Redox profile in liver of *Leporinus macrocephalus* exposed to different dissolved oxygen levels

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Abstract Aquatic organisms are continuously exposed to environmental variations, which can lead to physiological and biochemical alterations. *Leporinus macrocephalus*, known as piavuçu, is a migratory species that may be exposed to variations in dissolved oxygen levels. Studies evaluating oxidative changes undergone by this species in these conditions are scarce. Therefore, this investigation aimed at evaluating oxidative alterations in *L. macrocephalus* exposed to different oxygen levels for 96 h: 6.12 ± 0.18, 3.99 ± 0.17, 3.22 ± 0.17, 2.47 ± 0.30 and 0.710 ± 0.07 mg L^{-1}. At the end of the experimental period, fish were euthanized and livers used to determine lipid hydroperoxides, thiobarbituric acid reactive substances, catalase, glutathione-S-transferase, superoxide dismutase and thiol groups, which are an indirect measure of reduced glutathione. Results indicated a decrease in the studied parameters in hypoxic situations, suggesting a possible metabolic depression.

Keywords Oxidative stress · Hypoxia · Lipid peroxidation · Antioxidants

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Introduction

The aquatic environment presents natural daily and/or seasonal variations in dissolved oxygen levels. Such changes are due to high temperatures associated with organic decay and consumption by aquatic organisms, as well as anthropogenic action (Diaz and Rosemberg 1995; Wu 1999; Sampaio et al. 2008). The fluctuations in aquatic oxygen levels can affect the distribution of ectotherms, especially fish (Lushchak and Bagnyukova 2007). Some environmental conditions may lead to an imbalance between the generation of reactive oxygen species (ROS), also referred to as pro-oxidants, and the rates of antioxidants, whose action protects the cell against oxidation and ROS. If ROS is

produced to excess, and/or there is a decline in the levels of antioxidants, oxidative stress takes place (Sies 1991). Detrimental effects such as oxidation of proteins, DNA, steroid components and unsaturated lipid peroxidation in cell membranes may arise in the body from oxidative damage (Martínez-Alvarez et al. 2005). Energy limitations are observed in organisms living in places with oxygen deprivation. One of the consequences from this situation is the alteration in the redox state of the cell. When oxygen, the final acceptor of the electron transport chain, is available, the intermediate carriers are reduced, what can change such redox-dependent reactions as lipid peroxidation and ROS generation (Oliveira et al. 2005).

Biomarkers of environmental conditions include antioxidant and detoxification enzymes, as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST). They are located within different cellular compartments in most vertebrate tissues though the highest activity is seen in the liver which is the major organ for biotransformation of xenobiotics and amelioration of ROS (Zakharov et al. 1993; Lemaire et al. 1994; Monferrán et al. 2011).

Aquatic hypoxia triggers a complex set of physiological and biochemical alterations in fish, including decreased metabolic rate (DallaVia et al. 1994; Terova et al. 2008), increased ventilation rate, hematocrit and hemoglobin O_2 affinity (Jensen et al. 1993; Terova et al. 2008) and increased anaerobic respiration (Virani and Rees 2000). Most of these parameters are related to changes in the expression of the gene that encodes proteins with relevant physiological functions through activation of hypoxia inducible factor-1 (HIF) (Terova et al. 2008). HIF-1 is a heterodimer composed of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (Wang et al. 1995; Semenza 2007). Redox reactions have an important role in stability, DNA binding and phosphorylation of HIF-1α protein in salmonid cells. Thus, environmental disturbances involving oxidative stress may influence HIF-1α protein function and consequent gene expression (Nikinmaa et al. 2004).

Apparently, each teleost species responds distinctively to hypoxia: for example, lipid peroxidation decreased in liver of *Leporinus elongatus* while antioxidants remained stable or increased after 28 h hypoxia (Wilhelm Filho et al. 2005). There was an increase in CAT activity in liver of *Carassius auratus*

exposed to 8 h anoxia but other antioxidants and lipid peroxidation did not alter (Lushchak et al. 2001). In *Piaractus mesopotamicus*, antioxidants defenses such as CAT and GPx decreased after 48 h hypoxia whereas lipid peroxidation and SOD did not change significantly (Sampaio et al. 2008).

