Neotropical Ichthyology, 16(1): e170063, 2018
DOI: 10.1590/1982-0224-20170063

Journal homepage: www.scielo.br/ni
Published online: 15 March 2018 (ISSN 1982-0224)
Printed: 31 March 2018 (ISSN 1679-6225)

Original article

Temperature affects the hypoxia tolerance of neotropical Cichlid *Geophagus brasiliensis*

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Oxygen and temperature are the most limiting factors in aquatic environments. Several species are exposed to variations of these factors in water because of physical, chemical and biological processes. The objective of this study was to evaluate the metabolic profile and the tolerance to the hypoxia of *Geophagus brasiliensis* exposed to changes in temperature and oxygen availability. The fish were exposed to 20 and 90% of oxygen saturation combined with different temperatures (20°, 24° and 28° C) for 8 h. Hepatic and muscular glycogen, as well as the activities of lactate dehydrogenase (LDH), malate dehydrogenase (MDH), citrate synthase (CS) and their ratios were evaluated. Both glycogen and MDH activity showed a significant difference in the liver. While CS showed increased activity only in the heart. The increase in LDH activity in the white muscle shows the importance of the anaerobic pathway as energy source in this tissue. The MDH / LDH ratio increased in all tissues, while CS / LDH increased in the liver and decreased in the heart. Based on the results of the present study it may be concluded that this species used the anaerobic metabolism as the main strategy for hypoxia tolerance.

Keywords: Aerobic and anaerobic pathways, Behavior, Metabolism, Oxygen.

O oxigênio e a temperatura são os fatores mais limitantes em ambientes aquáticos. Várias espécies são expostas a variações destes fatores na água como resultado de processos físicos, químicos e biológicos. O estudo objetivou avaliar o perfil metabólico e a tolerância à hipóxia de *Geophagus brasiliensis* expostos a alterações na temperatura e disponibilidade de oxigênio. Os peixes foram expostos a 20% e 90% de saturação de oxigênio combinadas com diferentes temperaturas (20 ° C, 24 ° C e 28 ° C) durante 8h. Foram avaliados o glicogénio hepático e muscular, assim como as atividades das enzimas lactato desidrogenase (LDH), malato desidrogenase (MDH), citrato sintase (CS) e suas razões. Tanto o glicogênio quanto a atividade da MDH apresentaram diferença significativa no fígado. Enquanto a CS apresentou aumento de sua atividade apenas no coração. O aumento da atividade LDH no músculo branco mostra a importância da via anaeróbia como fonte de energia neste tecido. A razão MDH/LDH aumentou em todos os tecidos, enquanto CS/LDH apresentou aumento no fígado e diminuição no coração. Os resultados obtidos permitem concluir que esta espécie utilizou o metabolismo anaeróbio como principal estratégia de tolerância à hipóxia.

Palavras chave: Comportamento, Metabolismo, Oxigênio, Vias aeróbicas e anaeróbicas.

Introduction

Oxygen is one of the most limiting factors of the aquatic environment, and its depletion has been associated with the wide participation of human activities in environmental eutrophication (Diaz, Rosenberg, 2008). The availability of this gas in the water is essential for aquatic organisms, and it can influence their ecology, behavior and physiology (Martinez *et al.*, 2011).

Biochemical and physiological changes such as changes in lactate dehydrogenase, anaerobic ATP production, increasing of gill ventilation, and reducing heartbeat frequency (McNeil, Closs, 2007) can occur after a situation of intense hypoxia. Metabolic suppression, carried out by reducing the demand for O_2 and the use of anaerobic metabolism, is one of the most effective hypoxia tolerance strategies utilized by some species (Bickler, Buck, 2007).

The way how environmental physicochemical parameters, especially temperature, influence aquatic ecosystems is already known. They can directly influence the ecophysiological responses of organisms at different

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levels at the ecosystem (Pörtner, Peck, 2010), especially ectothermic organisms, which are characterized by dependence on the heat of their environment to regulate their body temperature. Especially the temperature can act like a "ecological driving force" in the aquatic environment, influencing all biological parameters (Guderley, 2004), for example, a change of the temperature may affect the fish tolerance to hypoxia and the energy consumption (Yang *et al*., 2015). The tolerance limits and the ability of species to adapt to new temperature regimes are determining factors for the success of a population that is subjected to changes in this variable (Pörtner, Knust, 2007).

