

Oxidative stress parameters in juvenile Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839) (Pleuronectiformes: Paralichthyidae) exposed to cold and heat shocks

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The aim of this study was to determine oxidative stress parameters in the liver and gill of Brazilian flounder juveniles (307.0 ± 16.0 g and 30.0 ± 4.0 cm) submitted to different water temperature (17.1, 23.0 and 28.8°C) for 72 h and maintained at salinity 25‰. After the acclimation of 7 days, in 23°C, fish were transferred to 200 L tanks containing seawater (salinity 25‰) at 28.8°C (heat shock), 17.1°C (cold shock) or 23.0°C (control), five replicates (five fish tank⁻¹). The sampled collection occurred in 0 (pre-challenge), 3, 24, 48 and 72 h after temperature shock. Flounder exposed to 17.1°C and 28.8°C showed significantly higher TBARS levels and GST activity in the liver post-exposition (PE) in relation to the control (23°C). CAT activity in liver present a significantly increase at 17.1°C, in first 48 h, and subsequently decrease in 72 h PE in relation to 28.8°C. The gills of flounder showed significantly higher TBARS levels, GST and CAT activity when submitted at 17.1 and 28.8°C in relation to 23.0°C. There were observed changes in lipid peroxidation levels (LPO), CAT and GST activities in the liver and gill of Brazilian flounder in response to reactive oxygen species (ROS) produced by thermal shocks.

O objetivo deste estudo foi determinar os parâmetros de estresse oxidativo no fígado e brânquias de juvenis de linguado (307,0 ± 16,0 g e 30,0 ± 4,0 cm) submetidos a diferentes temperaturas da água (17,1, 23,0 e 28,8°C) por 72 h e mantidos na salinidade de 25‰. Após uma aclimação de sete dias, em 23°C, os peixes foram transferidos para tanques de 200 L contendo água do mar (salinidade 25‰) em 28,8°C (choque quente), 17,1°C (choque frio) ou 23,0°C (controle), cinco repetições (cinco peixes/tanque). A coleta de amostras ocorreu em 0 (pré-exposição), 3, 24, 48 e 72 h após o choque térmico. O linguado exposto a 17,1°C e 28,8°C apresentaram um significativo aumento dos níveis de TBARS e atividade da GST no fígado pós-exposição (PE) em relação ao controle (23°C). A atividade da CAT no fígado apresentou um aumento significativo em 17,1°C, nas primeiras 48 h, e subsequente diminuição em 72 h PE em relação a 28,8°C. As brânquias do linguado apresentaram significativo aumento dos níveis de TBARS e atividade da GST e CAT quando submetidos a 17,1°C e 28,8°C em relação a 23,0°C. Foram observadas alterações nos níveis de peroxidação lipídica (LPO) e atividade de GST e CAT no fígado e brânquias de linguado em resposta as espécies reativas de oxigênio (ROS) produzidas pelo choque térmico.

Keywords: Catalase, GST, Lipid peroxidation, TBARS, Thermal shocks.

Introduction

Aquatic animals are constantly exposed to temperature variations in the natural environmental and culture farms in the State of Rio Grande do Sul, south of Brazil, due to seasonal thermal amplitudes. Temperature variations occur very frequently in estuarine environmental, where this species inhabits (Laguna dos Patos estuary, southern Brazil). In this habitat, temperature can vary from 10 to 30°C due to seasonal fluctuations or influence of climate

change (Garcia *et al.*, 2003). These climate alterations in recent years tend to contribute in changes which may result in injury for animal integrity, in addition the different species that inhabit the estuary can present alterations in cellular biomarkers. This includes gene and protein changes, metabolism, energy, immune, endocrine, neural and even behavioral changes that will first try to overcome that situation and then compensate for the imbalances produced by either the stressor or the consequences generated by their responses (Tort, 2011).

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The flounder *Paralichthys orbignyanus* (Valenciennes, 1839) occurs in estuarine and coastal waters from Rio de Janeiro (Brazil) to Mar del Plata (Argentina) (Figueiredo & Menezes, 2000). This species is an important item for local fisheries in the south Brazil, and presents all characteristics essential for aquaculture (Sampaio *et al.*, 2007). It is known for tolerance to a wide range of temperature (Wasielesky *et al.*, 1998) and salinity (Sampaio & Bianchini, 2002), nitrogenous compounds (Bianchini *et al.*, 1996), and acid stress (Wasielesky *et al.*, 1997).