Leporinus macrocephalus (Garavello and Britski 1988) (Anastomidae) is distributed throughout Paraguay and Paraná rivers basins. It is a migratory species, performing long ascending displacement during its pre-reproductive period (Reynalte-Tataje and Zaniboni Filho 2010). This species is bound to face frequent different dissolved oxygen levels in the water due to its migratory and distributional characteristics. Studies evaluating oxidative changes suffered by this species in these conditions are inexistent. Thus, the aim of this work was to analyze oxidative parameters in *L. macrocephalus* exposed to different dissolved oxygen levels.

Materials and methods

Experimental protocol

Fish (186.24 $\pm$ 7.26 g) were allocated in five groups ($n = 12$) and acclimated to laboratory conditions for 14 days in 250-L tanks under controlled temperature (23 $\pm$ 1.0°C) and dissolved oxygen levels (6.12 $\pm$ 0.18 mg L^{-1}). After this period, the animals were subjected to low levels of dissolved oxygen for 96 h: 3.99 $\pm$ 0.17, 3.22 $\pm$ 0.17, 2.47 $\pm$ 0.30 and 0.71 $\pm$ 0.07 mg L^{-1}. The control group was kept at 6.12 $\pm$ 0.18 mg L^{-1} O_2. Little variation was detected in the levels of oxygen as the laboratory environmental conditions were relatively stable, but the necessary adjustments were carried out by adding nitrogen and/or air to the tanks four times a day between 7 a.m. and 8 p.m. Fish were fed commercial food once a day at 8 a.m. and siphoning was performed 30 min later. Subsequently, an average 25% replacement of tank water previously prepared with the adequate dissolved oxygen level was performed, followed by the verification of the oxygen level.

Water parameters

Both at the beginning and at the end of the experiment, water alkalinity (37.23 $\pm$ 0.7 mg L^{-1} CaCO$_3$), water

hardness (29.07 ± 0.61 mg L^{-1} $CaCO_3$) and total ammonia (4.82 ± 0.53 mg L^{-1}) were determined by the sulfuric acid (H_2SO_4), the ethylenediaminetetraacetic acid (EDTA) titrimetric and the direct nesslerization methods, respectively according to Greenberg et al. (1976). Dissolved oxygen levels were monitored by means of an oxymeter (YSI model Y 5512) and water pH (7.15 ± 0.09) with a Quimix 400A pH meter.

Tissue collection

At the end of the experimental period, animals were euthanized by sectioning the spinal cord and livers dissected out and frozen to determine oxidative stress parameters: lipid hydroperoxide, thiobarbituric acid reactive substances (TBARS), CAT, SOD and GST. Livers were homogenized in a medium containing 120 mM potassium chloride (KCl) and 30 mM buffer phosphate (pH 7.4) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at $1,110 \times g$ in a refrigerated centrifuge for 20 min and supernatants separated for oxidative analyses (Buege and Aust 1978). Samples used to determine lipid hydroperoxides by xilenol orange method were homogenized in 30 mM buffer phosphate and butylated hydroxytoluene (BHT) 40 mM, followed by centrifugation at $1,110 \times g$ in refrigerated centrifuge for 20 min (Jiang et al. 1991). Samples for the determination of thiol groups were homogenized in trichloroacetic acid (TCA) 20% and centrifuged at $1,110 \times g$ in a refrigerated centrifuge for 10 min (Ellman 1959).

