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Physiological and metabolic adjustments of *Hoplosternum littorale* (Teleostei, Callichthyidae) during starvation

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ABSTRACT

All animals face the possibility of limitations in food resources that could ultimately lead to mortality caused by starvation. The primary goal of this study was to characterize the various physiological strategies that allow fish to survive starvation. A multiparametric approach, including morphological biomarkers, blood plasma metabolites, oxidative stress and energy reserves, was used to assess starvation effects on the fish *Hoplosternum littorale*. Adult specimens were maintained at four experimental groups: control (fed ad libitum), and starved (not fed) fish for 7 and 28 days. Significant changes were observed not only after 28 days, but also after 7 days of starvation. In the shorter period, the hepatosomatic index as well as plasma triglycerides and glucose were significantly lower in starved fish than in the control ones. These results were accompanied by reduced lipid, glycogen and protein reserves in liver and diminished glycogen content in muscle, suggesting the need of these macromolecules as fuel sources. In addition, increased antioxidant enzyme activities were observed in gills, without evidence of oxidative stress in any of the evaluated tissues. Most significant differences were found in 28-days starved fish: total body weight together with the hepatosomatic index was lower when compared to control fish. The plasmatic metabolites tested (glucose, triglyceride, cholesterol and protein), all energy reserves in liver and glycogen content in muscle decreased in 28-days starved fish. Lipid oxidative damage was reported in liver, kidney and brain, and antioxidant enzymes (GST, GR, GPx and CAT) were activated in gills. According to the multivariate analysis, oxidative stress markers and metabolic parameters were key biomarkers that contributed in separating starved from fed fish. Our study allowed an integrated assessment of the fish response to this particular condition.

1. Introduction

In their natural environment organisms rarely experience optimal conditions. On the contrary, for most of their lifespan, they have to cope with adverse conditions caused by natural stressors, involving pathogens, extreme temperatures, oxygen depletion and starvation (Holmstrup et al., 2010).

Fish, in general, have such ability to withstand long periods of fasting, that for many species, it is actually part of their lives (Navarro and Gutiérrez, 1995; Vigliano et al., 2002). Several factors like seasonal fluctuations, reproductive process or availability of prey are responsible for the natural process of fasting (Caruso et al., 2010; Pérez-Jiménez et al., 2012). In some neotropical environments, as the river-floodplain lake systems of the Middle Paraná, fishes often undergo periods of poor food supply, especially due to extreme fluctuations in the rainfall regime. Isolated lakes are generated during prolonged drought periods with high probability of desiccation and scarce food availability (Drago, 2007). Besides, farmed fish may also experience fast situations emerging from routine procedures in aquaculture, as those used to avoid risks of overproduction (Krogdahl and Bakke-McKellep, 2005). These periods of unfavorable feeding conditions, both natural and artificially imposed, cause physiological effects that may vary considerably in relation to fish species, age, nutritional state, and food deprivation length (Navarro and Gutiérrez, 1995).

Macromolecules have dual roles in living systems: during periods of positive energy balance, their concentrations are established by their functional and structural roles whereas their

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energetic value leads to their degradation during periods of negative energy balance (Guderley et al., 2003). Fish usually cope with food deprivation by redirecting their energy reserves toward vital processes in detriment of growth (Sumpter et al., 1991). The dynamics of endogenous energy utilization can be discerned in part by monitoring morphological indexes such as the hepatosomatic index (HSI) (Jensen, 1979) and the condition factor (CF) (Bulow, 1970) and they were reported as valuable environmental risk assessment tool (Sanchez et al., 2008; Van der Oost et al., 2003). The mobilization of energy reserves stored in liver and skeletal muscles enables fish to cope with the increased energy demand associated with stress (Menezes et al., 2015). Even more, fasting tolerance is related to reserves in tissues and their consumption rate (Barcellos et al., 2010; Liew et al., 2012). However, if energy reserves are insufficient, structural biomass can also be consumed to some extent (Rios et al., 2011).

Under stress, animal organisms produce increasing quantities of reactive oxygen species (ROS) which induce antioxidant systems. When stress is severe, the ROS production can overload the antioxidant defenses, and this unbalance balance leads to cell damages. The ROS are detoxified by a set of antioxidant enzymes that protect macromolecules such as proteins, lipids and nucleic acids against damage (Lushchak, 2011). Previous studies have reported that antioxidant defenses in fish are activated by starvation (Furné et al., 2009; Morales et al., 2004). The levels of MDA, a metabolite derived from lipid peroxidation, and the activity of some antioxidant enzymes have been described as valuable biomarkers of pro-oxidant situations in fish (Bacchetta et al., 2014; Cazenave et al., 2014).

Thus, effects of starvation have been evaluated by individual analysis of serum chemistry (Pérez-Jiménez et al., 2012), hematology (Caruso et al., 2010), oxidative stress (Pascual et al., 2003) or digestive tract function and structure (Zeng et al., 2012). However, in order to obtain a holistic and integrative overview of how starvation affects fish health, the assessment of a battery of complementary biomarkers could be recommended.

Hoplosternum littorale is a fish with a wide geographical distribution in the Río de la Plata basin. It inhabits a variety of lentic environments where physical and chemical conditions usually make colonization by other fish species difficult (Affonso and Rantin, 2005; Liotta, 2005). These conditions are mostly related to oxygen availability, environmental temperature variations and anthropogenic factors (Affonso, 2001; Brauner et al., 1999; Duncan, 1998; Silva, 1992). Even more, fish that experience periods of drought such as *H. littorale* may stand up long-term food deprivation. Fasting tolerance of adult *H. littorale* raise questions about their physiology and metabolism. Studies concerning its biological mechanisms to face these nutritional challenges are not available.

