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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

dinitrophenyl thioether which can be detected at 340 nm; as described by Habig et al. (1974). Catalase activity (CAT; EC 1.11.1.6) was measured according to the method of Beutler (1982) following the decomposition of H_2O_2 at 240 nm ($\varepsilon = 0.071\ mM^{-1}\ cm^{-1}$). The assay mixture consisted of 1 M Tris-HCl; 5 mM EDTA (pH 8.0); 10 mM H_2O_2 and enzyme extract. Glutathione reductase activity (GR; EC 1.6.4.2) was determined as described by Tanaka et al. (1994) by measuring the oxidation of NADPH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.5); 20 mM GSSG; 2 mM NADPH and enzyme extract.

Lipid peroxidation (LPO) was determined by measuring the formation of thiobarbituric reactive substances (TBARS); according to Fatima et al. (2000). The rate LPO was measured spectrophotometrically at 535 nm and was expressed as nanomoles of TBARS formed per hour; per milligram of proteins (nmol TBARS mg $prot^{-1}$). Total amount of protein in the enzyme and LPO extracts were quantified according to the principle of protein–dye binding (Bradford, 1976) using bovine serum albumin as a standard. All measurements were performed in triplicate.

2.4. Statistical analyses

All data were reported as means ± standard deviation. Shapiro-Wilks test was applied to evaluate normality while Levene test was used to test the homogeneity of variance. For statistical comparisons of data among treatments; one way analysis of variance (ANOVA) followed by a Multiple Comparison Test (Tukey) were performed. Kruskal–Wallis test was applied to those variables with non-normal distribution or variance heterogeneity. P-values below 0.05 were regarded as significant. In addition; principal component analysis (PCA) was performed in order to get a comprehensive view of the results; and to define the most important parameters involved in thermal stress. To minimize the number of empty spaces in the dataset; multivariate analysis was carried out taking into account five (10 and 33 °C) or eight (25 °C) cases (individuals with all variables measured). All statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2015).

3. Results

No mortality occurred during any of the experimental challenges. Fish exposed for 21d at 10 °C seemed to decrease swimming activity.

3.1. Morphometric biomarkers

Morphometric biomarkers as well as body weight and total and standard length are summarized in Table 1. A significant reduction in HSI was observed in fish after acute and chronic exposure to the higher temperature tested (33 °C); in comparison to the control group. CF was not altered by the different water temperature or periods of exposition tested.

3.2. Hematology

Hematological parameters of *H. littorale* exposed 1d or 21d to different temperatures are shown in Table 2. No significant changes were observed after 1d exposure to any of the temperatures tested. After chronic exposure at 33 °C; a significant reduction of MCV and MCH was observed when compared to control group.

3.3. Blood metabolites

Fig. 1 shows blood metabolites of H. littorale exposed 1d or 21d to different temperatures. Both periods of exposure led to significant changes in blood metabolites levels. After acute exposure; higher plasma glucose levels were observed at 10 °C and decreased at 33 °C ($p < 0.05$). Contrary; fish chronically exposed to 10 °C showed significantly lower levels of blood glucose and no changes at 33 °C; when it was compared with control group. Triglycerides significantly increased at 10 °C after 1d and 21d exposure while 33 °C had no influence in this plasma metabolite. Total plasma cholesterol levels were not influenced by temperatures tested in this study. Total protein plasma concentration decreased after long-term exposure to 10 °C and 33 °C ($p < 0.05$).

3.4. Energy reserves

Energy reserves in liver and muscle are shown in Table 3. Temperature had significant effects on liver. The greatest changes were observed at 10 °C. After short-term exposure glycogen reserves significantly decreased and lipid content increased. These results were also observed after long-term exposure to this temperature; where protein levels also showed increased values compared to control group. At 33 °C glycogen levels decreased after 1d and 21d exposure and no changes were observed in lipid content. Protein levels significantly increased only after chronic exposure to 33 °C. No differences were observed in muscle at any temperature or period of exposure tested.

3.5. Oxidative stress

Oxidative status of different tissues are presented in Table 4 (antioxidant enzymatic activities) and Fig. 2 (lipid peroxidation). In liver; an acute period of both temperature exposures caused significant changes in all enzyme tested without modifications in LPO levels. At 10 °C there was an inhibition of GST and GR activities while at 33 °C GR and CAT showed the highest activities ($p < 0.05$). After 21d of exposure changes were observed at 10 °C with significantly higher LPO levels and decreased CAT activity versus control group. In kidney; acute exposure at 10 °C decreased GST and CAT activities without evidence of lipid peroxidation; while chronic exposure lead to higher LPO levels without changes in enzyme activities. When fish were exposed at 33 °C for 21d the lowest level of LPO was observed with activation of GST activity ($p < 0.05$) compared to control group. Gills showed that 1d exposure at 10 °C decreased GST and CAT activities without lipid peroxidation. When fish were exposed for the same period

Table 1
Morphometric biomarkers of *Hoplosternum littorale* exposed to different temperatures during 1d or 21d.