Pro-oxidants assay

Lipid peroxidation was monitored through two methods: determination of lipid hydroperoxide and TBARS. The former was performed with a modified version of the method by Jiang et al. (1991). This technique can detect the primary products of peroxidation, using Fe^{2+} oxidation by lipid hydroperoxides in acid medium in the presence of xylenol orange dye, forming a complex with Fe^{3+}. Reading was performed in spectrophotometer at 560 nm. Results are reported as nmol mg protein^{-1}. TBARS, in turn, was determined according to Wills (1987), measuring the end products of lipid peroxidation such as malondialdehyde. Aliquots of the supernatant were added to a Pyrex tube containing TCA 10% and thiobarbituric acid (TBA) 0.67% and incubated at 100°C for 45 min. The mixture was allowed to cool on ice for 5 min and then centrifuged at $1,000 \times g$ for 5 min, in order to extract the resulting chromogen (Schiff's base). The absorbance of the organic phase was determined at 535 nm in a spectrophotometer. Results are reported as nmol mg protein^{-1}.

Protein assay

Tissue proteins were quantified based on Lowry et al. (1951) using bovine albumin as standard. Reading was performed in spectrophotometer at 625 nm.

Antioxidant defenses assay

CAT activity was evaluated by measuring the decrease in the absorption at 240 nm in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4) and 2 mM hydrogen peroxide (H_2O_2), thereby determining the pseudo-first-order reaction constant (k') of the decrease in H_2O_2 absorption, as outlined in Boveris and Chance (1973). Results are reported as pmol mg protein^{-1}.

Total SOD activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine/sodium hydroxide (pH 10.2). Enzyme activity is expressed as SOD units mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adenochrome formation, as described by Misra and Fridovich (1972).

GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined spectrophotometrically at 340 nm according to Habig et al. (1974). The assay was performed at 25°C using 100 mM potassium phosphate buffer, pH 6.5, with reduced glutathione (GSH) and CDNB (dissolved in ethanol) at a final concentration of 1 mM each. Activity was calculated from the changes in absorbance at 340 nm. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 pmol of CDNB with GSH per minute at 25°C. The enzymatic activity is expressed as pmol min^{-1} mg protein^{-1}.

Thiol groups are nonenzymatic antioxidants and represent an indirect measure of GSH. The method is based on GSH reacting with 5,5′-ditio-bis-2-nitrobenzoic (DTNB) acid. The final product formed is the yellow 2-nitro-5-mercapto-benzoic (TNB) acid. The

samples were read spectrophotometrically at 412 nm (Ellman 1959). The content of thiol groups is expressed as μmol mg protein^{-1}.

Statistical analysis

Statistical analysis was performed with Software GraphPad® Instat 3.0. Bartlet's method was used to determine if the data were parametric and Kolmogorov & Smirnov's to test homoscedasticity. Since the data showed a normal distribution, analyses were carried out with one-way ANOVA followed by Dunnett's test. Regression analysis was done using SigmaPlot® 11.0. Minimum significance was 95% ($P < 0.05$).

Results

No mortality was observed in any of the treatments but *L. macrocephalus* exposed to 0.71 ± 0.07 mg L^{-1} swam near the waterline.

Results indicate a significant decrease in hydroperoxide levels determined by xylenol orange in liver: 39.21% (1.47 ± 0.44 nmol mg protein^{-1}) in *L. macrocephalus* exposed to 3.22 mg L^{-1} O$_2$ and 68.79% (1.91 ± 0.40 nmol mg protein^{-1}) in those kept at 0.71 mg L^{-1} O$_2$ ($P < 0.05$), showing significant lipoperoxidation in this tissue when compared to control (6.12 ± 2.39 nmol mg protein^{-1}) (Fig. 1a). Lipid peroxides measured by TBARS, content of total thiol groups and CAT activity decreased according to the reduction of dissolved oxygen levels (Figs. 1b, 2a, b). No statistically significant difference was seen for SOD ($P < 0.05$) (Fig. 2c). GST activity decreased significantly, 39.01 and 49.51%, in the groups exposed to 2.47 mg L^{-1} (4.14 ± 0.25 pmol min^{-1} mg protein^{-1}) and 0.71 mg L^{-1} O$_2$ (3.45 ± 0.16 pmol min^{-1} mg protein^{-1}), respectively comparing to control (6.84 ± 0.6 pmol min^{-1} mg protein^{-1}) (Fig. 2d). A positive correlation was found between the levels of thiol groups and hydroperoxide groups in the liver ($r^2 = 0.908$, $P < 0.05$) (Fig. 3); thus, a reduction in both pro-oxidants and antioxidants was observed in this tissue.