We aimed to assess the physiological and metabolic effects of acute and prolonged starvation on *H. littorale*. We analyzed multiple responses simultaneously, such as morphological, hematological, and biochemical indexes, oxidative stress parameters and energy reserves, in order to obtain an integrated multibiomarker approach of this particular condition.

2. Materials and methods

2.1. Fish

Adults *H. littorale* ($n = 64$; 10.6 ± 0.5 cm standard length; 36.0 ± 4.9 g body weight) were obtained from a local fish farm. For acclimation purpose, fish were held in 150-L tanks containing well aerated dechlorinated water for 4 weeks, and were fed three times a day to apparent satiation with dry commercial pellets (Crude protein 40%, Fat 10%, Carbohydrate 10%. Shulet brand, Shulet S.A. 108/A/E, Buenos Aires).

Both acclimation and experimental periods were carried out in 12:12 h light–dark cycles and temperature was 25 ± 1 °C.

2.2. Experimental design

Experiments were conducted under semi-static conditions. The aquarium water was renewed daily by siphoned. The water conditions were: pH 6.9 ± 0.2, total hardness 48 ± 0.1 ppm CO_3Ca, oxygen levels 7.5 mg/L and temperature 25 ± 1 °C.

Tests were conducted in glass aquaria containing 10 L of aquarium water. Control and test groups were replicated four times. Fish (two per aquarium) were randomly distributed into four experimental groups: control fish fed ad libitum for 7 and 28 days (C7, C28), and fish starved for 7 and 28 days (S7, S28). All aquaria were covered with a thin black plastic that prevented stress brought about by management practices in adjacent aquaria. At the end of each period, sampling was performed early in the morning.

2.3. Biomarkers

Prior to blood sampling and dissection, fish were anaesthetized in benzocaine 100 mg/L as described by Parma de Croux (1990). Body weight (g) and total and standard length (cm) were recorded for each individual. Blood was collected immediately from the caudal vessel, and plasma was separated via centrifugation (at $1400 \times g$ for 10 min). The brain, liver, kidney, gill and muscle were dissected and quickly frozen in liquid nitrogen and subsequently stored at −80 °C until biochemical determinations. Before freezing, the wet weight of the liver was determined. All experiments were conducted in accordance with national and institutional guidelines (Human and Animal Research Committee of the School of Biochemistry, National University of Littoral, Argentina; CONICET, 2005) for the protection of animal welfare.

2.3.1. Morphometric biomarkers

Condition factor (CF) was calculated according to Goede and Barton (1990): $CF = BW/L^3 \times 100$, with BW = body weight (g), L = total length (cm). The hepatosomatic index (HSI) was calculated as: $HSI = LW/BW \times 100$, with LW = liver weight (g).

2.3.2. Hematology

Red blood cells (RBC) counts were performed with a Neubauer chamber. Hematocrit (Ht) values were determined by the micromethod using capillary tubes and centrifuged at 1409 *g* for 10 min. Hemoglobin concentration (Hb) was measured by the cyanmethemoglobin method at 546 nm (Houston, 1990). Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated from primary indexes (Cazenave et al., 2005).

2.3.3. Blood metabolites

Plasma levels of total protein, cholesterol, triglycerides and glucose were determined with enzymatic colorimetric methods using the appropriate kits and according to the protocols of the manufacturer (Wiener Lab®). Briefly, total plasma protein concentration was measured by a kit reagent containing EDTA/Cu complex in an alkaline medium that reacts with peptide bonds to yield a purple-blue complex (Gasbarro et al., 1972). Plasma levels of total cholesterol and triglycerides were analyzed by using standard enzymatic-colorimetric test (Fossatti and Prencipe, 1982; McGowan et al., 1983; Trinder, 1969). Finally, plasma glucose was assayed by a colorimetric test, based on the glucose oxidase method

(Henry et al., 1974). All biochemical analyses were measured in triplicate.

2.3.4. Oxidative stress

Oxidative stress in different tissues was assessed by both antioxidant enzyme activities and lipid peroxidation levels.

For enzyme extracts preparation, tissues were homogenized in an ice-cold 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM 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.

Enzyme activities were assayed spectrophotometrically. The activity of soluble glutathione *S*-transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. Catalase activity (CAT) was determined according to Beutler (1982). The enzymatic activity was calculated in terms of the enzyme extract protein content (Bradford, 1976), and is reported as mU mg prot^{-1} (GST, GR and GPx) or U mg prot^{-1} (CAT). Each enzymatic assay was carried out by triplicate.

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}).

2.3.5. Energy reserves

Glycogen, lipid and protein content were measured in liver and muscle. Glycogen was estimated according to Seifter et al. (1950). Briefly, 20 mg of hepatic and 60 mg of muscle tissues were treated with 1 ml KOH 30% and 0.5 ml KOH 60% at 100 °C. After alkaline tissue disruption, glycogen was precipitated by ethanol and glucose was determined using the anthrone reagent method. Lipid content was extracted using chloroform: methanol (2:1) by the method described by Folch et al. (1957) and total protein concentration was estimated in tissue homogenates according to Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Statistical analyses

All values are given as means ± standard deviation. Data were first tested for normality and homogeneity of variance using Shapiro Wilks and Levene's test, respectively. For statistical comparisons of data among 4 groups, 1-way ANOVA followed by Tukey's post-test was used for normally distributed data, and the Kruskal Wallis test for non-normally distributed data. Differences among means were considered significant when $p < 0.05$. 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 starvation physiology. The matrix data included individuals with 40 variables measured. Variables that had not a normal distribution were transformed using Log_{10} and tested again, prior to parametric analysis. All statistical analyses were performed by the InfoStat software (Di Rienzo et al., 2012).