An increase in hypoxic zones by stratification of water bodies, together with the increased oxygen demand from organisms due to an increase in metabolic rate, are some of the consequences of temperature change (Stramma *et al.*, 2008). Oxygen and temperature can also interact to form the "thermal oxygen pressure", wherein the organism, through a "trade-off", faces a confrontation between changing their physiology depending on either temperature or the oxygen level (Taylor *et al.*, 2007). Recently, the hypoxic conditions have been aggravated because of intensification of global warming (Roze *et al*., 2013), making the effect of temperature on hypoxia tolerance in fish an important subject to investigate (He *et al*., 2015) as hypoxia tolerance can differ between different species (Borowiec *et al*., 2016).

Geophagus brasiliensis (Quoy & Gaimard, 1824) has great variety in its morphology, and its occurrence covers a wide geographical area in South American, including tropical and subtropical regions. Therefore, the populations of this fish are exposed to different environmental conditions including variations in water temperature and oxygen concentration. Perazzo *et al.* (2013) suggested that speciation events for this species were due to this environmental variability. Because environmental adaptation plays an important role in determining species distributions among heterogeneous environments, and is important to know the adaptive characteristics of *G. brasiliensis* considering that this species is widely distributed in different environmental conditions. Thus, this study aimed to measure the tolerance of *G. brasiliensis* to hypoxia at different temperatures through its physiological and metabolic responses.

Material and Methods

Experimental fish. *Geophagus brasiliensis* (8.31 ± 0.87g) were purchased from a private fish farm located in Guarapari, Espirito Santo State, Brazil and taken to the laboratory, where they were kept in standard acclimatization conditions for 30 days (1,000L tank, natural photoperiod, fed twice a day, 70% of the water exchanged once a week, physicochemical parameters monitored daily) at 28°C. After this period, the fish were individually transferred to 30L aquaria and were acclimatized for 14 days at the following experimental temperatures: 20°C (20 ± 0.09°C), 24°C (24.12 ± 0.21°C) and 28°C (28.04 ± 0.11°C) under normoxia conditions (90% ± 4.05% oxygen saturation). The decrease of the temperature was performed progressively (2°C per day) from 28°C to 24 or 20°C. This process was controlled using a 110W resistor places in the aquaria coupled to a digital thermostat accurate to one decimal place. These temperatures represent the temperature range in which the species is found in the wild (Rantin, Petersen, 1985).

The fish were fed twice a day with commercial fish feed with 45% crude protein. The aquariums were cleaned daily, and 70% of the water was exchanged weekly. Total ammonia (1.34 ± 0.21 mg/L), nitrite (0.06 ± 0.03 mg/L) and hardness (69.6 ± 6.92 mg $CaCO_3$/L) were evaluated according to APHA (1998) twice a week, before and after the water exchange.

After identification, the voucher specimen was deposited in the ichthyological collection of the Museu de Biologia Professor Mello Leitão, Santa Teresa - ES (MBML - PEIXES 12643). The experiment was authorized by the animal experimentation committee of the institution (CEUA - UVV), in accordance to research protocol n°. 305/2014.

Experimental design. Fish from each temperature group were individually transferred to an experimental aquarium containing 10 L of water and constant aeration at the same temperature as their acclimatization. The fish remained there for 24 hours before starting the experiment. A 150 W thermostat was used to reach and maintain the desired experimental temperature. An oxygen reading chamber was used to monitor the oxygen that was given in mg/L. The chamber was connected on one side to a submerged pump inside the aquarium; on another side, the probe of a Multiparameter device was inserted that performed the readings of percentage of oxygen saturation and temperature throughout the experiment, thus forming a closed water circulation system.