Discussion

Organisms with aquatic respiration suffer permanent interference of dissolved oxygen levels, what may
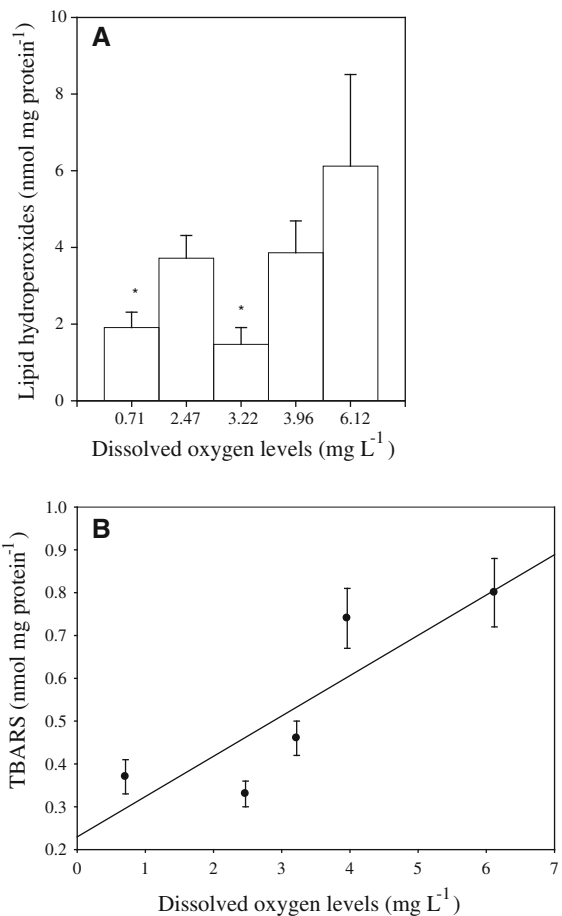
Fig. 1 (**a**) Determination of lipid hydroperoxides by xylenol orange. (**b**) Relationship between thiobarbituric acid reactive substances and dissolved oxygen levels in which *Leporinus macrocephalus* was kept for 96 h, according to equation $y = 0.2298 + 0.0941x$ ($r^2 = 0.740$ $P < 0.05$), where y is the level of lipid peroxidation through TBARS (nmol mg protein^{-1}) and x the dissolved oxygen levels (mg L^{-1}). *Significant difference from control by one-way ANOVA and Dunnett test ($P < 0.05$)