3. Results

Our results showed that starvation had no influence on the survival rate of *H. littorale*. There was no mortality at the end of the experimental periods (7 days and 28 days of starvation). However, a decreased swimming activity was observed (not quantified) in 28-days starved fish.

3.1. Morphometric biomarkers

Condition indexes, body weight and total and standard body length for 7-days and 28-days control and starved *H. littorale* are shown in Table 1. At the end of 28-days starvation period, fish showed a significant total weight loss when compared to control group. Starvation during 7 and 28 days caused a 1.7 and 2.6 fold reduction of HSI, respectively (Table 1).

3.2. Hematology

Hematological parameters of 7-days and 28-days starved *H. littorale* are shown in Table 2. None of the parameters evaluated differed among treatments.

3.3. Blood metabolites

As shown in Fig. 1, both starvation periods led to decreased levels in blood metabolites. 7-days starved fish showed only diminished glucose and triglyceride plasma levels. The main changes were observed after 28 days of food deprivation, where all tested metabolites significantly decreased (glucose, triglyceride, cholesterol and total protein).

3.4. Oxidative stress

The effect of starvation on several antioxidant enzyme activities in liver, kidney, gills and brain is presented in Table 3. Fig. 2 shows lipid peroxidation levels in the same mentioned tissues.

A 7-days period of starvation provoked only significant increased GST and GPx activities in gills, compared to the respective control group. After 28 days of starvation, an inhibition of liver GST and CAT activities, together with increased kidney CAT activity and an activation of all antioxidant enzymes in gills were observed. LPO levels were significantly higher in liver, kidney and brain of fish starved for 28 days (Fig. 2).

Table 1
Somatic data and condition indexes in 7-days and 28-days control and starved *Hoplosternum littorale*.

	7-days period		28-days period	
	Control	Starved	Control	Starved
Total weight (g)	35.95 ± 2.18	35.82 ± 1.38	41.23 ± 3.16	33.20 ± 1.64*
Total length (cm)	13.77 ± 0.64	13.82 ± 0.33	14.35 ± 0.47	13.84 ± 0.70
Standard length (cm)	10.80 ± 0.54	10.58 ± 0.33	11.05 ± 0.41	10.46 ± 0.57
Condition factor	1.37 ± 0.06	1.36 ± 0.06	1.39 ± 0.13	1.25 ± 0.17
Hepatosomatic index	1.99 ± 0.53	1.18 ± 0.31*	2.18 ± 0.32	0.83 ± 0.14*

Values are expressed as mean ± SD.
* $p < 0.05$ 7-days starved vs. 7-days control or 28-days starved vs. 28-days control.

Table 2
Hematological parameters in 7-days and 28-days control and starved *Hoplosternum littorale*.

	7-days period		28-days period	
	Control	Starved	Control	Starved
RBC ($10^6/\mu L$)	1.50 ± 0.29	1.60 ± 0.40	1.87 ± 0.66	1.47 ± 0.45
Ht (%)	33.80 ± 4.40	40.47 ± 6.65	41.00 ± 6.22	38.12 ± 6.59
Hb (g/dL)	7.66 ± 1.63	9.39 ± 1.80	11.43 ± 2.49	11.09 ± 2.56
MCV (μm^3)	229.67 ± 29.41	262.10 ± 53.27	230.53 ± 12.86	277.64 ± 80.94
MCH (pg)	52.37 ± 11.97	61.41 ± 17.69	54.08 ± 11.51	67.49 ± 26.27
MCHC (%)	22.71 ± 4.19	23.27 ± 3.14	22.74 ± 5.75	28.85 ± 6.33

Values are expressed as mean ± SD.

Table 3
Antioxidant enzyme activities measured in liver, kidney, gills and brain of 7-days and 28-days control and starved *Hoplosternum littorale*.

	7-days period		28-days period	
	Control	Starved	Control	Starved
Liver				
GST	1545.22 ± 464.71	1251.42 ± 151.16	2061.80 ± 102.17	1238.38 ± 251.27*
GR	32.32 ± 6.16	33.15 ± 5.98	33.21 ± 5.35	33.46 ± 7.50
GPx	587.11 ± 138.96	840.64 ± 234.67	687.02 ± 81.48	735.74 ± 189.29
CAT	86.56 ± 36.57	51.19 ± 16.74	148.22 ± 27.46	73.87 ± 28.93*
Kidney				
GST	1288.48 ± 350.98	1443.33 ± 245.62	1215.14 ± 292.98	1647.62 ± 687.39
GR	62.73 ± 14.54	67.66 ± 10.11	57.77 ± 19.17	83.34 ± 28.69
GPx	789.96 ± 188.18	840.64 ± 234.67	735.86 ± 226.90	1051.95 ± 505.69
CAT	3.15 ± 0.76	3.83 ± 1.77	4.48 ± 1.03	7.20 ± 2.65*
Gills				
GST	576.10 ± 169.59	892.45 ± 221.56*	484.58 ± 137.64	2006.55 ± 567.20*
GR	24.72 ± 3.12	33.01 ± 8.45	22.07 ± 2.94	98.05 ± 36.93*
GPx	180.17 ± 35.66	303.5 ± 81.66*	223.26 ± 40.64	581.04 ± 79.71*
CAT	8.15 ± 3.81	15.06 ± 5.07	10.55 ± 3.91	31.19 ± 12.79*
Brain				
GST	2618.55 ± 420.47	2732.27 ± 749.68	2173.15 ± 627.44	3085.41 ± 909.30
GR	109.96 ± 26.12	87.27 ± 38.21	64.19 ± 27.16	102.39 ± 52.14
GPx	458.02 ± 179.04	492.63 ± 131.34	341.68 ± 107.33	520.93 ± 169.82
CAT	47.56 ± 10.52	41.77 ± 17.19	32.92 ± 13.08	39.33 ± 11.12

Values are expressed as means ± SD. Activity expressed in mU mg $prot^{-1}$ (GST, GR and GPx) or U mg $prot^{-1}$ (CAT). GST: glutathione S-transferase, GR: glutathione reductase, GPx: glutathione peroxidase; CAT: catalase.
* $p < 0.05$ 7-days starved vs. 7-days control or 28-days starved vs. 28-days control.