Different oxygen conditions, normoxia (90% ± 3.41% oxygen saturation) and hypoxia (20% ± 2.68% oxygen saturation), were combined with different temperatures, 20°C (20.7 ± 0.37°C), 24°C (23.7 ± 0.84°C) and 28°C (28.5 ± 0.7°C), totaling six treatments with eight replicates for each (total n = 48). Each experiment lasted 8 hours (Chippari-Gomes *et al.*, 2005). For treatments with hypoxic conditions, the aeration was removed at the beginning of the experiment, and the aquarium was completely closed, thus preventing any gaseous exchange with the environment. The oxygen was gradually reduced (from 25% saturation per hour) using the introduction of nitrogen gas bubbles in the water according to Chippari-Gomes *et al.* (2005). Once the desired 20% of oxygen saturation was achieved, the nitrogen introduction stopped and the eight experimental hours started (Chippari-Gomes *et al.*, 2005).

At the end of the experiment, the animals were anesthetized with eugenol (100 mg/L) and the blood sample was collected using a syringe contained EDTA for the plasma lactate determination. For these analyses, we used a Kit by Katal (K11B). The fish were euthanized by spinal cord section and the liver, heart and white muscle tissues were excised. Part of the liver and muscle were weighed and stored in test tubes containing 1 mL of KOH (potassium hydroxide) for glycogen analysis. The rest of these tissues and the heart were immediately stored in a -80°C freezer for further analysis.

The determination of concentration of hepatic and white muscle glycogen was performed according to Dubois *et al.* (1956) with modifications. The reading was made in a spectrophotometer at 480 nm and the concentration was expressed in μmol g^{-1}.

Heart, white muscle and liver tissues were homogenized with EDTA buffer (ethylene diamino tetraacetic acid) 1 mM containing 1% Triton X-100 (octylphenoxypolyethoxyethanol) and 20 mM Hepes (hydroxyethylpiperazine etanosulfuric acid) and had the pH adjusted to 7.4 for citrate synthase enzyme (CS). For lactate dehydrogenase (LDH) and malate dehydrogenase enzymes (MDH), 150 mM imidazole buffer containing 1 mM EDTA and 1% Triton X-100 (pH 7.4) was used. The homogenate was used at a 1:3 dilution for liver and muscle and 1:9 dilution for heart. They were centrifuged at 13,000 g for 15 minutes in a centrifuge Eppendorf 5417R at 4°C. The enzyme assays were performed with the extract (supernatant portion) at 25°C. Enzyme levels were measured by oxidation of NADH at 340 nm (molar extinction coefficient mM = 6.22) for LDH and MDH enzymes and via the oxidation of DTNB at 412 nm (molar extinction coefficient mM = 13.6) for CS enzyme.

Enzyme activity levels were determined according to techniques reviewed by Driedzic, Almeida-Val (1996) with modifications. The activity of LDH and MDH enzymes was obtained in 0.15 mM NADH, 1 mM KCN in 50 mM imidazole, pH 7.4. The LDH enzyme reactions were initiated with the addition of 1 mM pyruvic acid, and MDH enzyme was initiated with the addition of 0.5 mM oxalacetic acid. The CS enzyme activity was obtained in 0.25 mM DTNB, 75 mM Tris, and pH 8.0. The reactions were initiated with the addition of 0.04 mM acetyl CoA and 0.5 mM oxalacetic acid. The amount of protein was quantified by Bradford method (Bradford, 1976).

Statistical analysis. All data were evaluated for normality of the residuals using the Shapiro-Wilk test and the data that did not have normal distribution were normalized by transformation to log10 or 1+log10. For enzyme activity and glycogen level analysis as function of temperature and oxygen conditions as independent variables, two-way ANOVA was used ($p < 0.05$). Analyses were performed using the program SigmaStat version 12.5 (Systat Software, Inc.).

Results

The muscle glycogen concentration did not differ between treatments. However, in liver, the glycogen concentration showed significant reductions at 24°C and 28°C in hypoxia (Tab. 1). The lactate concentration in blood plasma did not differ between treatments (Tab. 1).

In white muscle and heart tissues, the activity of the enzyme MDH did not differ among the different conditions of oxygen and temperatures (Figs. 1b,c, respectively; Tab. 2). However, its activity in the liver showed a significant reduction in hypoxia but without a difference between the temperatures (Fig. 1a; Tab. 2).

LDH enzyme activity did not differ between the different conditions of oxygen and temperatures in the liver and heart tissues (Figs. 2a,c, respectively; Tab. 2). In muscle, however, there was a significant difference in the activity of this enzyme at 28°C, where the normoxic condition showed greater activity than hypoxia (Fig. 2b; Tab. 2).