In another species of flounder *Platichthys stellatus*, higher temperatures cause an increase in oxygen consumption. As a consequence, there is an increase of ventilation volume, cardiac output, and ventilation-reperfusion ratio (Watters & Smith, 1973). However, a sharp temperature increase or decrease can cause heat shock, thus stimulating numerous changes in different fish species. Changing temperature can lead to metabolic activation, which combined with an increase in oxygen consumption, initiates the oxidative stress process (Storey, 1996; Hermes-Lima, 2004). Cells subjected to heat shock can respond to it by an increase in the antioxidant defenses, particularly the antioxidant and associated enzymes (Hermes-Lima, 2004).

Oxidative stress is defined as an unbalanced state between pro-oxidants and antioxidants, resulting in elevated production of reactive oxygen species (ROS) and free radicals, agents potentially deleterious to the organism (Halliwell, 1992; Matés *et al.*, 1999; Halliwell & Gutteridge, 1999). This process can be induced by a large variety of conditions, including nutritional imbalance, exposure to chemical and physical environmental agents, strenuous physical activities, injury, and hereditary disorders (Chow, 1991). Imbalance between pro-oxidant and antioxidant levels can induce damage to DNA and RNA, inducing mutations. In addition, it causes oxidation of thiol groups (-SH) in enzymes and other proteins, therefore causing lipid peroxidation, which may induce changes in membrane permeability, loss of secretory function, and even cell death. Tissue damage and the subsequent release of pro oxidants results in a series of events at the intracellular level that leads to oxidative stress (Halliwell, 1992; Halliwell & Gutteridge, 1999), which can be detected through measurement of oxidative stress parameters.

This study is necessary due to changes that occur in the estuarine environment, with alterations in water temperature in a short period of time. These alterations may decrease the levels of dissolved oxygen in the water and consequently an unbalance in the ratio pro/antioxidants, featuring a state of oxidative stress. Therefore, the aim of this study was to determine oxidative stress parameters (CAT and GST) and lipid peroxidation in the liver and gill of juvenile Brazilian flounder exposed to heat and cold shocks.

Material and Methods

Experimental fish and acclimation. Sixty-five Brazilian flounder (307.0 ± 16.0 g and 30.0 ± 4.0 cm) produced and reared at the Aquaculture Marine Station of the Universidade Federal do Rio Grande (FURG) in south Brazil, were acclimated during 16 days in three tanks (500 L) equipped with recirculating aquaculture systems. The fish were fed twice daily with commercial diet (Supra Salmonídeos, 46% crude protein) until apparent satiation. Water temperature and salinity were maintained at $23.0 \pm 0.4^\circ\text{C}$ and 25‰, respectively.

Temperature exposure and tanks management. After the acclimation period under the control temperature (23°C), fish were randomly transferred to 200 L tanks containing seawater at 29°C (heat shock), 17°C (cold shock) or 23°C (control). Four tanks were used for each temperature and five fish were placed in each tank. Others five fish were placed in one tank, in the same acclimation conditions, which formed a new group (0 h or pre-challenge).

The maintenance of the lower experimental temperature (17°C) was performed adding PET bottles with ice inside the tanks, when necessary. The other experimental temperatures (23 and 29°C) were maintained by submerged heaters as described above. Once a day, 50% of the water volume of the tanks corresponding to 48 and 72 h was renewed with water storage in reservoirs kept under the same conditions of temperature and salinity of the experimental tanks. During the experimental period, temperatures remained 17.1 ± 0.1 , 23.0 ± 0.2 and $28.8 \pm 0.1^\circ\text{C}$ and salinity remained 25‰ for all treatments. Fish were not fed for about 24 h prior to challenge neither during the temperature exposure. Alkalinity (172.4 ± 5.9 mg $\text{CaCO}_3 \text{ L}^{-1}$) (Baumgarten *et al.*, 1996), total ammonia (1.38 ± 0.03 mg L^{-1}) (Unesco, 1983), nitrite (0.05 ± 0.02) (Bendschneider & Robinson, 1952), pH (8.2 ± 0.4) and dissolved oxygen (5.9 ± 0.8 mg L^{-1}) (multi-parameter YSI 556) remained in suitable conditions to the specie during all experimental period.

Tissues collection. Each challenge protocol (including the control treatment) was applied to five replicates (five fish tank⁻¹) where each tank accounted a sampling point: 3, 24, 48 and 72 h after temperature shock. Group pre-challenge (N = five fish) was sampled before the exposure to the temperature shock.

At each sampling point, fish were netted in a single pass with a large net to minimize sampling effort and stress associated with repeat netting. Fish were immediately sedated with benzocaine (50 ppm) (Henrifarma Produtos Químicos e Farmacêuticos LTDA, Brazil) and euthanized by single cranial pithing following tissues collection (liver and gills). After, the tissues were stored in a -80°C freezer for subsequent analysis.