	1d exposure			21d exposure		
	10 °C	25 °C	33 °C	10 °C	25 °C	33 °C
Total weigth (g)	23.25 ± 3.34	20.18 ± 3.79	19.33 ± 1.37	26.14 ± 3.69	26.00 ± 9.74	23.51 ± 3.15
Total length (cm)	11.55 ± 0.59	11.16 ± 0.66	10.78 ± 0.47	12.60 ± 0.72	11.79 ± 1.46	10.91 ± 0.49
Standard length (cm)	8.81 ± 0.46	8.64 ± 0.69	8.38 ± 0.32	9.46 ± 0.59	9.15 ± 1.25	8.58 ± 0.20
Condition Factor	1.53 ± 0.18	1.43 ± 0.07	1.39 ± 0.12	1.45 ± 0.07	1.52 ± 0.12	1.41 ± 0.11
Hepatosomatic Index	1.73 ± 0.36	1.53 ± 0.36	1.20 ± 0.41*	1.67 ± 0.28	1.65 ± 0.56	1.07 ± 0.15*

Values are expressed as mean ± SD. Eight individuals were sampled at each temperature point. Groups significantly different from controls are marked with an asterisk ($p < 0.05$).

Table 2
Hematological parameters of *Hoplosternum littorale* exposed to different temperatures during 1d or 21d.

	1d exposure			21d exposure		
	10 °C	25 °C	33 °C	10 °C	25 °C	33 °C
RBC (10^6/μL)	1.30 ± 0.35	1.47 ± 0.40	1.31 ± 0.29	1.79 ± 0.40	1.95 ± 0.40	2.18 ± 0.37
Ht (%)	26.99 ± 3.09	29.2 ± 4.4	29.8 ± 6.18	51.77 ± 8.74	41.7 ± 13.3	41.32 ± 6.05
Hb (g/dL)	7.61 ± 1.52	7.88 ± 1.96	6.90 ± 1.56	11.12 ± 3.12	10.00 ± 3.44	9.33 ± 0.94
MCV (μm^3)	224.2 ± 53.0	208.5 ± 39.8	223.9 ± 25.1	293.1 ± 26.3	216.2 ± 42.0	191.2 ± 24.2*
MCH (pg)	64.42 ± 16.01	55.04 ± 13.05	46.56 ± 6.16	62.00 ± 8.08	50.51 ± 11.14	43.29 ± 7.44*
MCHC (%)	28.14 ± 4.35	26.75 ± 5.22	21.27 ± 2.30	21.21 ± 2.59	22.76 ± 3.30	23.42 ± 2.25

Values are expressed as mean ± SD. Eight individuals were sampled at each temperature point. Groups significantly different from controls are marked with an asterisk ($p < 0.05$). RBC: red blood cells, Ht: hematocrite, Hb: hemoglobin concentration, MCV: mean cell volume, MCH: mean cell hemoglobin, MCHC: mean cell hemoglobin concentration.