The CS enzyme activity did not show difference between treatments in liver and muscle tissues (Figs. 3a,b, respectively; Tab. 2). In the heart, there was a significant increase in the activity at 24°C and 28°C in normoxia (Fig. 3c; Tab. 2).

The ratio between the activities of the enzymes MDH/LDH and CS/LDH was performed. As the ratio of enzymes MDH/LDH, the muscle showed an increase in hypoxia at 28°C. In liver and heart, this increase appeared at the temperatures 24°C and 28°C in hypoxia. Referring to ratio between the enzymes CS/LDH, no difference was observed in muscle. However, in the liver an increase was shown at 24°C and 28°C in hypoxia, unlike the heart that showed a reduction at these temperatures and an increase at 20°C in the same oxygen concentration (Tab. 3).

Tab. 1. Two-way ANOVA of liver glycogen and blood lactate in *Geophagus brasiliensis* exposed to 20°C, 24°C and 28°C in normoxia (90% oxygen saturation) and hypoxia (20% oxygen saturation) conditions for 8 hours.

Condition	Temperature (ºC)		
	20°C	24°C	28°C
Liver Glycogen			
Normoxia	587.1±101.9[a]	846.6±192.0[b]	853.7±88.6[b]
Hypoxia	715.8±173.2[a]	559.7±220.1[*a]	427.7±177.7[*b]
Plasma Lactate			
Normoxia	0.98 ± 0.76	0.62 ± 0.61	0.54 ± 0.18
Hypoxia	0.86 ± 0.89	0.77 ± 0.65	0.73 ± 0.36
Two-Way ANOVA Glycogen			
Source of Variation	df	F	*p*
Temperature	2	0.652	0.526
Condition	1	16.558	<0.001
Temperature X Condition	2	12.123	<0.001
Two-Way ANOVA Lactate			
Source of Variation	df	F	*p*
Temperature	2	0.924	0.405
Condition	1	0.314	0.578
Temperature X Condition	2	0.612	0.547

