 2000. Developments in recirculation system for Mediterranean fish species. Aquacult. Eng. 22, 17–31.

Boyd, C.E., 1979. Water Quality in Warm Water Fish Ponds. Agricultural Experiment Station, Auburn University, Auburn, Al.

Boyd, C.E., 1990. Water Quality in Ponds for Aquaculture. Alabama Agricultural Experiment Station, Auburn University, Auburn, Al.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bragigand, V., Berthet, B., 2003. Some methodological aspects of metallothionein evaluation. Comp. Biochem. Physiol. A 134, 55–61.

Button, S.B., Hostetler, H.P., Mair, D.M., 1977. Copper dispersal in water supply reservoir. Water Res. 11, 539–544.

Canli, M., Stagg, R.M., Rodger, G., 1997. The induction of metallothionein in tissues of the Norway lobster *Nephrops norvegicus* following exposure to cadmium, copper and zinc: the relationships between metallothionein and the metals. Environ. Pollut. 96, 343–350.

Carbonell, G., Tarazona, J.V., 1993. A proposed method to diagnose acute copper poisoning in cultured rainbow trout (*Oncorhynchus mykiss*). Sci. Total Environ. Suppl. 1329–1334.

Carvalho, C.S., Araújo, H.S.S., Fernandes, M.N., 2004. Hepatic metallothionein in a teleost (*Prochilodus scrofa*) exposed to copper at pH 4.5 and pH 8.0. Comp. Biochem. Physiol. B 137, 225–234.

Cassillas, E., Smith, L.S., 1977. Effect of stress on blood-coagulation and hematology in rainbow-trout (*Salmo gairdneri*). J. Fish Biol. 10, 481–491.

CETESB (Companhia de Tecnologia de Saneamento Ambiental), 2010. Relatório de qualidade das águas superficiais no estado de São Paulo 2009. CETESB, São Paulo.

Crocker, C.E., Cech, J.J., 1998. Effects of hypercarbia on blood-gas and acid–base status in the white sturgeon, *Acipenser transmontanus*. J. Comp. Physiol. 168, 50–60.

Crocker, C.E., Farrell, A.P., Gamperl, A.K., Cech, J.J., 2000. Cardiorespiratory response of white sturgeon to environmental hypercarbia. Am. J. Physiol. Regul. Integr. 279, 617–628.

Crouch, R.K., Gandy, S.E., Kimsey, G., Galbraith, R.A., Galbraith, M.P., Buse, M.G., 1981. The inhibition of islet superoxide dismutase by diabetogenic drugs. Diabetes 30, 235–241.

Dang, Z. 2000. Adaptative stress responses in fish gills. PhD Thesis. Catholic University of Nijmegen, Nijmegen, Netherlands.

Devlin, T.M., 1997. Manual de Bioquímica com Correlações Clínicas, 4th ed. Edgard Blucher, São Paulo, SP.

Dubois, M.G., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–358.

EFSA (European Food Safety Authority), 2008. Scientific report of EFSA prepared by Working Group on seabass/seabream welfare on Animal Welfare Aspects of Husbandry Systems for Farmed European seabass and gilthead seabream. Annex I. EFSA J. 844, 1–89.

English, T.E., Storey, K.B., 2003. Freezing and anoxia stresses induce expression of metallothionein in the foot muscle and hepatopancreas of the marine gastropod *Littorina littorea*. J. Exp. Biol. 206, 2517–2524.

Ferreira, A.B.G., Amadeu, S.L., Soares, V.M., 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on *Daphnia magna*. Aquat. Toxicol. 89, 28–39.

Fivelstad, S., 1999. Vannkvalitet i Norske Smoltanlegg: Vannkvalitet, Oksygenering, pH og Karbondioksyd (in Norwegian), pp. 1–12.

Fivelstad, S., Haavik, H., Lovik, G., Olsen, A.B., 1998. Sublethal effects and safe levels of carbon dioxide for Atlantic salmon postsmolts (*Salmo salar* L.). Aquaculture 160, 305–316.