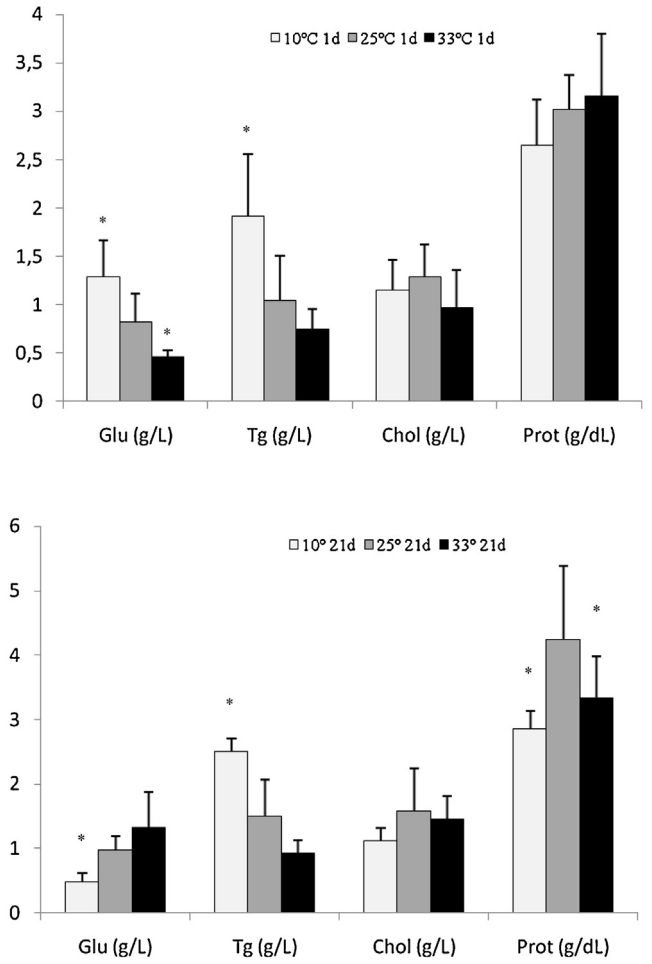

Fig. 1. Blood metabolites of *Hoplosternum littorale* exposed to different temperatures during 1d or 21d.Values are expressed as mean ± SD. Eight individuals were sampled at each temperature point. Groups significantly different from controls are marked with an asterisk ($p < 0.05$). Glu: glucose, Tg: triglyceride, Chol: cholesterol and Prot: total protein.

at 33 °C; GR activity increased and LPO levels decreased ($p < 0.05$). This tissue showed no changes in enzymes activities after long-term exposure to the different temperatures tested in this work. Only LPO levels significantly changed: higher values were observed at 10 °C while 33 °C decreased lipid peroxidation compared to control group. Brain tissue showed no changes in enzyme activities or lipid peroxidation at any period or temperature tested.

3.6. Multi-biomarker approach

Two components were extracted by applying the PCA (Fig. 3). Principal components may be interpreted for eigenvalues of the data matrix higher than 1 (Legendre and Legendre, 1979). The PCA indicated that 9 eigenvalues were higher than 1. Correlation coefficients are significant when they are higher than √d/n (d: number of principal components; n: number of variables). Therefore; correlation coefficients > 0.47 were indicative of a good representation of the variables with principal component axes. The components accounted for 35.6% of the original dataset variance. The first principal component (PC1) explained 20.8% of the variance; and showed significant positive loadings for HSI; hematological parameters associated to red blood cells; LPO; plasmatic triglycerides and lipid content in liver; among others. On the contrary; a negative correlation was found mainly for antioxidant enzymes activities in liver and GST in kidney. PC2 (14.8% of the total variance) showed positive loadings for MCV; gills antioxidant enzymes (CAT and GST); and protein content in liver; and negative correlations were found mainly for plasmatic glucose and protein levels and glycogen content in liver.

4. Discussion

Changes in the environmental temperature induce a series of behavioral and physiological responses in an ectothermic organism as strategies for re-establish homeostasis (Foss et al., 2012). Fishes enter into metabolic depression in response to low water temperatures to alleviate the effects of temperature on metabolism. This has performance consequences at whole organism level (Alzaid et al., 2015; Costa et al., 2013). We observed that fish exposed to 10 °C seemed to diminish food intake and became quiescent. The fish were apathetic; stayed at the bottom of the tank and showed lethargy; despite their reactions to external stimuli were normal. On the other hand; fish exposed to heat stress showed a stable; but in somewhat agitated state. However; no erratic swimming was observed at any of the periods tested. These behaviors were not observed in the control tank. Other authors also observed that swimming activity of fish is directly proportional to water temperature (Peterson and Anderson, 1969; Claireaux et al., 1995; Beyan et al., 2015)

4.1. Morphometric biomarkers

Water temperature significantly affects some physiological fish processes such as growth and metabolism (Lermen et al., 2004). Non-optimal water temperature and insufficient food have demonstrated to inhibit fish growth (Fine et al., 1996). In the same way; the CF is often used to express the overall wellbeing of fish and serve as an initial screening biomarker to indicate the nutritional state of them (Neff and Cargnelli, 2004). As is it shown in Table 1; no detrimental effects of temperatures tested on fish morphometric biomarkers were observed. Total weight; standard length and CF showed similar values among groups. Even though the amount of food ingested by fish was not estimated; exposure to different temperatures did not reduce these biomarkers probably because food was provided in sufficient quantity

Table 3
Tissue energy reserves of *Hoplosternum littorale* exposed to different temperatures during 1d or 21d.