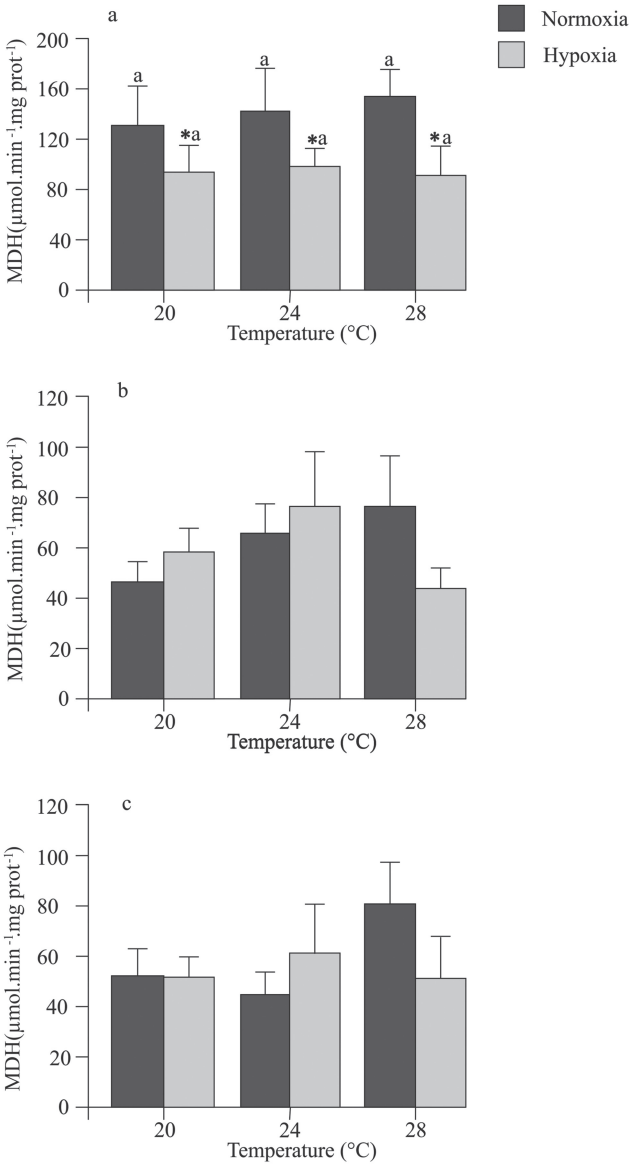

Fig. 1. Malate Dehydrogenase enzyme activity of *Geophagus brasiliensis* exposed to normoxic (90% oxygen saturation) and hypoxia (20% oxygen saturation) conditions for 8 hours at 20°C, 24°C and 28°C. **a.** in liver; **b.** in white muscle; and **c.** in heart. Asterisks indicates significant differences between treatments at the same temperature, $p < 0.05$. Different lowercase letters indicate significant differences for the same treatment at the temperatures studied, $p < 0.05$.

Tab. 2. Two-way ANOVA of Malate Dehydrogenase (MDH), Lactate Dehydrogenase (LDH) and Cytrate Synthase (CS) activity in *Geophagus brasiliensis* exposed to 20°C, 24°C and 28°C in normoxia (90% oxygen saturation) and hypoxia (20% oxygen saturation) conditions for 8 hours.

Source of Variation	df	F	*p*
Malate Dhydrogenase (MDH)			
Liver			
Temperature	2	0.075	0.928
Condition	1	5.687	0.022
Temperature X Condition	2	0.264	0.769
Muscle			
Temperature	2	0.810	0.451
Condition	1	0.198	0.659
Temperature X Condition	2	2.065	0.140
Heart			
Temperature	2	0.462	0.633
Condition	1	0.455	0.504
Temperature X Condition	2	1.009	0.374
Lactate Dehydrogense (LDH)			
Liver			
Temperature	2	0.296	0.746
Condition	1	1.437	0.239
Temperature X Condition	2	1.154	0.327
Muscle			
Temperature	2	3.170	0.055
Condition	1	4.509	0.041
Temperature X Condition	2	3.544	0.040
Heart			
Temperature	2	0.974	0,388
Condition	1	1.890	0.179
Temperature X Condition	2	1.601	0.217
Citrate Synthase (CS)			
Liver			
Temperature	2	0.420	0.660
Condition	1	1.055	0.310
Temperature X Condition	2	0.018	0.982
Muscle			
Temperature	2	0.0888	0.915
Condition	1	0.0050	0.944
Temperature X Condition	2	0.949	0.395
Heart			
Temperature	2	2.583	0.089
Condition	1	24.646	<0.001
Temperature X Condition	2	5.343	0.009

Discussion

Factors such as temperature and oxygen availability may not be stressful when isolated, but the interaction between these parameters can cause a stressful situation and cause serious impacts on fish (McBryan *et al*., 2013). In normal oxygen conditions where energy demand is low, carbohydrate is stored mainly in the form of glycogen in the liver. In hypoxic conditions and the consequently greater energy requirements, glycogen is rapidly used for the maintenance of basal functions and to supply anaerobic metabolism, thus reducing its concentration in tissues (Chippari-Gomes *et al*., 2005). At lower temperatures fish may improve their hypoxia tolerance because of the reduced energy consumption and high concentration of glycogen (Yang *et al*., 2015). The significant reduction of glycogen in the liver under hypoxic conditions at highest temperatures (24°C and 28°C) corroborates this hypothesis.