Fivelstad, S., Olsen, A.B., Asgard, T., Baeverfjord, G., Rasmussen, T., Vindheim, T., Stefansson, S., 2003a. Long-term sublethal effects of carbon dioxide on Atlantic salmon smolts (*Salmo salar* L.): ion regulation, haematology, element composition, nephrocalcinosis and growth parameters. Aquaculture 215, 301–319.

Fivelstad, S., Olsen, A.B., Waagbo, R., Zeitz, S., Hosfeld, C.D., Stefansson, S., 2003b. A major water quality problem in smolt farms: combined effects of carbon dioxide and reduced pH and aluminium on Atlantic salmon (*Salmo salar* L.) smolts: physiology and growth. Aquaculture 215, 339–357.

Fivelstad, S., Waagbo, R., Stefansson, S., Olsen, A.B., 2007. Impacts of elevated water carbon dioxide partial pressure at two temperatures on Atlantic salmon (*Salmo salar* L.) parr growth and haematology. Aquaculture 269, 241–249.

Florindo, L.H., 2002. O papel dos quimiorreceptores de O_2 e CO_2/pH no controle dos reflexos cardio-respiratórios e da respiração na superfície aquática (ASR) em tambaqui, *Colossoma macropomum*: Respostas a longo prazo durante a hipóxia e a hipercarbia. Ph.D. Thesis. PPGCF, Federal University of São Carlos, SP, Brazil.

Florindo, L.H., Reid, S.G., Kalinin, A.L., Milsom, W.K., Rantin, F.T., 2004. Cardiorespiratory reflexes and aquatic surface respiration in the neotropical fish tambaqui (*Colossoma macropomum*): acute responses to hypercarbia. J. Comp. Physiol. 174 B, 319–328.

Gallaugher, P., Farell, A.P., 1998. Hematocrit and blood–oxygen-carrying capacity. Fish Physiol. 17, 185–227.

Gentzkow, C.J., Masen, J.M., 1942. An accurate method for the determination of blood urea nitrogen by direct nesslerization. J. Biol. Chem. 143, 531–544.

Gilmour, K.M., 1998. Gas Exchange, In: Evans, D.H. (Ed.), The Physiology of Fishes, 2nd ed. CRC Press, Boca Raton, pp. 101–127.

Good, C., Davidson, J., Welsh, C., Snekvik, K., Summerfelt, S., 2010. The effects of carbon dioxide on performance and histopathology of rainbow trout *Oncorhynchus mykiss* in water recirculation aquaculture systems. Aquacult. Eng. 42, 51–56.

Grosell, M., Nielsen, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp. Biochem. Physiol. B 133, 287–303.

Gül, S., Belge-Kurutas, E., Yildiz, E., Sahan, A., Doran, F., 2004. Pollution correlated modifications of liver antioxidant systems and histopathology of fish (*Cyprinidae*) living in Seyhan Dam Lake, Turkey. Environ. Int. 30, 605–609.

Hafeman, D.G., Sunde, R.A., Hoekstra, W.G., 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr. 104, 580–587.

Handy, R.D., Eddy, F.B., Baines, H., 2002. Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. Biochim. Biophys. Acta Biomembr. 1566, 104–115.

Harrower, J.R., Brown, C.H., 1972. Blood lactic acid. A micromethod adapted to field collection of microliter samples. J. Appl. Physiol. 32 (5), 709–711.

Hosfeld, C.D., Engevik, A., Mollan, T., Lunde, T.M., Waagbo, R., Olsen, A.B., Breck, O., Stefansson, S., Fivelstad, S., 2008. Long-term separate and combined effects of environmental hypercarbia and hyperoxia in Atlantic salmon (*Salmo salar* L.) smolts. Aquaculture 280, 146–153.

Jain, N.C., 1986. Schalm's Veterinary Hematology. Lea e Febiger, Philadelphia, PA.

Jiang, Z.Y., Wollard, A.C.S., Wolf, S.P., 1991. Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids 26, 853–856.

Kägi, J.H.R., 1993. Evolution, structure and chemical activity of class I metallothioneins: an overview. In: Suzuki, K.T., Imura, N., Kimura, M. (Eds.), Metallothioneins III: Biological Roles and Medical Implications. Birkhauser Verlag, Basel, pp. 29–55.