influence their survival and behavior, among other aspects. Each species displays different strategies in order to cope with low levels of dissolved oxygen according to the environment it is set in. Studies carried out with species that inhabit essentially the same region as *L. macrocephalus* have demonstrated that they can endure low dissolved oxygen levels: the lethal concentration (LC$_{50-96h}$) of dissolved oxygen is 0.52 mg L^{-1} for *Rhamdia quelen* (Braun et al. 2006), 0.38 mg L^{-1} for *Hoplias malabaricus* and 0.36 mg L^{-1} for *Pimelodus maculatus* (Parma de Croux 1994). LC$_{50}$ of dissolved oxygen for *L. macrocephalus* has not yet
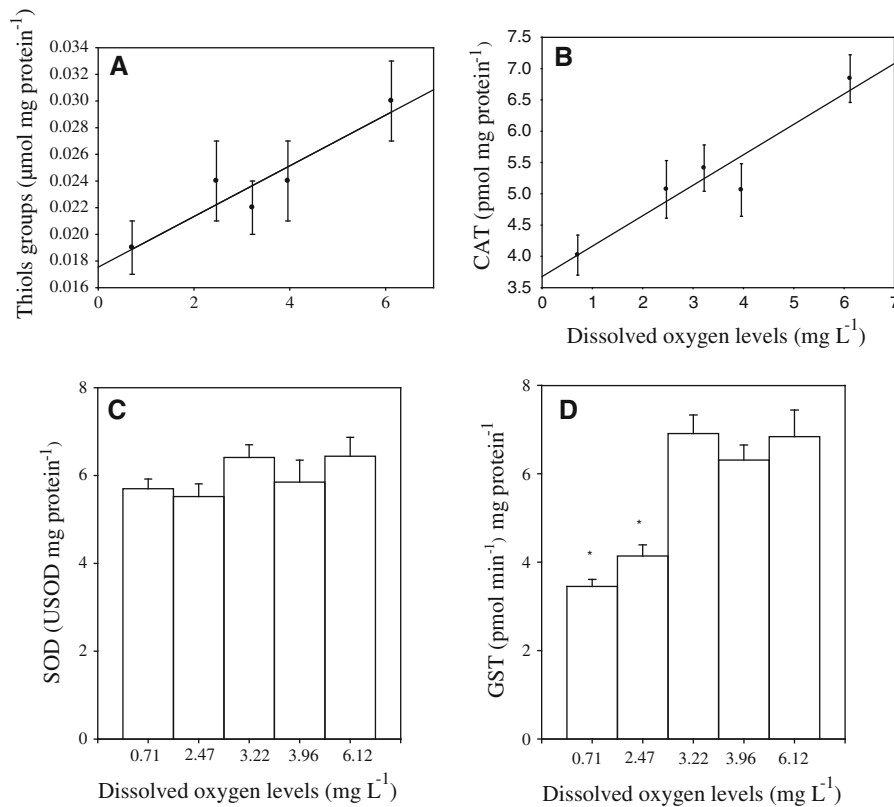
Fig. 2 (**a**) Relationship between thiol groups content in liver and dissolved oxygen levels in which *L. macrocephalus* was kept for 96 h, according to equation $y = 0.0172 + 0.022x$ ($r^2 = 0.881$), where y is the thiol groups content (µmol protein^{-1}) and x the dissolved oxygen levels (mg L^{-1}). (**b**) Relationship between catalase activity and dissolved oxygen levels in which *L. macrocephalus* was kept for 96 h, according to

equation $3.676 + 0.486x$ ($r^2 = 0.9046$), where y is the catalase activity (pmol mg protein^{-1}) and x the dissolved oxygen levels (mg L^{-1}). Activity of superoxide dismutase (**c**) and glutathione-S-transferase (**d**) in liver of *L. macrocephalus* exposed to different oxygen levels. *Significant difference from control by one-way ANOVA and Dunnett test ($P < 0.05$)
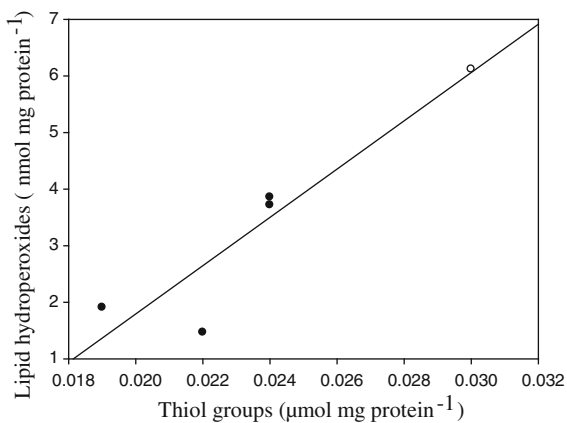

Fig. 3 Correlation of thiol group levels with lipid hydroperoxides in liver of *Leporinus macrocephalus* exposed to different oxygen levels for 96 h

been analyzed. Nonetheless, no mortality was detected throughout the experiment.

