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Effect of thermal stress on metabolic and oxidative stress biomarkers of *Hoplosternum littorale* (Teleostei, Callichthyidae)

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ABSTRACT

The present study aimed to investigate in *Hoplosternum littorale* (Hancock, 1828) the effects of different water temperatures (10 °C, 25 °C-control group- and 33 °C) on physiologic and metabolic traits following acute (1 day) and chronic (21 days) exposures. We analyzed several biomarker responses in order to achieve a comprehensive survey of fish physiology and metabolism under the effect of this natural stressor. We measured morphological indices, biochemical and hematological parameters as well as oxidative stress markers. To evaluate energy consumption, muscle and hepatic total lipid, protein and glycogen concentrations were also quantified. Extreme temperatures exposures clearly resulted in metabolic adjustments, being liver energy reserves and plasma metabolites the most sensitive parameters detecting those changes. We observed reduced hepatosomatic index after acute and chronic exposure to 33 °C while glycogen levels decreased at both temperatures and time of exposure tested. Additionally, acute and chronic exposures to 10 °C increased liver lipid content and plasma triglycerides. Total protein concentration was higher in liver and lower in plasma after chronic exposures to 10 °C and 33 °C. Acute exposition at both temperatures caused significant changes in antioxidant enzymes tested in the different tissues without oxidative damage to lipids. Antioxidant defenses in fish failed to protect them when they were exposed for 21 days to 10 °C, promoting higher lipid peroxidation in liver, kidney and gills. According to multivariate analysis, oxidative stress and metabolic biomarkers clearly differentiated fish exposed chronically to 10 °C. Taken together, these results demonstrated that cold exposure was more stressful for *H. littorale* than heat stress. However, this species could cope with variations in temperature, allowing physiological processes and biochemical reactions to proceed efficiently at different temperatures and times of exposure. Our study showed the ability of *H. littorale* to resist a wide range of environmental temperatures and contributes for the understanding of how this species is adapted to environments with highly variable physicochemical conditions.

1. Introduction

Reasonable evidence demonstrated that alteration of aquatic ecosystems is increasing. The severity; frequency of occurrence and spatial scale of environmental stressors have raised in the last few decades (IPCC, 2012). Due to rapid human population growth and global warming; the problem of natural stress is likely to become worse in the coming years. Thermal impact on the aquatic environment is receiving growing attention. Given the increasing threat of climate change; for which a rise of mean and variance of temperature (IPCC, 2014) as well as extreme climatic events are expected (Sulmon et al., 2015); thermal stress is one of the most important environmental challenges that fishes may face (Pörtner and Peck, 2010).

The Middle Paraná River is a complex floodplain. In this neotropical environment; associated systems are regulated by flood pulse regime and hydrological connectivity in relation to principal channels. Shallow lakes become isolated due to prolonged drought periods. As the physicochemical conditions of these ecosystems are highly variable; they turn into stressful environments for fishes (Drago, 2007). Surface waters typically exceed 30 °C for sustained periods during the summer. In addition; fish mortality has been commonly observed in lotic and lentic water bodies of the flood valley of Middle Paraná River; following winter peaks of temperatures lower than 5 °C (Bonetto et al., 1967; Dioni and Reartes, 1975; González Naya et al., 2011; Gómez, 2014).

Changes in the surrounding environment may disrupt homeostasis and can damage biological functions. As fish are ectothermic animals; when environmental temperature changes it induce compensatory responses. Its purpose is to reduce the effects of the stressor on

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metabolism and they can involve a set of behavioral and physiological adjustments (Morris et al., 2013). Different cellular processes are involved in the maintenance of physiological homeostasis within the temperature range of a species. The most important of them is the antioxidant system and the alterations of energetic metabolism. These mechanisms allow an organism to overcome the negative consequences of thermal stress; including the accumulation of free radicals; protein degradation and energy depletion (Hofmann, 2005; Werner et al., 2006; Afonso et al., 2008; Blier, 2014; Madeira et al., 2016).

When temperature variations are substantial; fish have been shown to move to different areas of water or to refuges to avoid thermal stress (Howell et al., 2010); but fish in shallow lakes and streams often lack of refuge options. *Hoplosternum littorale*; endemic to neotropical freshwaters; generally inhabits shallow and lentic environments where they experience great seasonal changes in water physicochemical conditions (Winemiller, 1987). Additionally; the aerial respiration of *H. littorale*; combined with their tolerance to a wide range of environmental conditions (Affonso, 2001); increases the chances of this species to survive in environments subjected to thermal changes. Its success in occupying extremes environments and their wide distribution all over South America make this species an attractive model to study the physiology and metabolism of fish under thermal stress. We have recently demonstrated the utility of a battery of biomarkers as tools to monitor both an environmental and anthropogenic stressors (inanition and heavy metals; respectively) in this fish species (Rossi et al., 2015; Ale et al., 2016). In the present study we aimed to investigate in *H. littorale* the effects of different water temperatures on physiologic and metabolic traits following acute (1 day) and chronic (21 days) exposure. We analyzed several biomarker responses in order to reach a comprehensive survey of fish physiology and metabolism under the effect of this natural stressor. We measured morphological indices; biochemical and hematological parameters as well as oxidative stress markers. To evaluate energy consumption due to thermal stress; muscle and hepatic total lipid; protein and glycogen concentrations were also quantified.