	1d exposure			21d exposure		
	10 °C	25 °C	33 °C	10 °C	25 °C	33 °C
Liver						
glycogen (μmol/g tissue)	184.5 ± 67.6*	426.2 ± 117.4	303.6 ± 29.1*	153.0 ± 53.2*	531.9 ± 149.5	278.8 ± 57.7*
lipid (μmol/g tissue)	13.3 ± 3.1*	9.7 ± 0.9	8.8 ± 1.3	19.1 ± 5.5*	9.3 ± 4.7	8.1 ± 1.3
protein (mg/g tissue)	116.1 ± 8.4	120.6 ± 19.6	134.2 ± 16.9	139.7 ± 18.2*	114.5 ± 8.3	133.7 ± 27.9*
Muscle						
glycogen (μmol/g tissue)	0.52 ± 0.23	0.74 ± 0.28	0.59 ± 0.20	0.82 ± 0.31	0.51 ± 0.15	0.78 ± 0.30
lipid (μmol/g tissue)	4.44 ± 1.33	3.75 ± 0.95	3.46 ± 0.80	5.08 ± 1.87	4.16 ± 0.62	3.63 ± 0.50
protein (mg/g tissue)	137.8 ± 3.2	119.9 ± 43.4	137.3 ± 56.0	150.0 ± 29.3	141.0 ± 22.1	114.1 ± 42.0

Values are expressed as means ± SD. Eight individuals were sampled at each temperature point. Groups significantly different from controls are marked with an asterisk ($p < 0.05$).

to support the metabolic demand. Viant et al. (2003) showed no changes on growth rates in juvenile steelhead trout after 5 °C elevation in temperature within 3 weeks.

HSI can be strongly affected by water temperature in fish and it can indicate its health status (Ma et al., 2015). In our study; acute and prolonged exposition at 33 °C induced a significant reduction in HSI when compared to control fish. Since no significant difference was found in total fish weight; these results could indicate that energy stores within the liver were rapidly mobilized in response to heat stress. Gandar et al. (2016) observed a substantial decrease of the HSI; although not significant; with temperature raising in *Carassius auratus* suggesting that this result may indicate a growing energy requirement due to thermal acclimation.

4.2. Hematology

Blood parameters can indicate the health status of an individual (Harikrishnan et al., 2011) as the composition of fish blood is influenced by metabolism; nutritional status and diseases (He et al., 2007). Hematological parameters of *H. littorale* were not altered following acute thermal stress. We only observed a reduction in MCV and MCH levels after 21d of exposure to 33 °C. Oxygen is transported to fish cells by Hb. The concentration of this protein and the number of RBC are strongly related to oxygen supply (Ma et al., 2015). A reduced quantity of erythrocytes and a decreased hemoglobin level lead to a deteriorated oxygen supply at high temperatures (Zarejabad et al., 2010). In this study; RBC count and Hb were not affected by any temperature or period of exposition tested; demonstrating that oxygen supply was enough for the metabolic functions of fish. Similar results were found by Lermen et al. (2004) in *Rhamdia quelen* and by Zarejabad et al. (2010) in great sturgeon exposed to different temperature regimes. In adittion; Ruane et al. (2001) concluded that if there is no increase in Ht values; there is no need for extra oxygen in the fish organism. Ht can be used as a reliable criterion for determination of organism oxygen demand (Radoslav et al., 2013) and in this study; no changes among temperature treatments were observed in Ht values. These results also show good tolerance of the species to this range of temperature variation.

The MCV reveals the status or size of the red blood cells and reflects cell division during erythropoiesis. The decrease in MCV observed in fish exposed chronically to 33 °C indicates that erythrocytes have shrunk. This microcytosis evidences a high percentage of immature red blood cells in the general circulation of the fish (Milligan and Wood, 1982). Accordingly; Houston and Murad (1992) observed that erythropoiesis is stimulated by temperature in goldfish. In some fish species catecholamines induce erythrocyte swelling leading to an increase in MCV under thermal stress (Jensen 2004; Radoslav et al., 2013) and Hb concentration tended to increase as temperature increased (Zhang, 1991; Ma et al., 2015). The differences with the present results can be attributed to other factors such as size and nutritional status of fish; the

Table 4
Antioxidant enzyme activities measured in liver, kidney, gills and brain of *Hoplosternum littorale* exposed to different temperatures during 1d or 21d.