Enzymatic assay. Liver and gill tissues were homogenized in 1.15% (w v⁻¹) KCl solution containing 1mM Phenylmethylsulphonyl-Fluoride (PMSF). Homogenates were centrifuged at 1000 g for 10 min to eliminate nuclei and cell debris and the supernatant fraction obtained was frozen at -80°C for further measurements (Azambuja *et al.*, 2011). Supernatants were used for analysis of thiobarbituric acid reactive substances (TBARS), catalase (CAT 1.11.1.6) and glutathione transferase (GST EC 2.5.1.18). Lipid peroxidation was measured by TBARS using methods described by Buege & Aust (1978). Briefly, absorbance measurements at 535 nm were used to measure the reaction between thiobarbituric acid and lipoperoxidation (LPO) products, resulting in the formation of a chromogen (Schiff's base). Results were reported as nmol mg⁻¹ protein. The protein content of homogenates was measured using methods described in Lowry *et al.* (1951), using bovine serum albumin (BAS) as the standard. CAT activity was determined by according to methods described by Boveris & Chance (1973), in which H₂O₂ loss is followed spectrophotometrically at 240 nm. Results were reported as pmol mg/protein. GST activity was determined spectrophotometrically at 340 nm using the method described in Habig *et al.* (1974). Activity was calculated by monitoring changes in absorbance at 340 nm using an extinction coefficient of 9.6 mmol cm⁻¹. One

unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1µmol of CDNB (1-chloro-2,4-dinitrobenzene) with GSH per min at 25°C.

Statistical analysis. Data are reported as means ± SEM (N=5). Homogeneity of variances among groups was tested with the Levene test. Data for TBARS, CAT and GST had homogeneous variances and comparisons between different treatments were made using two-way analysis of variance and the Dunnet test. Analysis was performed using Statistica 7.0 software and minimum significance levels were set at 0.05.

Results

Oxidative stress parameters from control fish did not show any significant differences at the time of collection and were therefore pooled and considered as 0 h or pre-challenge. Flounder exposed to 17.1°C showed higher TBARS levels in the liver (81.7%) compared to those fish kept at 28.8°C in the period of 3 h post-exposition (PE). The inverse occurs in the period of 24 h PE when the TBARS levels in the liver were higher (148.5%) in 28.8°C in relation to 17.1°C. TBARS levels in 48 h PE showed higher activity in fishes exposed to 17.1 (97.6 %) and 28.8°C (83.3%) compared to 23.0°C (Fig. 1a).