2. Materials and methods

2.1. Fish

Experiments were carried out in accordance with national and institutional guidelines (CONICET, 2005) for the protection of animal welfare.

Fish were obtained from a local fish farm. Adults *H. littorale* (n = 48; 9.15 ± 1.22 cm standard length; 25.17 ± 10.74 g body weight) were acclimatized for 4 weeks in 150-L plastic tanks filled with dechlorinated and artificially aerated tap water. During this period; fish were fed three times a day with dry commercial pellets (Crude protein 40%; Fat 10%; Carbohydrate 10%. Shulet brand; Shulet S.A.; 108/A/E; Buenos Aires). Acclimation and experimental periods were carried out in the Bioassay Laboratory at the Instituto Nacional de Limnología (CONICET; Argentina) with 12 h light/12 h dark photoperiod regime provided by artificial illumination (60 Lux). The physicochemical parameters of the water were: dissolved oxygen 6.67 ± 0.63 mg L^{-1}; pH 6.15 ± 0.32; total hardness 43.8 ± 0.1 ppm $CaCO_3$; and temperature 25 ± 1 °C.

2.2. Experimental design

After the acclimation period had concluded; heat and cold temperature challenges were conducted in 10 L glass aquaria under semi-static conditions. Temperatures were chosen (10; 25 and 33 °C) according to the temperature ranges of the flood valley of Middle Paraná River (Mayora et al., 2013). A number of two fish per aquarium were randomly distributed into six experimental groups: fish exposed to 10 °C; 25 °C (control group) and 33 °C for 1 and 21 days (10 °C 1d; 10 °C 21d; 25 °C 1d; 25 °C 21d; 33 °C 1d; 33 °C 21d). Test groups were replicated four times (n = 8 fish per group). Temperatures were raised or lowered in an environmental chamber at a rate of 1 °C day^{-1} and tanks were examined twice daily (once in the morning and once in the evening). Fish were fed daily *ad libitum* during the challenges.

2.3. Biomarkers

At the end of each challenge; fish were anaesthetized in benzocaine 100 mg/L according to Parma de Croux (1990). For each fish its weight (g); as well as total and standard length (cm); was registered. Once blood was collected from the caudal vessel; plasma was separated by centrifugation at 1400 *g* for 5 min in order to measure blood metabolites (see 2.3.3). Tissues (liver; muscle; brain; gills and kidney) were dissected and immediately frozen in liquid nitrogen. They were kept at −80 °C until biochemical processing. The wet weight of the liver was recorded before freezing.

2.3.1. Morphometric biomarkers

Condition factor (CF) and hepatosomatic index (HSI) were calculated according to Goede and Barton (1990).

2.3.2. Hematology

A Neubauer chamber was used in order to count red blood cells (RBC). Hematocrit (Ht) values were determined by the micro method and hemoglobin concentration (Hb) was measured according to Houston (1990). Mean cell volume (MCV); mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated as previously described (Cazenave et al., 2005).

2.3.3. Blood metabolites

Commercial kits were used for plasma metabolites measurement. Total plasma protein concentration was measured by the kit Proteínas Totales AA (Wiener Lab®). Protein peptidic bonds react with the cupric ion in alkaline medium; rendering a purple-blue complex violet with a maximum absorption at 540 nm. Plasma levels of total cholesterol and triglycerides were analyzed by the kits Colestat enzimático and TG Color GPO/PAP AA respectively (Wiener Lab®); which used a standard enzymatic-colorimetric test by the Trinder reaction. Finally; plasma glucose was assayed by the kit Glicemia enzimática (Wiener Lab®) based on the glucose oxidase/peroxidase method.

2.3.4. Energy reserves

Glycogen was estimated according to Seifter et al. (1950). Briefly; tissues were digested with KOH 30% and KOH 60% in a boiling water bath. After alkaline disruption; glycogen was precipitated by ethanol with liberated glucose being measured by the anthrone reagent method. Lipid content was estimated according to Folch et al. (1957). Briefly; tissues were manually homogenized in 9 vols of ice-cold distillated water and extraction of total lipids was performed using chloroform: methanol (2:1). Total protein concentration was estimated according to Lowry et al. (1951) using bovine serum albumin as standard.

2.3.5. Oxidative stress

Oxidative stress in liver; kidney; gills and brain was assessed by both antioxidant enzyme activities and lipid peroxidation levels. For enzyme extracts preparation; tissues were processed from each individual (not pooled); according to Bacchetta et al. (2014). Briefly; tissues were homogenized in an ice-cold 0.1 M sodium phosphate buffer (pH 6.5) containing 20% (v/v) glycerol; 1 mM Ethylenediaminetetraacetic acid (EDTA) and 1.4 mM dithioerythritol. The homogenates were centrifuged at 20,000*g* (4 °C) for 30 min and the supernatant (enzyme extract) was collected and stored at −80 °C for enzyme measurement.

The activity of the enzyme glutathione-S-transferase (GST; EC 2.5.1.18) was determined following the conjugation of reduced glutathione with 1-chloro-2; 4-dinitrobenzene (CDNB) that produces a