Physiological changes have been observed in fish exposed to hypoxia. *Ictalurus punctactus* kept for 7 days in moderate hypoxia (2 mg L^{-1} O$_2$) increased ventilation rate and heart rate, mechanisms triggered in order to maintain a constant oxygen partial pressure in the respiratory cascade (Burleson et al. 2002). Exposure of *C. auratus*, which had a remarkable tolerance against oxygen deprivation, to anoxia, led to metabolic depression and conversion of lactate produced by anaerobic glycolysis into ethanol, which is excreted via the gills (Shoubridge and Hochachka 1980, 1983; Van Waversveld et al. 1989; Roesner et al. 2008). Silver catfish juveniles exposed to very low oxygen levels (0.40 ± 0.05 mg L^{-1}) resort to

strategies in an attempt to minimize hypoxia effects, like searching for oxygen near the water surface and increasing opercular beat rate (Braun et al. 2006). A similar behavior was observed in *L. macrocephalus* exposed to 0.71 ± 0.07 mg L^{-1}. Such behaviors can be observed in culture tanks to identify critical hypoxic conditions when oxymeters are not available (Braun et al. 2006).

As well as behavioral changes, oxidative alterations may also be induced by hypoxia. Biological oxidation is a primitive process (Kelly et al. 1998). Oxidation of lipids is the most commonly used approach in free radical research field because many organisms, especially the aquatic ones, contain high amounts of lipids with polyunsaturated fatty acid residues, a substrate for oxidation. Since lipids are oxidized usually through the formation of peroxides, the process of their formation has been called lipid peroxidation (Lushchak and Bagnyukova 2006). The lipid hydroperoxides detected by xylenol orange arise from the damage caused by lipoperoxidation in its early stage while the final products of lipoperoxidation are seen through TBARS. In the present experiment both methods have displayed a decrease in the lowest levels of dissolved oxygen. The reduction in prooxidant rates found in this study may be related to the fact that in hypoxic conditions there is a decline in the metabolic rate, what makes the electron chain transport, the primary and biggest source of ROS, to be blocked or to operate at low intensity (Lushchak and Bagnyukova 2006), thus decreasing oxidant levels and consequently tissue damage. This mechanism is a possible explanation for our findings.

In order to attenuate deleterious effects of biological oxidation, aerobic organisms have developed antioxidant defense mechanisms. The nonenzymatic antioxidants are the first components of this defense line, with the enzymatic antioxidant mechanisms evolving only later (Kelly et al. 1998). Nonenzymatic antioxidants, such as thiols, act mainly by suppressing, eliminating and inactivating free radicals (Ribeiro et al. 2005). One of the main nonprotein thiols is glutathione, which in its reduced form (GSH) is involved in the protection of the membrane against lipoperoxidation (Lushchak and Bagnyukova 2006). It acts on the nonenzymatic reaction against ROS, such as the superoxide anion and hydroxyl radical, and also as electron donor for reducing peroxides in the reaction catalyzed by GPx (Martínez-Alvarez et al.

2005), hence helping to reduce oxidative stress. Glutathione has been found to play an important role in the antioxidant system in fish: many investigated species have shown maintenance of their GSH levels even in prolonged stress conditions (Lushchak and Bagnyukova 2006). The current investigation found a decrease in the content of total thiol groups only in *L. macrocephalus* exposed to the lowest dissolved oxygen level. Similarly, common carps *Cyprinus carpio* exposed to 0.9 mg L^{-1} O_2 for 5.5 h presented lower lipid peroxides in liver while the total content of GSH did not change significantly (Lushchak et al. 2005).

The enzymatic antioxidant system prevents the oxygen cascade reaction by means of intercepting or inactivating ROS intermediates and stagnating lipoperoxidation, with a fundamental role in restraining oxygen toxicity when the levels of the other antioxidant components are scarce (Martínez-Álvarez et al. 2005). The activity of the enzymatic antioxidants CAT and GST decreased in liver of *L. macrocephalus* exposed to 96 h hypoxia. Similarly, *Perccottus glenii* individuals subjected to 0.4 mg L^{-1} O_2 presented lower TBARS, CAT and thiol groups than those maintained in normoxia (Lushchak and Bagnyukova 2007). Likewise, a significant reduction in CAT activity in liver of *P. mesopotamicus* exposed to 2.2 mg L^{-1} O_2 for 48 h was also observed (Sampaio et al. 2008). The mechanism most likely accountable for the diminution in CAT activity is also related to the blockage of the electron chain transport or its low intensity operation. Less oxygen molecules would undergo partial reduction, diminishing also the production of hydrogen peroxide which would be decomposed by the enzyme catalase. This would consequently lead to a decrease in the activity of the enzyme (Lushchak and Bagnyukova 2007).