Kruger, N.J., 1994. The Bradford method for protein quantification. Meth. Mol. Biol. 32, 9–15.

Lai, J.C.C., Kakuta, I., Mok, H.O.L., Rummer, J.L., Randall, D., 2006. Effects of moderate substantial hyperoxia on erythropoietin levels in rainbow trout kidney and spleen. J. Exp. Biol. 209, 2734–2738.

Larsen, B.K., Pörtner, H.-O., Jensen, F.B., 1997. Extra-and intracellular acid–base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercarbia and copper. Mar. Biol. 128, 337–346.

Leung, K.M.Y., Furness, R.W., 2001. Metallothionein induction and condition index of dogwhelks *Nucella lapillus* (L.) exposed to cadmium and hydrogen peroxide. Chemosphere 44, 321–325.

Leung, K.M.Y., Svavarsson, J., Crane, M., Morritt, D., 2002. Influence of static and fluctuating salinity on cadmium uptake and metallothionein expression by the dogwhelk *Nucella lapillus* (L.). J. Exp. Mar. Biol. Ecol. 274, 175–189.

Li, J., Quabius, E.S., Wendelaar Bonga, S.E., Flik, G., Lock, R.A.C., 1998. Effects of waterbone copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquat. Toxicol. 43, 1–11.

Lu, G.D., 1939. The metabolism of piruvic acid in normal and vitamin B-deficient state. A rapid specific and sensitive method for estimation of blood pyruvate. Biochem. J. 33, 249–254.

MacIntyre, C., Ellis, T., North, B.P., Turnbull, J.F., 2008. The Influences of Water Quality on the Welfare of Farmed Trout: A Review. In: Branson, E. (Ed.), Fish Welfare. Blackwell Scientific Publications, London, pp. 150–178.

Mastin, B.J., Rodgers Jr., J.H., 2000. Toxicity and bioavailability of copper herbicides (Clearigate, Cutrine-Plus, copper sulfate) to freshwater animals. Arch. Environ. Toxicol. 39, 445–451.

Matés, J.M., 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153, 83–104.

Mayer, O.H., Leach, A., Kling, P., Olsson, P., Hogstrand, C., 2003. Activation of the rainbow trout metallothionein — a promoter by silver and zinc. Comp. Biochem. Physiol. B 134, 181–188.