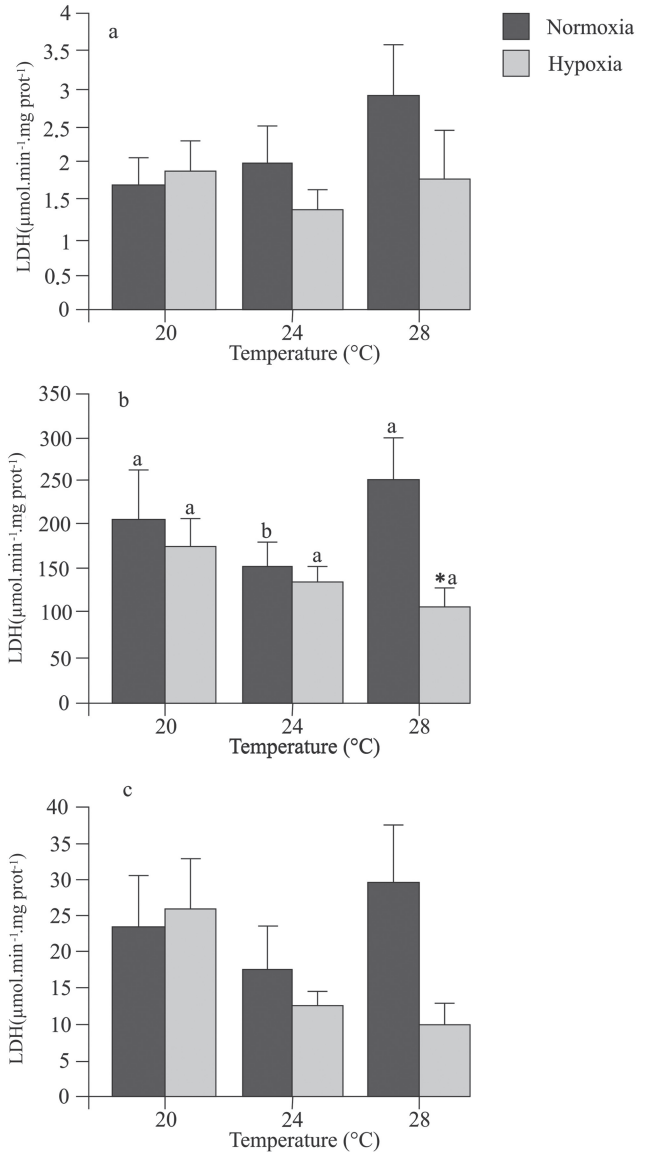

Fig. 2. Lactate Dehydrogenase enzyme activity of *Geophagus brasiliensis* exposed to normoxic (90% oxygen saturation) and hypoxic (20% oxygen saturation) conditions for 8 hours at 20°C, 24°C and 28°C. **a.** in liver; **b.** in white muscle; and **c.** in heart. Asterisks indicates significant differences between treatments at the same temperature, $p < 0.05$. Different lowercase letters indicate significant differences for the same treatment at the temperatures studied, $p < 0.05$.

