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

Fernanda Garcia Sampaio [a], Cheila de Lima Boijink [b], Laila Romagueira Bichara dos Santos [c], Eliane Tie Oba [d], Ana Lúcia Kalinin [a,e], Alfredo José Barreto Luiz [f], Francisco Tadeu Rantin [a,e,*]

[a] Brazilian Agricultural Research Corporation, EMBRAPA, Environment, Ministry of Agriculture, Livestock and Food Supply, Jaguariúna, SP, Brazil
[b] Brazilian Agricultural Research Corporation, EMBRAPA, West Amazon, Ministry of Agriculture, Livestock and Food Supply, Manaus, AM, Brazil
[c] Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, SP, Brazil
[d] Brazilian Agricultural Research Corporation, EMBRAPA, Amapá, Ministry of Agriculture, Livestock and Food Supply, Macapá, AP, Brazil
[e] National Institute of Science and Technology, Comparative Physiology (FAPESP/CNPq), Brazil
[f] Brazilian Agricultural Research Corporation, EMBRAPA, Environment, Ministry of Agriculture, Livestock and Food Supply, Jaguariúna, SP, Brazil

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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.

* Corresponding author at: Federal University of São Carlos, Department of Physiological Sciences, Via Washington Luiz, km 235, 13565-905 São Carlos, SP, Brazil. Tel.: +55 16 3351 8314; fax: +55 16 3351 8401.
E-mail address: ftrantin@ufscar.br (F.T. Rantin).

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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