SOD activity remained stable in liver of *L. macrocephalus* exposed to hypoxia, as it was observed in liver of *C. carpio* exposed for 5.5 h–0.9 mg L^{-1} O_2 (Lushchak et al. 2005), *C. auratus* after 8 h anoxia (Lushchak et al. 2001) and *P. glenii* subjected to 0.4 mg L^{-1} O_2 hypoxia for 10 h (Lushchak and Bagnyukova 2007). The maintenance of SOD activity in spite of hypoxia can be attributed to preventive mechanisms developed by the animal in order to attenuate oxidative damage in a possible future reoxygenation (Lushchak et al. 2001).

In addition to the enzymes aforementioned, GST has an important role in minimizing the accumulation

of the toxic products of lipid peroxidation, including malodialdehyde, hydroxynonenal and lipid hydroperoxides (Prohaska 1980; Hermes-Lima et al. 1998; Lushchak et al. 2001). In hypoxia experiments, GST activity remained stable in liver of *P. glenii* (Lushchak and Bagnyukova 2007), *C. carpio* (Lushchak et al. 2005) and *C. auratus* (Lushchak et al. 2001). The decrease in GST levels observed in *L. macrocephalus* could also be related to a decrease in its substrate, GSH, which is a nonprotein thiol. As previously stated, there was a reduction in thiol groups, of which glutathione is the main compound. Furthermore, lipid peroxidation decreased in *L. macrocephalus* and consequently the accumulation of toxic products also decreased, thereby reducing the activity of GST, a detoxifying enzyme.

The significant decline in hydroperoxide levels, along with the action of the thiol groups in suppressing free radicals, led to attenuation of cell damage. The positive correlation found between the levels of thiols and hydroperoxydes in liver of *L. macrocephalus* supports its connection with protection of cell membranes against lipoperoxidation, as previously suggested by Lushchak and Bagnyukova (2006).

There are no studies analyzing metabolic pathways in *L. macrocephalus*. An earlier experiment of Bolner (2007) with *Leporinus obtusidens* exposed to different dissolved oxygen levels showed that glucose levels increased in liver according to the reduction in dissolved oxygen levels but glycogen decreased with the decrease in dissolved oxygen levels. Lactate levels remained steady in liver while in muscle there was an increase according to the reduction in dissolved oxygen levels. These data suggest the use of the anaerobic pathway by this species to face low dissolved oxygen concentrations.

Wilhelm Filho et al. (2005) and Wilhelm Filho (2007) pointed out that mechanisms of hypoxia followed by reoxygenation are common in nature. Therefore, the vertebrates, mainly fish, respond to hypoxia by suffering firstly a drastic decrease in antioxidants, what lasts for a short period of time. After that fish may restore the levels of antioxidant in detriment of other functions, such as growth and weight gain. So, in the model of acute hypoxia employed in this investigation, the results fit perfectly with the proposed initial response to hypoxia, constituting an important model for acute studies and biomarker of aquatic environmental quality.

Thus, each species, taking into account characteristics of its biology and habitat, responds to hypoxia in the most advantageous way in order to avoid harm especially in its vital functions, ensuring its survival in extreme situations.

Conclusions

The results allow concluding that *L. macrocephalus* is quite resistant to hypoxia. This study suggests that acute hypoxic conditions in aquatic environment may lead to a decline in metabolic rates in fish, triggering a decrease in the pro-oxidants levels alongside a reduction in the antioxidant levels. This may be a mechanism developed by the organisms to cope with adverse environmental conditions or different ecological niches. However, further studies are necessary to analyze more extensively the redox profile of *L. macrocephalus* exposed to low dissolved oxygen levels.

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