3.5. Energy reserves

Proximate composition of liver and muscle, as well as liver weight is shown in Table 4. There was an obvious impact of starvation on liver weight and glycogen reserves. They were significantly reduced in both experimental periods in comparison with the respective control specimens. The decrease percentage in glycogen levels after 7 days of starvation reached 71% and 70% in liver and muscle, respectively. After 28 days of food deprivation they were 97% in liver and 65% in muscle. Significant differences were observed in lipid and protein concentrations only in liver, whereas no differences were observed in muscle.

3.6. Integrated analysis

Fig. 3 and Table 5 show that 45.4% of overall variance is explained by the first two principal components. According to Legendre and Legendre (1979), interpretation of principal components may be done for eigen values of the data matrix higher than 1. The PCA indicated that 10 eigen values were higher than 1; moreover

Table 4
Liver weight and tissue metabolites (liver and muscle) of 7-days and 28-days control and starved *Hoplosternum littorale*.

	7-days period		28-days period	
	Control	Starved	Control	Starved
Liver				
Total weight (g)	0.73 ± 0.24	0.42 ± 0.09*	0.89 ± 0.13	0.27 ± 0.07*
Glycogen (μmol/g tissue)	574.10 ± 47.60	166.86 ± 63.56*	447.71 ± 59.98	13.36 ± 5.89*
Lipid (μmol/total liver wt)	9.13 ± 3.03	4.18 ± 0.88*	11.25 ± 2.31	3.07 ± 0.80*
Protein (mg/total liver wt)	115.74 ± 48.59	67.3 ± 9.58*	148.75 ± 14.69	43.36 ± 18.44*
Muscle				
Glycogen (μmol/g tissue)	2.27 ± 1.54	0.68 ± 0.27*	3.20 ± 1.40	1.12 ± 0.30*
Lipid (μmol/g tissue)	7.22 ± 2.19	5.53 ± 0.71	7.61 ± 3.20	5.89 ± 0.27
Protein (mg/g tissue)	153.93 ± 20.33	153.64 ± 40.64	180.54 ± 30.66	141.14 ± 30.47

Values are expressed as means ± SD.
* $p < 0.05$ 7-days starved vs. 7-days control or 28-days starved vs. 28-days control.

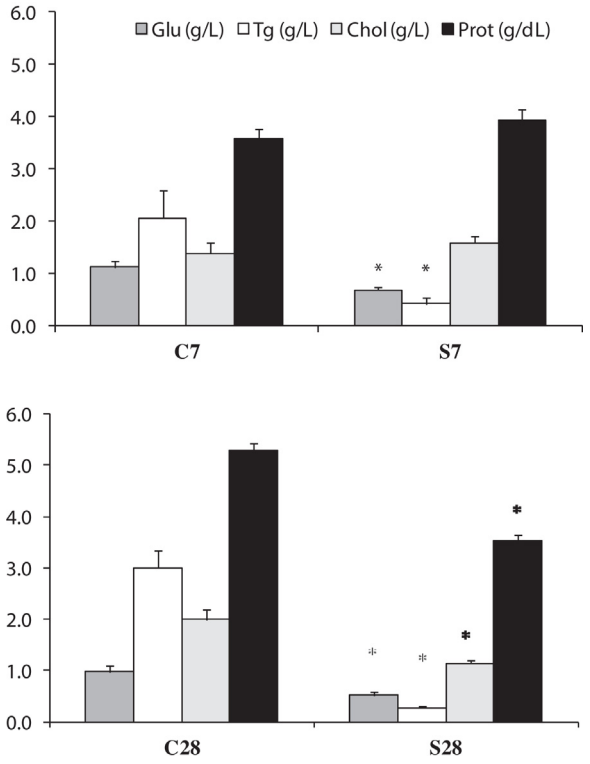

Fig. 1. Blood metabolites (glucose, triglyceride, cholesterol and total protein) of 7-days and 28-days control (C7 and C28) and starved (S7 and S28) *Hoplosternum littorale*. Values are expressed as mean ± SD. Concentration expressed in g/L (Glu, Tg and Chol) or g/dL (Prot). *$p < 0.05$ 7-days starved vs. 7-days control or 28-days starved vs. 28-days control. Glu: glucose, Tg: triglyceride, Chol: cholesterol and Prot: total protein.