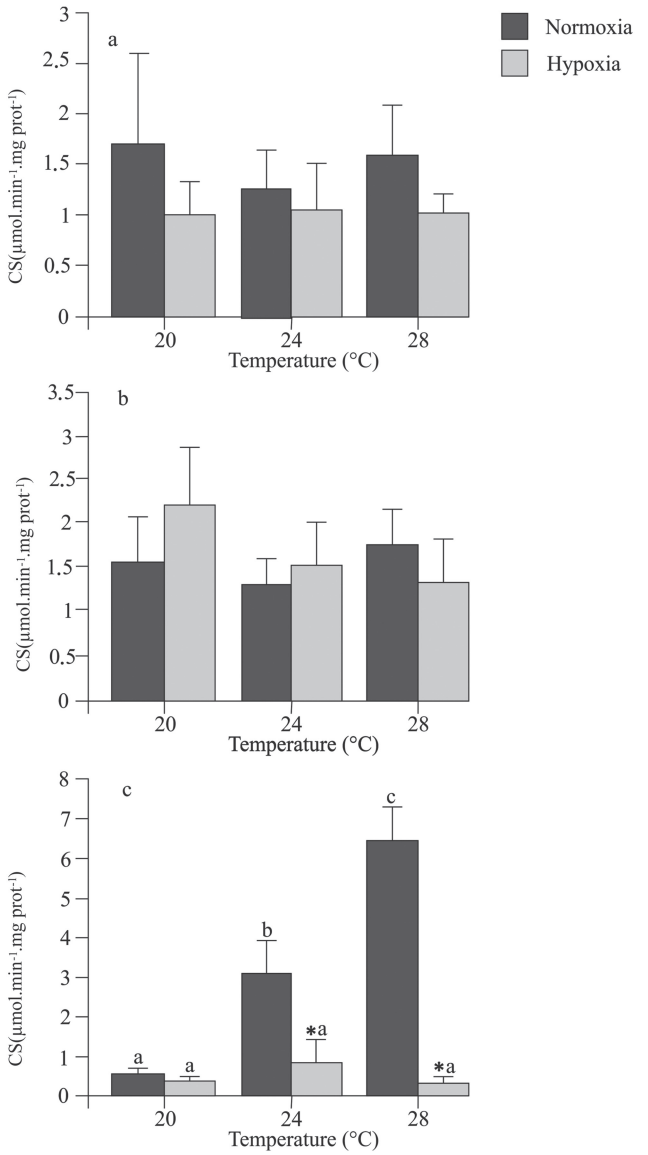

Fig. 3. Citrate Synthase enzyme activity of *Geophagus brasiliensis* exposed to normoxic (90% oxygen saturation) and hypoxic (20% oxygen saturation) conditions for 8 hours at 20°C, 24°C and 28°C. **a.** in liver; **b.** in white muscle; and **c.** in heart. Asterisks indicates significant differences between treatments at the same temperature, $p < 0.05$. Different lowercase letters indicate significant differences for the same treatment at the temperatures studied, $p < 0.05$.

Tab. 3. Enzimatic ratios in liver, muscle and heart in *Geophagus brasiliensis* exposed to 20°C, 24°C and 28°C in normoxia (90% oxygen saturation) and hypoxia (20% oxygen saturation) conditions for 8 hours. Temp. = Temperature; Cond. = Condition.

Temp. (°C)	Cond.	Liver		Muscle		Heart	
		MDH/LDH	CS/LDH	MDH/LDH	CS/LDH	MDH/LDH	CS/LDH
20	Normoxia	59.71	0.93	0.24	0.01	2.60	0.02
	Hypoxia	48.21	0.50	0.29	0.01	1.95	0.14
24	Normoxia	67.42	0.61	0.62	0.01	3.83	0.25
	Hypoxia	104.44	1.12	0.41	0.01	5.76	0.07
28	Normoxia	61.11	0.63	0.23	0.01	2.81	0.22
	Hypoxia	83.69	1.32	0.41	0.01	8.19	0.05

The lactate accumulation is usually response to hypoxia and indicate the use of anaerobic metabolism, since this is the final product of this metabolic pathway. However, some fish species like *G. brasiliensis* when in oxygen deprivation periods choose not to use this pathway, which seems to be an important metabolic adjustment to prevent the acidosis caused by the lactate accumulation (Omlin, Weber, 2010), and the ability to reduce acidosis by the reduction of energy demand by metabolic suppression has been showed by tolerant species (Jackson, 2004).

The significant reduction of MDH enzyme activity in the liver when in hypoxic condition indicates the ability of metabolic suppression of this species is a way of saving energy, and so it consigns it to higher energy requirement organs such as the heart. Kumar, Gopesh (2015) found a similar result, they also observed the reduction of MDH activity in the liver of *Clarias batrachus* in hypoxia condition. Additionally, because this is an enzyme that participates in the production of glucose (gluconeogenesis) and the liver is an important organ in this process, the reduction of MDH activity in this tissue may indicate a reduction in glycogen storage, which corroborates with the reduction in its reserve under the influence of hypoxia observed in this study.

LDH is a major enzyme in the activation of anaerobic metabolism. Environmental anaerobiosis, stimulated by changes in environmental conditions such as oxygen, occurs gradually and does not require a rapid activation of anaerobic metabolism. Organisms that are more tolerant to low oxygen concentration save energy by reducing the demand for ATP combined with the deletion of certain physiological functions. Thus, these organisms tend to have a preference to tolerate situations of low oxygen supply, hoping this will be available again, and to develop anaerobic pathways that yield more ATP in relation to the fermentation of lactate. These pathways final release products such as ethanol and acetate, unlike lactate, can be excreted easily preventing tissue acidosis (Hochachka *et al.*, 1993).

As the major determinants of survival of fish in hypoxia are metabolic suppression, a large stock of glycogen in tissues and the ability to avoid acidosis (Lutz, Nilsson, 1997), the absence of increased LDH activity in the liver in hypoxic condition together with the glycogen storage mobilization in this condition indicates the ability of *G. brasiliensis* to prevent acidosis caused by lactate accumulation in the liver.

