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Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish *Prochilodus lineatus*☆

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Abstract

We investigated the effect of copper on liver key enzymes of the anaerobic glucose metabolism (hexokinase, HK; phosphofructokinase, PFK; pyruvate kinase, PK; lactate dehydrogenase, LDH) as well as of the pentose pathway (glycose-6-phosphate dehydrogenase, G6PDH) from the fish *Prochilodus lineatus*. The fish were acclimated at either 20 °C or 30 °C at pH 7.0, transferred to water at pH 4.5 or 8.0, and exposed to 96 h-CL_{50} copper concentrations. Copper accumulation in liver was higher in fish acclimated at 20 °C and maintained in water pH 8.0. Three-way analysis of variance revealed a significant effect of temperature on all enzymes, a significant effect of pH on all enzymes except for PK, and a significant effect of copper on only PFK, and LDH in pH 4.5 at 20 °C and, at 30 °C, on PFK and PK at pH 4.5 and 8.0, HK at pH 4.5 and G6PDH at pH 8.0. There were significant interactions between treatments for many enzymes. These changes suggest that the activity of enzymes in question is modified by a change in ambient water. At least at 30 °C, the overall reduction in the glycolytic enzyme activities of copper-exposed fish seems to reduce energy availability via glucose metabolism, thereby contributing to enhance copper toxic effects.

Keywords: Copper; Fish; Glycolytic enzymes; Liver; *Prochilodus lineatus*; Water pH; Temperature

1. Introduction

Copper is a trace element which is essential to the function of specific proteins and enzymes. However, at high concentrations it may be toxic to organisms. The increasing industrial activities and the use of $CuSO_4$ as a fungicide in agricultural practices as well as in the control of algae and pathogens in fish culture ponds have increased the copper concentration in aquatic systems. Furthermore, occasional accidents have aggravated this situation by suddenly introducing substantial amounts of copper into aquatic environments, which may be accompanied by changes in water pH, depending on the type of industrial effluent in question.

The toxicity of copper to fish has been well documented. In addition to its acute lethality (Pickering and Henderson, 1966; Cusimano et al., 1986; Carvalho and Fernandes, 2006), a wide range of toxicological responses of several organs to this metal has been reported in a number of fish species (Wood, 2001; Dautremepuits et al., 2004). Copper alters the function of the gills and liver (Karan et al., 1998; Grosell et al., 2002; Dautremepuits et al., 2004) by causing severe histological changes in these organs (Mazon et al., 2002a,b; Sola et al., 1995; Arellano et al., 1999; Fernandes and Mazon, 2003; van Heerden et al., 2004). The toxic action of copper may be direct on the cellular components or occur by inducing stress responses (Wendelaar Bonga, 1997; Mazon et al., 2004). In both cases, the physiological and morphological disruption of cellular integrity is a consequence of the impairment in the biochemical systems.

Copper uptake in freshwater fish occurs mainly by the gills, followed by the skin and intestine. The liver is the major organ

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in which copper homeostasis occurs. Copper is accumulated in the liver to be excreted via the bile, even though gills and kidneys also participate in its excretion (Grosell et al., 1998; Mazon and Fernandes, 1999). Copper toxicity depends on chemical and physical characteristics of the water. Temperature, pH, hardness, and alkalinity are the main factors influencing copper bioavailability in aquatic environments (Tao et al., 2001). For example, ambient pH may promote copper uptake by the gills depending on water hardness and alkalinity (Tao et al., 2001), while the temperature may enhance the copper effects on enzyme activity by changing the metabolic rate, kinetics, and structural properties of the enzymes (Somero, 1995, 2004).

To supply the energy demand for detoxification and repair processes in fish exposed to copper, the glycogen/glucose is mobilized in the liver. Inside the cells, each metabolic pathway is continuously regulated in order to maintain homeostasis and, in general, few key enzymes control the metabolic flux (Brooks and Storey, 1995). In the anaerobic metabolism of glucose such key enzymes as hexokinase (HK) and phosphofructokinase (PFK) are at the beginning of the glycolytic sequence and the pyruvate kinase (PK) and lactate dehydrogenase (LDH) are at the terminal sequence of the glycolytic pathway. The glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme of the pentose-phosphate shunt. Although direct effects of copper on certain glycolytic enzymes have been demonstrated in extracts of mammalian and fish tissues (Lai and Blass, 1984; Isani et al., 1994; Gul et al., 2004), little is known about the effect of copper on these enzymes after exposing the whole animal to the metal (Tóth et al., 1996; Beaumont et al., 2000; Antognelli et al., 2003).