Mazeaud, F., Maral, J., Michelson, A.M., 1979. Distribution of superoxide dismutase and glutathione peroxide in the carp: erythrocytic manganese SOD. Biochem. Biophys. Res. Commun. 84, 1161–1168.
Mazon, A.F., Fernandez, M.N., 1999. Toxicity and differential tissue accumulation of copper in the tropical freshwater fish, *Prochilodus scrofa* (Phrocilodontidae). Bull. Environ. Contam. Toxicol. 63, 797–804.
Mazon, A.F., Cerqueira, C.C.C., Fernandes, M.N., 2002. Gill cellular changes induced by copper exposure in the South American tropical freshwater fish *Prochilodus scrofa*. Environ. Res. 88 A, 52–63.
McKenzie, D.J., Dalla Valle, A.Z., Taylor, E.W., Steffensen, J.F., 2002. Tolerance of acute hypercapnic acidosis by the European eel (*Anguilla anguilla*). J. Comp. Physiol. 172 B, 339–346.
McKenzie, D.J., Dalla Valle, A.Z., Piccolella, M., Taylor, E.W., Steffensen, J.F., 2003. Tolerance of chronic hypercapnia by the European eel (*Anguilla anguilla*). J. Exp. Biol. 206, 1717–1726.
Mills, G.C., 1959. The purification and properties of glutathione peroxidase of erythrocytes. J. Biol. Chem. 234, 502–506.
Moore, R.L., Powell, L.J., Goodwin, D.C., 2008. The kinetic properties producing the perfunctory pH profiles of catalase-peroxidases. Biochim. Biophys. Acta 1784, 900–907.
Moran, D., Wells, R.M.G., Pether, S.J., 2008. Low stress response exhibited by juvenile yellowtail kingfish (*Seriola lalandi* Valenciennes) exposed to hypercarbic conditions associated with transportation. Aquacult. Res. 39, 1399–1407.
Mosleh, Y.Y., Paris-Palacios, S., Arnoult, F., Couderchet, M., Biagianti-Risbourg, S., Vernet, G., 2004. Metallothionein induction in aquatic oligochaete *Tubifex tubifex* exposed to herbicide isoproturon. Environ. Toxicol. 19, 88–93.
Mouneyrac, C., Amiard, J.C., Amiard-Triquet, C., 1998. Effects of natural factors (salinity and body weight) on cadmium, copper, zinc and metallothionein-like protein levels in resident populations of oysters *Crassostrea gigas* from a polluted estuary. Mar. Ecol. Prog. Ser. 162, 125–135.
MPA (Ministério da Pesca e Aquicultura), 2010. Boletim estatístico da pesca e aquicultura. Brasil, 2008–2009.
Nascimento, I.A., Pereira, S.A., Leite, M.B.N.L., 2006. Biomarcadores como instrumentos preventivos de poluição. In: Zagatto, P.A., Bertoletti, E. (Eds.), Ecotoxicologia aquática: métodos e aplicações. Rima, São Carlos, pp. 413–432.
Nussey, G., Van Vuren, J.H.J., Du Preez, H.H., 1995. Effect of copper on the hematology and osmoregulation of the Mozambique tilapia, *Oreochromis mossambicus* (Cichidae). Comp. Biochem. Physiol. C 111, 369–380.
Otero, J., Toni, P., Garcia Morato, Y.V., 1983. Cu-Zn superoxide dismutase, Metodo para su determinación. Ver. Iberoam. Invest. Clin. 2, 121–127.
Pedrajas, J.R., Peinado, J., Lopez-Barea, J., 1995. Oxidative stress in fish exposed to model xenobiotics. Oxidatively modified forms of Cu, Zn superoxide dismutase as potential biomarkers. Chem. Biol. Interact. 98, 267–282.
Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., Wendelaar-Bonga, S.E., 1995. Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. Aquat. Toxicol. 32, 303–320.
Perry, S.F., Gilmour, K.M., 1996. Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercarbia in an elasmobranch (*Squalus acanthias*) and a teleost (*Oncorhynchus mykiss*). J. Exp. Biol. 199, 2105–2118.
Perry, S.F., Walsh, P.J., Mommsen, T.P., Moon, T.W., 1988. Metabolic consequences of hypercapnia in the rainbow trout *Salmo gairdneri*: β-adrenergic effects. Gen. Comp. Endocrinol. 69, 439–447.
Perry, S.F., Fritsche, R., Hoagland, T.M., Duff, D.W., Olson, K.R., 1999. The control of blood pressure during external hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 202, 2177–2190.
Petochi, T., Di Marco, P., Priori, A., Finoia, M.G., Mercatali, I., Marino, G., 2011. Coping strategy and stress response of European sea bass *Dicentrarchus labrax* to acute and chronic environmental hypercarbia under hyperoxic conditions. Aquaculture 315, 312–320.
Portz, D.E., Woodley, C.M., Cech, J.J., 2006. Stress-associated impacts of short-term holding on fishes. Rev. Fish Biol. Fish. 16, 125–170.