	1-day exposure			21-days exposure		
	10 °C	25 °C	33 °C	10 °C	25 °C	33 °C
Liver						
GST	742.6 ± 175.6*	1049.5 ± 186.9	1269.8 ± 149.0	1337.5 ± 212.9	1477.3 ± 214.6	1565.1 ± 691.7
GR	35.39 ± 7.56*	51.27 ± 7.02	70.20 ± 10.00*	41.92 ± 14.72	36.45 ± 2.10	43.76 ± 15.31
CAT	59.28 ± 12.85	54.51 ± 12.66	83.17 ± 20.85*	36.20 ± 9.40*	79.33 ± 15.55	66.33 ± 23.87
Kidney						
GST	788.2 ± 175.7*	992.7 ± 185.9	1177.6 ± 188.0	539.6 ± 92.1	821.2 ± 347.1	1126.2 ± 285.0*
GR	124.3 ± 26.4	150.5 ± 37.2	94.0 ± 17.1	100.8 ± 10.8	140.1 ± 67.8	110.8 ± 45.1
CAT	11.62 ± 4.79*	21.13 ± 8.37	25.52 ± 11.18	10.69 ± 3.49	17.15 ± 8.55	19.81 ± 5.96
Gills						
GST	369.9 ± 110.8*	568.7 ± 227.5	763.6 ± 30.13	631.7 ± 153.9	550.4 ± 271.2	863.1 ± 245.3
GR	34.26 ± 3.69	61.17 ± 26.64	90.27 ± 27.27*	60.89 ± 20.18	49.53 ± 22.86	61.31 ± 12.98
CAT	10.01 ± 0.64*	19.31 ± 9.21	21.84 ± 5.91	25.80 ± 9.65	18.92 ± 9.46	22.34 ± 7.00
Brain						
GST	1363.3 ± 130.8	1535.9 ± 779.4	955.4 ± 48.8	1103.2 ± 345.9	1411.5 ± 476.1	1301.6 ± 461.9
GR	99.7 ± 17.0	106.4 ± 11.3	98.0 ± 16.3	92.3 ± 27.8	120.1 ± 35.1	88.1 ± 34.5
CAT	38.13 ± 17.00	40.72 ± 20.90	22.41 ± 5.84	23.67 ± 7.35	28.99 ± 10.29	20.47 ± 4.26

Values are expressed as means ± SD. Eight individuals were sampled at 25 °C and five individuals were sampled at 10 °C and 33 °C. Enzyme activity is expressed in mU mg $prot^{-1}$ (GST and GR) or U mg $prot^{-1}$ (CAT). Groups significantly different from controls are marked with an asterisk ($p < 0.05$). GST: glutathione S-transferease, GR: glutathione reductase, CAT: catalase.

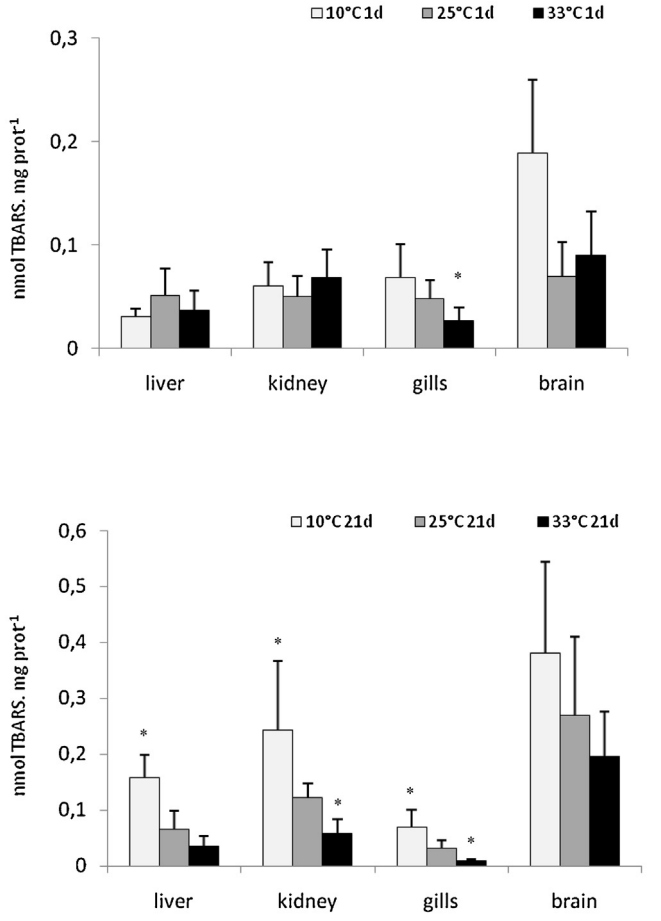

Fig. 2. Lipid peroxidation levels in liver, kidney, gills and brain of *Hoplosternum littorale* exposed to different temperatures during 1d or 21d.Values are expressed as mean ± SD. Eight individuals were sampled at each temperature point. Groups significantly different from controls are marked with an asterisk ($p < 0.05$).

duration of exposure and stress levels and also to inter-species differences (Smit et al., 1981; Steinhagen et al., 1990; Perry and Reid, 1992).Our results on hematologic characteristics of *H. littorale* at 25 °C are in agreement with those found by Affonso et al. (2004) and Rossi et al. (2015).