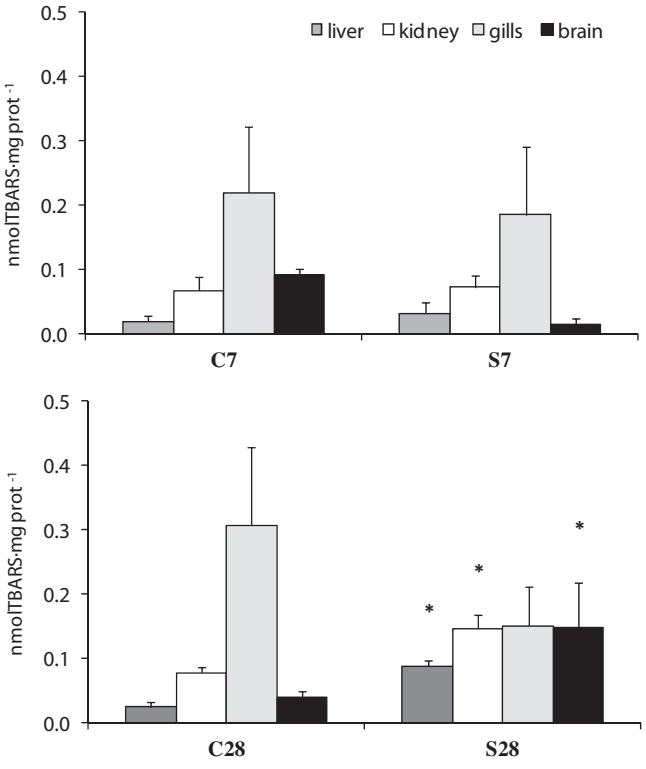

Fig. 2. Lipid peroxidation levels in liver, kidney, gills and brain of 7-days and 28-days control (C7 and C28) and starved (S7 and S28) *Hoplosternum littorale*. Values are expressed as mean ± SD. *$p < 0.05$ 7-days starved vs. 7-days control or 28-days starved vs. 28-days control.

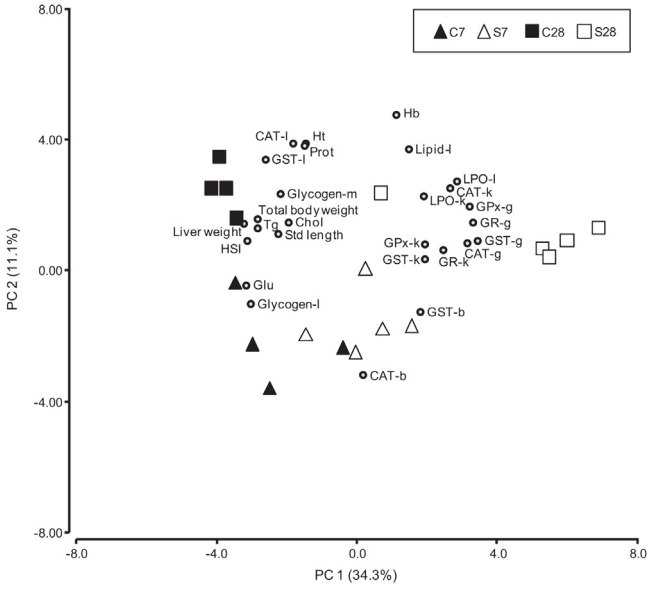

Fig. 3. Principal components analyses (PCA) of the biomarkers responses in 7-days and 28-days control (C7 and C28) and starved (S7 and S28) *Hoplosternum littorale*. Biomarkers with correlation coefficients >0.5 were represented in the PCA. Biomarkers abbreviations are explained in the text. Abbreviations for different tissues were followed by the corresponding letter: b: brain; g: gills; k: kidney; l: liver; m: muscle.

correlation coefficients are significant when they are higher than $\sqrt{d/n}$, d being the number of principal components and n the number of variables. Therefore, correlation coefficients >0.5 were indicative of a good representation of the variables with principal component axes. The first axis explained 34.3% (PC1, 34.3%) of the global inertia in the data and it ordinates mainly control and starved fish. Thus, on this first principal component, starved fish (on the right) were clearly distinguished from control (on the left), showing significant positive loadings for the antioxidant enzymes in gills (GST, GR, GPx, and CAT) and LPO in liver, among others. On the contrary, a negative correlation was found mainly for liver weight, glucose, HSI, liver glycogen, Tg and total body weight.

PC2 (11.1% of the total variance) showed a high individual variation for experimental periods (7 days of starvation below and 28 days of starvation above). This axis appears to be positively correlated with some hematological variables (Hb, Ht), antioxidant enzymes measured mainly in liver (CAT, GST) and hepatic lipids; and negatively for CAT measured in brain.

4. Discussion

4.1. Morphometric biomarkers

Fish energy requirements to maintain life processes during starvation are met by mobilizing the body energy reserves, which eventually leads to body weight loss (Navarro and Gutiérrez, 1995; Vigliano et al., 2002). It has also been reported that, as in mammals, fish physical activity declines during prolonged starvation (Guderley et al., 2003; Novak et al., 2005), avoiding body reserves waste and weight loss. We also observed a diminished swimming activity with a significant reduction in body mass after 28 days of starvation, suggesting that fish inactivity was not enough to prevent weight loss in *H. littorale*. Previous studies show that starvation reduces fish swimming ability and this might be due to decrease muscle enzyme activity, decrease concentrations of contractile proteins and loss of muscle mass (Martínez et al., 2003; Rios et al., 2011; Yu et al., 2010).

Table 5
Loading of variables on principal component analysis.