The fish *Prochilodus lineatus* is highly sensitive to copper (Mazon and Fernandes, 1999), and although this species is tolerant to changes in water pH, the toxicity of the metal to this fish may be strongly enhanced by ambient pH (Takasusuki et al., 2004; Carvalho et al., 2004a,b; Carvalho and Fernandes, 2006). The temperature acclimation of *P. lineatus* ranges from 15 °C to 35 °C (Barrionuevo and Fernandes, 1995, 1998; Fernandes et al., 1995), and the thermal tolerance zone is equivalent to 1046 °C in 20 g fish (Barrionuevo and Fernandes, 1995). *P. lineatus* displays partial oxygen compensation and a short time-course for adjustments of aerobic metabolism, irrespective of changes in water temperature (Fernandes et al., 1995; Barrionuevo and Fernandes, 1998).

In this context, this study investigated the effect of copper on the activity of the key enzymes of the anaerobic glucose metabolism (hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase) as well as on the activity of the glucose-6-phosphate dehydrogenase in the liver of the neotropical freshwater fish *P. lineatus* at extreme environmental conditions, i.e., in ambient pH 4.5 and 8.0, at 20 °C and 30 °C.

2. Materials and methods

2.1. Animals

Juvenile *P. lineatus* (Teleostei, Prochilodontidae) (body mass = 15–25 g; total length = 10–15 cm) from the Hydrobiology and Aquaculture Station of Furnas Hydroelectric Power Plant, São José da Barra, MG, Brazil, were maintained for 30 days in holding tanks (1000 L) at 25 ± 1 °C with a continuous flow of aerated dechlorinated tap water (water composition: pH = 7.0 ± 0.2; conductivity = 8.3 ± 0.3 μS; alkalinity = 23.7 ± 1.9 mg L^{-1} as $CaCO_3$; hardness = 24.5 ± 0.2 mg L^{-1} as $CaCO_3$). After this period, the water temperature was decreased or increased by 1 °C on alternate days until it reached 20 °C or 30 °C. The fish were maintained at these temperatures for 30 days prior to the experiments. The photoperiod was 12D: 12L. The fish were fed with balanced fish food suitable for this species (FRI-ACQUA 40, Fri-Ribe Rações, SP, Brazil). Water pH, hardness and alkalinity were identical to the mean values found in the natural habitat of *P. lineatus*, and the temperature was set to the lowest and highest average temperature for winter and summer, respectively (CETESB, 1995–2005).

2.2. Experimental design

Groups of fish (maximum of 1 g fish L^{-1}, $n = 8–10$ each group) were randomly taken from the acclimation tanks and transferred to static test glass-aquaria (200 L) with continuous aeration, total hardness equal 24 mg L^{-1} as $CaCO_3$ and temperature set at either 20 °C or 30 °C. To evaluate the influence of pH and temperature alone on the enzyme activities, three control groups were maintained in water free of copper at each acclimation temperature (20 °C and 30 °C). Control group 1 was transferred to aquaria with water reduced to pH 4.5, group 2 was transferred to aquaria with water pH 7.0, and control group 3 was transferred to aquaria with water pH increased to 8.0. To evaluate the effect of copper on the enzyme activities in fish maintained in water at either low or high pH, two experimental groups were exposed to copper at each acclimation temperature. Experimental group 1 was maintained in aquaria with water pH 4.5, and experimental group 2 was maintained in aquaria with water pH 8.0. All experiments were performed in duplicate. Each aquarium of copper-exposed groups received 98 ± 0.8 μg Cu L^{-1} in pH 4.5 and 16 ± 0.2 μg Cu L^{-1} in pH 8.0 at 20 °C; 88 ± 0.8 μg Cu L^{-1} in pH 4.5 and 14 ± 0.5 μg Cu L^{-1} in pH 8.0 at 30 °C, which are the nominal 96 h-LC_{50} copper concentration for *P. lineatus* in water pH 4.5 and pH 8.0 at 20 °C and 30 °C (Carvalho and Fernandes, 2006). Copper was added as $CuSO_4 \cdot 5H_2O$, and its concentration in water was determined by an Atomic Absorption Spectrophotometer (AA12/1475 Gemini), according to quality assurance and quality control (QA/QC) requirements and SRM 3114 NIST (USA) reference standards. No copper was detected in the aquaria of the control groups. The pH was decreased to 4.5 by adding concentrated sulfuric acid, and increased to 8.0 through the addition of 2.0 N sodium hydroxide. Copper concentration and water pH in the experimental aquaria were adjusted prior to fish transfer. Physical and chemical characteristics of aquaria water were kept constant throughout the 96 h period of experiment.

After 96 h, the fish from the control groups and the surviving fish from copper-exposed groups were removed from aquaria, rapidly killed by decapitation and the liver was dissected in ice. Liver samples were collected, frozen in liquid nitrogen and stored at −80 °C (not longer than 3 weeks).