4.3. Blood metabolites and energy reserves

Glucose is the major energy source for most of activities in fish. Liver glycogen is an important energy reserve that can be rapidly mobilized during acute stress; so its catabolism and plasmatic glucose levels are closely related (Ma et al., 2015). Increased glycemia reflects the conversion of glycogen into glucose due to glycogenolysis for energy use; at least during the initial stages of stress exposition. We observed increased blood glucose levels with decreased glycogen reserves in liver in *H. littorale* exposed to 10 °C for 1d. High glucose levels at low temperatures are associated to a slow metabolism and demonstrate sub-lethal stress (Best et al., 2001). Increasing glucose levels with decreasing water temperature have been reported by several authors for a variety of fish species (Leach and Taylor, 1977; Click and Engin, 2005). Umminger (1969) suggested that cold-induced hyperglycemia may serve as a mechanism for supplying substrate for essential reactions (i.e.; ATP production) or to increase the activity of metabolic pathways required for cold acclimation. As osmoregulatory mechanisms are often impaired during temperature stress; hyperglycemia may also help to maintain serum osmolarity in freshwater fish (Kindle and Whitmore 1986). The lower circulating levels of glucose on 21d 10 °C exposed *H. littorale* indicated that glucose was used to supply energy for fish to cope with chronic suboptimal thermal condition. All these results demonstrate that environmental temperature affects blood glucose levels; but according to Chavin and Young (1970) the pattern is inconsistent among several fish species; so other environmental and physiological factors may contribute to these variations.

Stress responses and preservation of homeostasis increase energetic costs (Jager et al., 2014). In the present study; hepatic glycogen levels were significantly lower in all temperatures and periods tested but no changes were observed in muscle glycogen reserves. Similar results in liver glycogen were observed by Lermen et al. (2004) in *R. quelen* exposed for 21d at 15 or 31 °C. This indicates that fish use liver glycogen to provide energy when they are exposed to low or high temperatures (Lermen et al., 2004; Chatzifotis et al., 2010; Viant, 2003). Low levels of hepatic glycogen may also be a result of acclimation to low water temperatures (Werner et al., 2006). Dunn et al. (1983) suggested that muscle glycogen reserves are conserved to save energy in this tissue for arousal and escape.

Elevation in plasma lipids can be due to alteration in their metabolism or to impaired clearance. This hyperlipidemia can leads to fatty liver and subsequently liver dysfunction (Lu et al., 2010; Javed and Usmani, 2015). Elevated triglyceride levels in plasma and liver

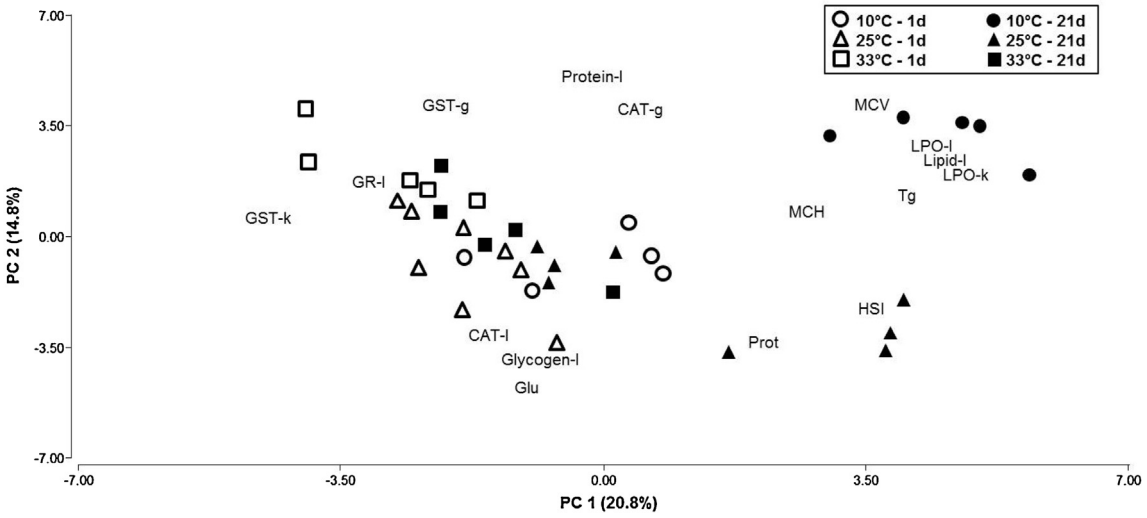

Fig. 3. Representation of the biomarkers (in letters) and individuals (in forms) onto the first factorial plane of the principal component analysis. Biomarkers with correlation coefficients > 0.47 were represented in the PCA. Biomarkers abbreviations are explained in the text. Enzymes and lipid peroxidation abbreviations for different organs were followed by the corresponding letter. −l: liver; −k: kidney; −g: gills; −b: brain.

were observed in fish exposed to 10 °C at both periods of time tested. This could indicate that fish accumulated triglycerides to cope with cold. Gracey et al. (2004) have studied transcriptional responses in liver tissue of common carp subjected to a progressive cooling regime. They identified enhance gene expression of enzymes and proteins involved in lipid biosynthetic processes.