	PC1	PC2
Eigen value	13.70	4.44
Variance (%)	34	11
Cumulative (%)	34	45
Total length	−0.30	0.40
Standard length	**−0.61**	0.17
Total weight	**−0.76**	0.20
Liver weight	**−0.88**	0.22
Hepatosomatic index	**−0.85**	0.14
Condition factor	−0.42	−0.04
RBC	−0.48	0.12
Ht	−0.39	**0.60**
Hb	0.31	**0.74**
MCH	0.36	0.46
MCV	0.25	0.14
MCHC	0.37	0.40
Plasma glucose	**−0.85**	−0.07
Plasma protein	−0.40	**0.59**
Plasma triglycerides	**−0.77**	0.24
Plasma cholesterol	**−0.51**	0.26
GPx-g	**0.88**	0.30
CAT-g	**0.86**	0.13
GST-g	**0.95**	0.14
GR-g	**0.91**	0.23
CAT-b	0.06	**−0.50**
GST-b	**0.50**	−0.20
GPx-b	0.44	−0.29
GR-b	0.27	−0.29
GPx-l	0.02	0.02
CAT-l	−0.49	**0.60**
GST-l	**−0.70**	**0.53**
GR-l	0.15	0.12
GPx-k	**0.53**	0.12
CAT-k	**0.73**	0.39
GST-k	**0.54**	0.05
GR-k	**0.68**	0.10
LPO-g	−0.38	0.19
LPO-b	0.36	0.25
LPO-l	**0.79**	0.42
LPO-k	**0.52**	0.35
Muscle glycogen	**−0.59**	0.36
Liver lipid	0.41	**0.58**
Liver protein	0.05	0.10
Liver glycogen	**−0.82**	−0.16

Significant correlation coefficients (>0.5) are showed in bold.

HSI reflects alterations in the metabolic activity of the liver, acting as an appropriate biomarker of the effect of an altered environment. Starvation induced a significant and immediate reduction in the HSI when compared to control fish. It lowered down 41% in 7-days and 62% in 28-days starved fish compared to the respective control group. These results could indicate that energy stores within the liver were rapidly mobilized in response to food deprivation. Decrease in HSI provoked by starvation was also verified in several teleosts (Barcellos et al., 2010; Bayir et al., 2011; Costas et al., 2011; Pérez-Jiménez et al., 2007).

4.2. Hematology

Hematologic analysis is a sensitive indicator of the physiological condition in animals. Blood values can vary widely among species and in response to habitat conditions (Hrubec et al., 2000). It is important to highlight that mean values recorded in *H. littorale* for hematological variables fall within the normal range reported by Affonso et al. (2004). Nevertheless, we found no significant responses in the hematological parameters of *H. littorale* in 7-days or 28-days starved fish. Rios et al. (2005, 2011) reported an anemia process in starved *Prochilodus lineatus* and *Hoplias malabaricus* after 35 and 30 days of food deprivation respectively. On the contrary, Caruso et al. (2011) observed no significant variation in haematocrit of *Dicentrarchus labrax* and *Pagellus bogaraveo* starved for 31 days. The different results on hematological variables are probably due to inter-species differences.

4.3. Blood metabolites

The blood glucose level is the most commonly measured physiological variable in starving animals. The reduction in plasma glucose levels induced by starvation in *H. littorale* after both experimental periods is in agreement with most studies performed in fish (Furné et al., 2012; Pérez-Jiménez et al., 2007, 2012; Soengas et al., 2006). The lower circulating levels of glucose on starved *H. littorale* may be due to utilization of glucose, an energetic substrate, to cope with starvation. Under food deprivation, glucose requirements for metabolic purposes may be satisfied by glycogen degradation (glycogenolysis) or by de novo glucose synthesis (gluconeogenesis) from non-glycoside substrates, such as lactate, glycerol, pyruvate and some amino acids (Polakof et al., 2012). In many fish species glycaemia during food deprivation is directly related to the capacity to mobilize liver glycogen, at least during the initial stages of starvation. The large reduction of liver glycogen induced in the present study suggests that this is a readily available energy reserve for *H. littorale*, as for most fish species (Metón et al., 2003; Pérez-Jiménez et al., 2007, 2012; Power et al., 2000; Soengas et al., 2006). Nevertheless, hepatic glycogen stores are almost depleted after 28 days of starvation being therefore unable to sustain significant rates of endogenous glucose production via glycogenolysis. In this period, the obtained glucose plasma levels could be due to an enhancement of endogenous basal gluconeogenesis. On this regard, Enes et al. (2009) observed in starved rainbow trout and gilthead sea bream liver, a significantly increased activity of key enzymes regulating gluconeogenesis. In addition, Rios et al. (2011) showed that the source of glucose for *P. lineatus* after 4 weeks of food deprivation might be amino acids from muscle breakdown. They observed that the total weight loss is caused by loss of muscle mass, indicating intense proteolysis. Although we did not measured loss of muscle mass, we did observed a total weight loss after 28 days of starvation, suggesting that this mechanism could be operating.

Previous studies have shown that plasma lipid concentration, particularly triglycerides and cholesterol, is highly dependent on fish nutritional or physiological state and on their developmental stage (Babin and Vernier, 1989). In the present study, a marked and significant decrease in plasma triglyceride levels has been observed. They were approximately threefold lower in 7-days and sevenfold lower in 28-days starved fish when compared with their control groups, reflecting the lack of dietary lipids availability. In addition, our results show significantly lower levels of plasma total cholesterol in 28-days starved *H. littorale*. In higher vertebrates, plasma triglyceride levels declines with the suppression of lipogenic enzymes activity (Shearer et al., 2012). Results for fatty acid synthase activity and a concomitant inhibition of Glucose-6-phosphate dehydrogenase (G_6PD) and malic enzyme activities reflect a down regulation of lipogenesis during food deprivation in fish, aiming to preserve plasma glucose levels (Navarro and Gutiérrez, 1995; Pérez-Jiménez et al., 2007). Both food deprivation and inhibition of fatty acid synthesis would be responsible for the reduction in plasma triglyceride and total cholesterol during starvation in fish (Costas et al., 2011; Mancera et al., 2008; Menezes et al., 2015; Peres et al., 2014; Pérez-Jiménez et al., 2007, 2012).