In muscle, however, LDH showed a significant increase in its activity in normoxia at 28°C, like Campos *et al.* (2017) observed for *Paracheirodon axelrodi* and *Paracheirodon simulans* at high temperatures, under normal oxygen conditions, demonstrating that the muscle works anaerobically. At higher temperatures, the production of ATP by aerobic pathways is reduced, although under normal oxygen conditions, which reflect a higher energy cost for the maintenance of homeostasis, the use of anaerobic metabolism is required (Pörtner, 2004). Thus, the major energy source in the white muscle of *G. brasiliensis*, observed by the expected increase in LDH activity because this is essentially anaerobic tissue, indicates an adaptive ability of the species to survive at higher temperatures and the important role of LDH enzyme in energy support of this tissue during periods when more energy is required (Hochachka, Somero, 2002). The activity of this enzyme showed a reduction in the same temperature under hypoxic condition in this study. The ability of muscle to maintain its function in hypoxic conditions by using the anaerobic metabolism, although this glycolytic pathway is reduced, has been observed in previous studies (Almeida-Val *et al.*, 1999).

When exposing individuals of *Clarias batachus* to hypoxia conditions Kumar, Gopesh (2015) observed that after 24 hours LDH activity increased in the heart and after 48 hours decreased to almost normal condition. Differing from the results obtained in the present study, where we did not find significant differences in the LDH activity in the heart, under normoxia or hypoxia.

The increase in the activity of the CS enzyme in the heart at 24°C and 28°C in normoxia indicates the extreme dependence on aerobic metabolism of this tissue. However, the significant reduction of its activity under the influence of hypoxia suggests the realization of metabolic suppression as a more advantageous option in relation to the activation of anaerobic metabolism during periods of low oxygen availability.

The increase in the ratio between the enzymes CS/LDH and especially MDH/LDH in the liver in hypoxic conditions at 24°C and 28°C, shows that this tissue potentiates the aerobic metabolism during low oxygen concentrations, indicating that this should be a strategy to avoid the accumulation of end products of anaerobic metabolism, such as lactic acid. Saavedra *et al*. (2016) also found high values for the MDH/LDH ratio, studying heart and liver from species of deep sea (*Beryx splendens* and *Hoplosthetus atlanticus*), where there are minimal oxygen conditions. The same pattern was observed in the heart of *G. brasiliensis* that showed an inhibition of anaerobic metabolism under hypoxia conditions in 24°C and 28°C, which also showed us that, this is an aerobic tissue, confirming the increase of CS activity at these same temperatures in normoxia.

It is important that more studies be done regarding metabolic changes resulting from global scale environmental changes, especially on temperature dependence in anaerobic metabolism (Sørensen *et al.*, 2014), so we can understand better how organisms adapt and what strategies they use in the face of new environmental conditions, since it is estimated that the temperature will increase from 1 to 7 ° C in the next 100 years (IPCC, 2014).

A change in the availability of dissolved oxygen in the water can generate serious impacts on the survival of some species and consequently on the diversity of species in a region, in the trophic relationships and in other ecological parameters (Ekau *et al*., 2010). The impact of hypoxic conditions on the environment may reduce local biodiversity, as well as environmental resilience and resistance (Ekau *et al*., 2010), and may influence the

nutrient cycling processes (Jennings, Wilson, 2009). The availability of oxygen also influences fish physiological parameters, such as growth, reproduction and food intake. The reduction in food intake is an important factor, since it consequently reduces the energy demand and the oxygen demand (Ekau *et al*., 2010).

Geophagus brasiliensis use the anaerobic metabolism as the main strategy for optimizing its strength in the absence of oxygen, because of mobilization of its energy reserves (*i.e.* LDH activity in white muscle). The synergistic effect of high temperature and low oxygen availability influences the mechanisms of the *G. brasiliensis*.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa e Inovação do Espírito Santo (FAPES). Levy Gomes is the recipient of a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors thank the Instituto Nacional da Mata Atlântica (INMA) for accepting the deposit of voucher specimen in Museu de Biologia Professor Mello Leitão.

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Submitted 25 May, 2017
Accepted 18 December, 2017 by Bernardo Baldisserotto