Fat accumulation can lead to liver hypertrophy (Lu et al., 2010) and could explain why HSI did not change when fish were exposed at 10 °C in our study: the decreased hepatic glycogen content was compensated by an increase in lipid reserves. When fish were exposed to 33 °C the intense utilization of hepatic glycogen reserves to maintain metabolism led to a great reduction in the HSI of *H. littorale*.

In the present study total plasma protein levels were significantly lower in fish exposed to 10 °C and 33 °C for 21d. These results could indicate that blood proteins were used as fuel source through proteolysis and subsequently amino acid oxidation (Mommsen et al., 1999; Di Marco et al., 2008). In addition; glycemia in fish exposed chronically to 33 °C suggests hepatic glucose production from released amino acids.

A redirection of energy away from growth and into maintenance and biological repair occurs in fish during a thermal stress (Iwama et al., 1998; Viant, 2003). Heat shock proteins (HSP) are an important family of proteins in thermal stress tolerance. They repair and prevent damage occurred from protein denaturation (Werner et al., 2005). Although we did not measured specifically HSP content we did observed that liver protein concentration was enhanced in fish exposed to 10 °C and 33 °C during 21d. This could be indicating an enhanced protein synthesis in this tissue. Werner et al. (2006) hypothesized that the HSP response to thermal stress would be directly correlated with a decrease in energy reserves; as protein synthesis and repair are energy-intensive processes. Viant et al. (2003) showed that increased expression of HSP in steelhead trout parr in response to chronic exposure at high temperature was associated with a decrease in liver glycogen. In the present study the observed increments in hepatic lipid and protein content could also be related with the abundance of available food. This indicates that fish were not under nutritional stress; so energetic changes were due to thermal conditions.

4.4. Oxidative stress

Temperature can greatly influence the response of oxidative stress biomarkers (Lushchak, 2011). As temperature increase cellular metabolism it can contribute to a greater production of reactive oxygen species (ROS) (Madeira et al., 2013). This can cause ROS triggering the activation of the enzymatic antioxidant defense system in order to prevent oxidative damage. However; activation of antioxidant mechanisms can fail to counteract oxidative damage (Sheriff et al., 2014). In the present study; no significant increase in LPO levels was observed when fish were exposed to 33 °C for any of the periods tested in the present study. Even more; lipid peroxidation was lower in gills (1d and 21d) and kidney (21d). We observed that fish respond to an acute (1d) temperature increment by the activation of antioxidant defenses; as showed CAT and GR activities in liver and GR activity in gills. An increment in temperature can also result in increased reaction rate of enzymes; and that increase would be enough to metabolize ROS (Almroth et al., 2015). Following chronic (21d) exposure to the highest temperature; only kidney GST activity significantly augmented. GSTs are a multigene super family of dimeric enzymes that are involved in the detoxification of pollutants and drugs as well as endogenous reactive compounds of cellular metabolism. Certain GST isozymes might act to conjugate metabolites arising from oxidative damage and have the ability to reduce lipid hydroperoxides (Regoli et al., 2011). The activation of this enzyme suggests an increment of fish capacity to withstand oxidative stress following 21d of high temperature exposure. In this work GST activity was determined using an unspecific technique and therefore obtained results should be considered with caution because they cannot exclusively being ascribed to an antioxidant function.

Low LPO levels observed in gills and kidney could indicate that degradation mechanisms of damaged lipids are taking place (Kohen and Nyska, 2002). Lipid peroxides can be removed via conversion to alcohols by phospholipid hydroperoxide gluthathione peroxidases or by the action of phospholipases; with the resultant free fatty acid peroxides rendered harmless by the action of glutathione peroxidases (Halliwell and Gutteridge, 1999; Schweikert and Burritt, 2012).

The return to basal levels of almost all antioxidant defenses could indicate that fish exposed to long-term higher temperature has acclimated to the warmer temperature and have established a new physiological steady state (Almroth et al., 2015). As elevated environmental temperature induce oxidative stress in many fish species (Parihar and Dubey, 1995; Heise et al., 2006; Lushchak and Bagnyukova, 2006; Bagnyukova et al., 2007); these results demonstrate the great tolerance of *H. littorale* to high water temperature.