Circulating protein levels have been used as indicators of the rates at which proteins are mobilized during starvation. In the present study, total plasma protein levels were significantly lower in 28-days starved fish, suggesting their role as fuel source in *H.*

littorale. In agreement with our results, total plasma protein levels significantly diminished in several starved fish species (Costas et al., 2011; Echevarría et al., 1997; Peres et al., 2013, 2014; Pérez-Jiménez et al., 2012). As plasma protein level is usually very stable in well-nourished animals, under malnutrition or stress conditions, altered plasma total protein levels often occur as consequence of amino acid oxidation or peripheral proteolysis (Di Marco et al., 2008; Mommsen et al., 1999).

4.4. Oxidative stress

Stressors may trigger a set of compensatory and adaptive responses in order to restore or readjust the altered processes to their normal levels and to cope with the adverse effects of stressors. We found that after 28 days of starvation, ROS generation is enhanced in *H. littorale* liver, kidney and brain, and that antioxidant defenses were inadequate for effective scavenging ROS, thus leading to the appearance of lipid peroxidation. Only gills preserved and even increased its capacity to cope with oxidative stress, preventing an increase in LPO. These results show that the experimental starvation periods caused a distinct and differential tissue response on the antioxidant capacity of *H. littorale*. Previous reports indicated that the antioxidant status and/or the responses to starvation vary from organ to organ. For example, Furné et al. (2009) observed that starved Adriatic sturgeon (*Acipenser naccarii*) and rainbow trout (*Oncorhynchus mykiss*) presented raised lipid peroxidation levels and diminished antioxidant competence in liver but found the opposite in muscle. In heart, no oxidative damage was detected during starvation in either species while CAT and superoxide dismutase (SOD) activities increased in trout. Accordingly to Guderley et al. (2003), muscle CAT and GST activities did not differ between fed and starved cod, but they did increase in liver. Antonopoulou et al. (2013) showed in starved sea bass, augmented CAT and SOD activity without changes in intestine GPx activity. They also found no variations in the activities of antioxidant enzymes and TBARS in liver while in red muscle SOD decreased and GPx and CAT activities remained unchanged.

The decreased hepatic GST and CAT activity observed in 28-days starved *H. littorale* might be the result of a failure in the cellular antioxidant defenses turning cells more sensitive to oxidative damage. The rise of lipid peroxidation levels found in our study accounts for such hypothesis. Previous studies in fish showed that starvation augments lipid peroxidation (Bayir et al., 2011; Morales et al., 2004; Pascual et al., 2003), and reduces GST and CAT activities in liver (Bayir et al., 2011; Blom et al., 2000; Furné et al., 2009; Pascual et al., 2003), which may be a consequence of the restriction of enzyme synthesis substrates. Several authors also observed decreased liver G_6PD activity in starved fish (Bayir et al., 2011; Metón et al., 2003; Morales et al., 2004). Lower G_6PD activity results in lower availability of NADPH, which is essential for CAT activity (Aras et al., 2009). Although we did not measured G_6PD activity in the present study, this could be the reason for the decreased CAT liver activity observed in fish starved for 28 days. On the other hand, as GST have a critical role against oxidative damage (Elia et al., 2003), the lower levels of this enzyme in fish liver, suggested a significant reduction of fish capacity to withstand oxidative stress following 28 days of starvation. The decreased activity of liver detoxification enzyme GST could be a general way to reduce the metabolic costs of starvation (Blom et al., 2000). Even more, the nutritional restriction that fish were subjected to could have influenced the supplement of sulfur amino acids and therefore the levels of reduced glutathione (GSH). As GST is a GSH-dependent enzyme, GSH level changes may also decrease GST activity (López-López et al., 2011; Pascual et al., 2003).

Lipid peroxidation levels indicated no oxidative stress in gills during any period of starvation tested. This may be partly because of the activation of the antioxidant enzymatic defense in the tissue. GST and GPx activities in gills increased significantly after both periods of starvation tested (Table 3), observing the highest values in fish starved for 28 days. The increased levels of GST indicate that this enzyme was induced by GSH conjugation. Jackson et al. (2002) have indicated that GST prevents lipid peroxidation and DNA damage by reducing DNA base hydroperoxides and lipid hydroperoxides; therefore, the increased GST activity observed in gills during the experimental periods suggests the detoxification of toxic products of lipid peroxidation, such as MDA. The augmented CAT and GPx activities observed in gills after 28 days of starvation in the present study are in agreement with Bayir et al. (2011). They observed increased SOD activity in the long term starved brown trout gills which resulted in higher H_2O_2 generation, which may be the reason for the high branchial CAT and GPx activities founded. They also observed that branchial GR activity increased in parallel with starvation, matching our results in the 28-day period. This suggests that pool of reduced glutathione in gills was maintained by enhanced rates of turnover (re-conversion of oxidized GSSG to GSH), and was available as substrate of GPx and GST.

After 28 days of starvation higher levels of CAT activity in kidney with increased LPO levels were found. The mitochondrial and peroxisomal location of CAT may facilitate a protective role, particularly when the mobilization of macromolecules renders cellular structures more susceptible to oxidative damage (Guderley et al., 2003). Nevertheless, the rise in LPO levels together with the unaltered activity of most antioxidant enzymes seems to indicate the incapacity of kidney to cope with a prolonged period of starvation.

Brain is particularly prone to suffer oxidative stress due to its high rate of oxidative metabolism, presence of autooxidizable neurotransmitters and cytochrome P450s, high levels of unsaturated fatty acids, and relatively modest antioxidant defenses (Halliwell and Gutteridge, 2007). We observed lipid oxidative damage in the brain of the 28-days starved fish probably due to the absence of cell protection by unchanged antioxidant enzyme activities, demonstrating that inherent anti-oxidative mechanisms were not activated.