Quabius, E.S., Balm, P.H.M., Wendelaar Bonga, S.E., 1997. Interrenal stress responsiveness of tilapia (*Oreochromis niloticus*) is impaired by dietary exposure to PCB 126. Gen. Comp. Endocrinol. 108, 472–482.
Ranzani-Paiva, M.J.T., Ishikawa, C.M., Das Eiras, A.A., Felizardo, N.N., 2000. Haemotological analysis of Chara, *Pseudoplatystoma fasciatum* in captivity. Aqua 2000: Responsible Aquaculture in the New Millennium, Nice, France, 2–6 May 2000: Special publication 28. European Aquaculture Society (590 pp.s).
Rodriguez-Ariza, A., Dorado, G., Pueyo, C., Lopez-Barea, J., 1991. Biochemical effects of environmental pollution in fishes from Spanish south-Atlantic littoral. Biochem. Soc. Trans. 19, 301S.
Rodriguez-Ariza, A., Abril, N., Navas, J.I., Dorado, G., Lopez-Barea, J., Pueyo, C., 1992. Metal, mutagenicity, and biochemical studies on bivalve molluscs from Spanish coasts. Environ. Mol. Mutagen. 19, 112–114.
Roesijadi, G., 1994. Metallothionein induction as a measure of response to metal exposure in aquatic animals. Environ. Health Perspect. 102, 91–95.
Ryu, S.-K., Park, J.-S., Lee, I.-S., 2003. Purification and characterization of a copper-binding protein from Asian periwinkle *Littorina brevicula*. Comp. Biochem. Physiol. C 134, 101–107.
Sampaio, F.G., 2008. Defesas antioxidantes e alterações bioquímicas no pacu. *Piaractus mesopotamicus* (Holmberg, 1887), em resposta à exposição ao cobre e ao meio ácido, isolados ou associados. PPGCF, Federal University of São Carlos, SP, Brazil.
Sampaio, F.G., de Lima, C.B., Oba, E.T., Santos, L.R.B., Kalinin, A.L., Rantin, F.T., 2008. Antioxidant defenses and biochemical changes in pacu (*Piaractus mesopotamicus*) in response to single and combined copper and hypoxia exposure. Comp. Biochem. Physiol. C 147, 43–51.
Sampaio, F.G., Boijink, C.L., Santos, L.R.B., Oba, E.T., Kalinin, A.L., Rantin, F.T., 2010. The combined effect of copper and low pH on antioxidant defenses and biochemical parameters in neotropical fish pacu, *Piaractus mesopotamicus* (Holmberg, 1887). Ecotoxicology 19, 963–976.
Sancho, E., Cerón, J.J., Ferrando, M.D., 2000. Cholinesterase activity and hematological parameters as biomarkers of sublethal molinate exposure in *Anguilla anguilla*. Ecotoxicol. Environ. Saf. 46, 81–86.
Serafim, A., Company, R.M., Bebianno, M.J., Langston, W.J., 2002. Effect of temperature and size on metallothionein synthesis in the gill of *Mytilus galloprovincialis* exposed to cadmium. Mar. Environ. Res. 54, 361–365.
Smart, G.R., 1981. Aspects of Water Quality Producing Stress in Intensive Fish Culture. In: Pickering, A.D. (Ed.), Stress and Fish. Academic Press, London, pp. 277–289.
Smith, E., Naidu, R., Alston, A.M., 2002. Heavy metals in the environment. Chemistry of inorganic arsenic in soils: II. Effect of phosphorus, sodium, and calcium on arsenic sorption. J. Environ. Qual. 31, 557–563.
Straus, D.L., Tucker, C.S., 1993. Acute toxicity of copper sulfate and chelated copper to channel catfish *Ictalurus puntatus*. J. World Aquacult. Soc. 24, 390–395.
Summerfelt, S.T., Vinci, B.J., Piedrahita, R.H., 2000. Oxygenation and carbon dioxide control in water reuse systems. Aquacult. Eng. 22, 87–108.
Thorarensen, H., Farrell, A.P., 2011. The biological requirements for post-smolt Atlantic salmon in closed-containment systems. Aquaculture 312, 1–14.
Tort, L., Pavlidis, M.A., Woo, N.Y.S., 2011. Stress and Welfare in Sparid Fish. In: Pavilidis, M., Mylonas, C.C. (Eds.), Sparid: Biology and Aquaculture of Gilthead Sea Bream and Other Species. Blackwell Publishing, Oxford, UK (Ed).
Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organism: an application to Mediterranean and Antartic mollusk. Mar. Environ. Res. 44, 69–84.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.
Wepener, V., Van Vuren, J.H., Du Preez, H.H., 1992. The effect of hexavalent chromium at different pH values on the hematology of *Tilapia sparrmanii* (Cichlidae). Comp. Biochem. Physiol. C 101, 375–381.
Wood, C.M., 2001. Toxic responses of the gill. In: Benson, W.H., Schlenk, D.W. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts. Taylor and Francis, Washington, pp. 1–87.
Yamamoto, K., 1987. Contraction of spleen in exercised cyprinid. Comp. Biochem. Physiol. A 87, 1083–1087.