Fish exposed to 10 °C during 1d showed a significant decrease of antioxidant enzyme activities but no oxidative lipid damage occurred. On the contrary; chronic exposure (21 d) produced the rise in LPO levels (in liver; gills and kidney) with unaltered activity of antioxidant enzymes. This seems to indicate the incapacity of fish to cope with a prolonged period of low temperature exposure. It has been stated that CAT activity can be inhibited by the fluctuation of superoxide radicals (Filho, 1997). This may actually be the case for the results observed in liver; as CAT activity was significantly lower in this tissue. The decrease in environmental temperature may cause oxidative stress in fish (Abele and Puntarulo, 2004; Malek et al., 2004). Lushchak (2011) speculated that temperature decrease weakens the systems of ROS elimination; and/or enhances ROS production. Oxidative stress may increase because of enhanced oxygen solubility at cold temperature (Weiss, 1970) or a decrease in mitochondrial membrane fluidity (Hazel, 1995) that disturb the transfer of electrons and enhance the production of ROS (Kammer et al., 2011). In addition; also the homeoviscous adaptation to maintain the proper cell membrane fluidity can enhance oxidative stress (Crockett, 2008).

Although brain is one of the most susceptible tissues to be affected by oxidative stress (Halliwell and Gutteridge, 2007); no significant changes in lipid peroxidation or in the protective antioxidant enzymes tested in the present study were observed. Tseng et al. (2011) showed that the concentration of cellular protein carbonyls in brain of zebrafish (*Danio rerio*) was significantly increased within 1 h after a cold shock. Additionally; they observed that the specific activity of superoxide dismutase (SOD) and the mRNA level of CAT were also increased. It is important to remark that fish in our study were exposed to the different temperatures tested after they were raised or lowered gradually (at a rate of 1 °C day^{-1}); providing the opportunity for a certain acclimatization to occur. However; an increasing trend (not significantly) in lipid peroxidation was observed in brain when fish were exposed to 10 °C during an acute or chronic period.

In the present study *H. littorale* showed a distinct and differential tissue response on the oxidative stress and antioxidant defenses when exposed to thermal stress. Antioxidant responses were evaluated through three enzymatic activities (CAT; GST; GR) in liver; kidney; gills and brain; but other antioxidant defenses not being assessed in the present study (i.e; SOD; Glutathione peroxidase; reduced glutathione; etc.) could also have been elicited in the different tissues. Previous reports indicated that temperature affects oxidative stress parameters and that responses are tissue specific and species specific (Kammer et al., 2011; Vinagre et al., 2014; Madeira et al., 2016).

4.5. Integrating analysis

Multivariate statistical techniques are promising approaches for analyzing large data sets and can identify biomarkers responsible for distinguishing among fishes exposed to different temperatures (Madeira et al., 2016). Interestingly; the PCA analysis clearly differentiated fish

exposed chronically to 10 °C; evidencing differential physiologic and metabolic responses among organisms under cold stress. Certain biomarkers showed greater sensitivity to low temperature exposure. Strong responses were observed in LPO levels in liver and kidney; as in liver lipid content and plasmatic triglycerides. These results evidence that chronic exposition to cold water temperature lead to oxidative stress and energy consumption in *H. littorale*. These fish are well adapted to elevated temperatures and can acclimate via metabolic adjustments but may be more vulnerable to cold temperatures; which is in accordance to their tropical origin. It is also interesting to highlight that the first axis allowed differentiating acute condition (empty patterns) from chronic condition (solid patterns). In addition; liver proved to be a target organ facing temperature changes. Liver energy reserves and blood metabolites were the most sensitive biomarkers detecting those changes. These results confirm its major role in most metabolic pathways.

5. Conclusions

Physiological strategies activated by *H. littorale* to face acute and chronic periods of thermal stress may have important implications in understanding how fish are well adapted to environments with highly variable physicochemical conditions. Thermal exposures in this species clearly resulted in a metabolic adjustment. The grade of temperature-induced biochemical stress responses in target organs was in the order liver > gills > kidney > brain; being liver energy reserves and blood metabolites the most sensitive parameters detecting those changes. Exposure to cold water had a higher impact on biomarkers in this species. Even more; only low temperature promoted oxidative damage in different tissues. Considering that *H. littorale* has a wide distribution; these results are according to a species derived from the tropics (Nico et al., 2011). Taking into account exposure time; acute changes were more evident although many of them exhibited a transient state showing that many adaptive processes in this fish species could be elicited. The present results are in agreement with the ability of *H. littorale* to resist a wide range of environmental temperatures. This work confirmed tolerance of this species to cope with prolonged exposures to elevated temperatures (probably lethal to other fish) showing *H. littorale* as a test species candidate in assessing the quality of freshwater in tropical ecosystems. Since thermal changes in natural freshwater systems directly influence survival and persistence of populations (Jesus et al., 2016); these studies become even more relevant in the face of changes in global temperatures.

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