4.5. Energy reserves

All successful starvation strategies should allow animals to maintain some acceptable level of physiological homeostasis under fasting. It must enable the animal to mobilize endogenous physiological fuels such as proteins, lipids, and carbohydrates to meet the energetic demands required to carry out the basic processes of life (McCue, 2010).

Liver is the most metabolically active tissue and, its energy store seems to be readily available, though it is not the most extensive in terms of energy. An obvious impact of starvation on glycogen reserves was found in the present study. The first 7 days of starvation triggered a mobilization of liver glycogen to meet energy demands. In fact, hepatic glycogen was the main energy source in both periods of starvation tested. It is known that glycogen depletion is a stress-induced response. The observed changes are consistent with the findings stating that hepatic glycogen is the main substrate mobilized in fish during starvation (Barcellos et al., 2010; Furné et al., 2012; Navarro and Gutiérrez, 1995; Pérez-Jiménez et al., 2007).

Liver glycogen contents are extremely variable in fish, representing 1–12% of liver fresh weight (Kaushik, 1999). The intense utilization of endogenous glycogen reserves to maintain metabolism during food deprivation leads to a great reduction in the wet mass of liver and, consequently, in the hepatosomatic index of *H. littorale*. There is a tendency for water to be stored in direct proportion to glycogen storage in the liver (MacKay and Bergman, 1932), meaning that any change in the glycogen content of the liver

could influence its wet mass. Several studies observed that liver weight was greatly reduced due to an intense mobilization of glycogen (Menezes et al., 2015; Peres et al., 2013, 2014; Pérez-Jiménez et al., 2007). The reduction of liver mass is an immediate effect of starvation that has been observed in many other species, including neotropical ones (Cook et al., 2000; German et al., 2010; Rios et al., 2002, 2011). Several studies have focused on the phenotypic flexibility of organ size and function in response to environmental changes such as food availability (Gao et al., 2008; German et al., 2010; Rios et al., 2002). This indicates that individuals can show substantial though reversible transformations in phenotypic traits (behavior, physiology and morphology) when environmental conditions change rapidly and over shorter timescales than a lifetime (Zeng et al., 2012).

In this study, significant differences were observed in protein liver concentrations when they were expressed as mg/total liver weight. According to Navarro and Gutiérrez (1995) only absolute values of total protein in the whole liver are meaningful physiological measurements. On the other hand, it is known that the use of hepatic lipids as an energy source during starvation depends on the species, the lipid-reserve tissue, and the strategy followed to mobilize other reserves such as carbohydrates (Furné et al., 2012). Our results on hepatic lipid content showed a significant reduction when expressed per total liver weight after 7 and 28 days of starvation.

The high percentages of reduction in muscle glycogen induced in the 7-days and 28-days starved fish (70% and 65% respectively) indicate that this reserve is used by *H. littorale* as fuel during such circumstances. Similar effects were observed by other authors (Pérez-Jiménez et al., 2012; Rios et al., 2006). Our data showed that the glycogen decrease was greater in liver than in muscle, as liver is the main organ for glycogen synthesis and storage.

To our knowledge, very little information is available about *H. littorale* metabolism. Luquet et al. (1991) observed in starved *H. littorale* that lipids played an important role in energy supply during the first phase but, in the long term, routine energy requirements were met mostly by protein mobilization. The difference with our work is that they evaluated changes in whole body composition though carcass analysis, while we examined energy reserves in muscle and liver tissue.

4.6. Integrated analysis

The principal component analysis (PCA) reduces the dimensionality of an observed dataset with many correlated variables by transforming them into a new set of variables, named principal components, retaining as much as possible the variation of the observed dataset (Jolliffe, 2005). It is used to take the most important information from the dataset reducing the number of dimensions described by their eigen values, loadings and scores (Chen et al., 2014). In the present study, data obtained in starved fish demonstrated the high sensitivity of certain biochemical parameters tested. Strong responses were seen in oxidative stress markers and metabolic parameters, which were key biomarkers in separating starved from control fish. Even more, our present results suggest that starvation may lead to sublethal stress on *H. littorale* in both starving treatments.

Gills showed one of the highest degrees of alteration among the investigated organs, since gill tissue plays a wide variety of physiological functions and has to rely on blood-borne substrates to maintain its high metabolic rate (Hoar and Randall, 1984). Because the gill is multifunctional, changes in this organ may lead to the impairment of several functions, including gas exchange, ion regulation and excretion of metabolites. Therefore, the development of high levels of antioxidant defenses seems to be the strategy used by gills to degrade H_2O_2 precursor of the highly reactive hydroxyl radical that may disrupt the molecular structure of cell membranes causing damage to proteins and other biomolecules, originating the free passage of water and solutes and impacting the osmoregulatory function of the gills (Cruz et al., 2015).

5. Conclusions

Knowing the physiological strategies activated by *H. littorale* to face acute and prolonged periods of starvation may have important implications in understanding how fish are well adapted to environments with highly variable food availability. Our results demonstrate the utility of a battery of biomarkers to assess metabolic and physiological responses triggered by starvation. Although the highest degree of alterations was observed after 28 days of treatment, significant changes were also found in the 7-days starved fish. Major changes included total weight, HSI and blood metabolites decrease, and energy reserves depletion. Oxidative stress occurred in all tissues after 28 days of starvation, except for gills which showed antioxidant defenses activation.

It is important to highlight that the scenario reported in our study may be worsened by the ongoing climatic changes, such as heat waves and prolonged droughts. Therefore, it is extremely important to know the physiological mechanisms related to starvation due to its effects on higher levels of biological organization, like diminished growth, survival probability and reproduction (Reddy and Leatherland, 1998). We consider the biomarkers tested here as useful tools to monitor future environmental issues.

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