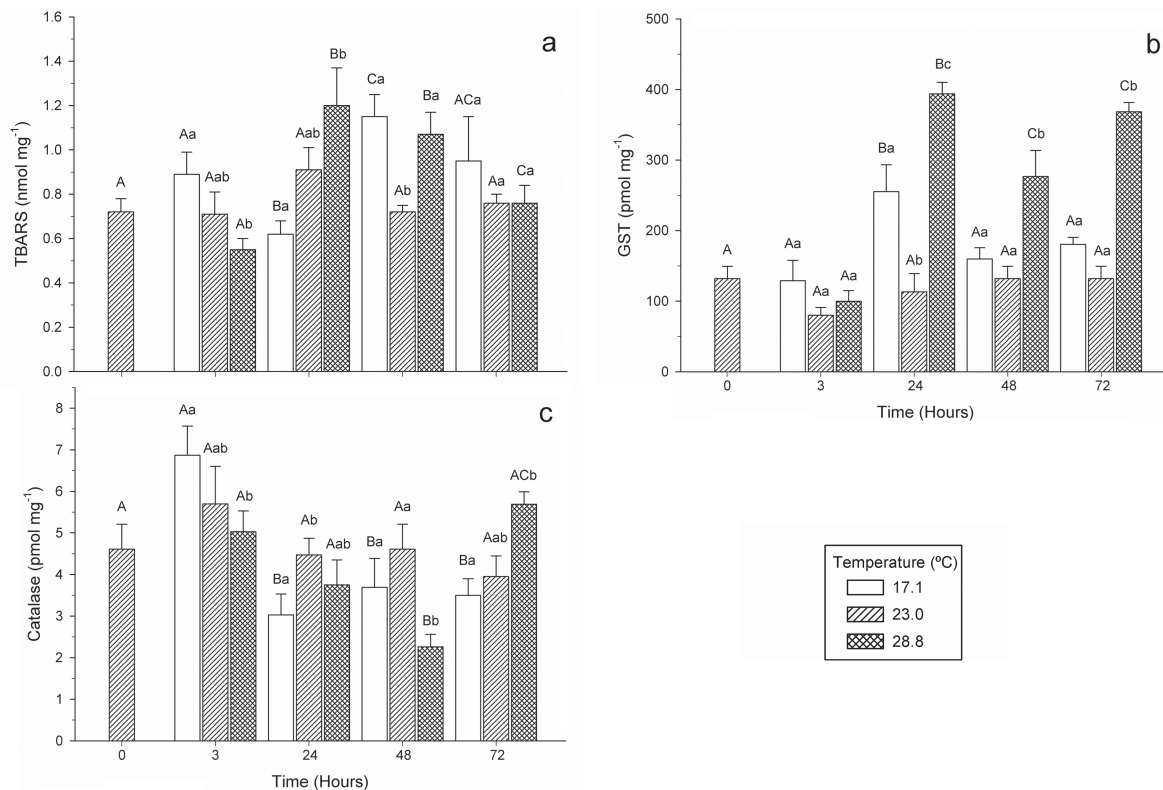


Fig. 1. (TBARS), (GST) and (CAT) activity in the liver of *Paralichthys orbignyanus* juveniles exposed to different temperatures (17.1, 23.0 and 28.8°C) as a function of time exposition (72 h). Values are expressed as means ± SEM, N=5. ^aLower case letters indicate significantly different at the different temperatures and same time ($P < 0.05$), determined by two-way ANOVA and by Dunnet test. ^ACapital letters indicate significantly different at the same temperatures and different times ($P < 0.05$), determined by two-way ANOVA and by Dunnet test.

TBARS activity in liver among all times in the same temperature of 17.1°C showed an increase, except in 24 h PE where there is a decrease (33%), in relation to the pre-challenge. This activity also demonstrates an increase in the periods of 24 and 48 h PE (124.7 and 92.7% respectively) in temperature of 28.8°C in relation to the pre-challenge and the same treatment and other periods.

In liver, GST activity was higher at 28.8°C in relation to 17.1 and 23.0°C in all experimental period PE, except in 3 h. GST activity in temperature of 17.1°C present an increase in 24 h PE in relation to 23.0°C. In 24 h PE, GST activity was higher in treatment of 17.1°C in relation to the control and same treatment in other times. This increase is also observed to 28.8°C in relation to the control and same temperature after 24 h PE (Fig. 1b).

Flounder exposed to 17.1°C present an increase CAT levels in liver in relation to 28.8°C at 3 and 48 h PE (62.5 and 80.7% respectively), and in the period of 24 and 72 h PE showed a decrease in this levels in relation to treatment of 23.0 (42.1%) and 28.8°C (57.3%), respectively. CAT activity showed a decrease in treatment of 17.1°C after 24 h in relation to the pre-challenge (66.0%) and same treatment in 3 h (221.4%). Flounder exposed to 28.8°C present a decrease in 48 h PE in relation to same treatments in the different times (Fig. 1c).

The gills of flounder showed higher TBARS activity at all experimental times when submitted at 17.1 (70.4, 54.6, 57.9 and 42.3%) and 28.8°C (69.1, 77.5, 61.3 and 43.2%) in relation to 23.0°C. There was also a higher in TBARS activity in temperature of 17.1 and 28.8°C, in the period of 3 h PE, when compared to the pre-challenge and same temperature over the time (Fig. 2a).

GST and CAT activity in gills of flounder fish showed a higher in the treatment with 17.1 and 28.8°C in relation to 23.0°C (3 h) respectively (Figs. 2b-c). There was also an increase in the enzymatic activity of GST in 17.1 (134.9%) and 23.0°C (122.8%) when compared to 28.8°C in 24 h (Fig. 2b). Inverse occurs in 23.0 (34.3%) and 28.8°C (47.6%) in relation to 17.1°C in 24 h when the CAT activity decrease (Fig. 2c). GST and CAT activity in 48 h presented a higher in 17.1°C in comparison to 23.0 and 28.8°C (Figs. 2b-c) and at the end (72 h) occurred an increase in GST activity in 17.1 (178.9%) and 23.0°C (139.4%) in relation to 28.8°C (Fig. 2b). In relation to the same temperature and different times, after 3 h GST and CAT activity showed a higher in the temperature of 17.1 and 28.8°C in comparison to the pre-challenge and the same treatment in 72 h (Figs. 2b-c). There is also an increase CAT activity in 28.8°C in the same treatment in the periods of 24 (78.6%), 48 (71.2%) and 72 h (54.8%) (Fig. 2c), in relation to 3 h.

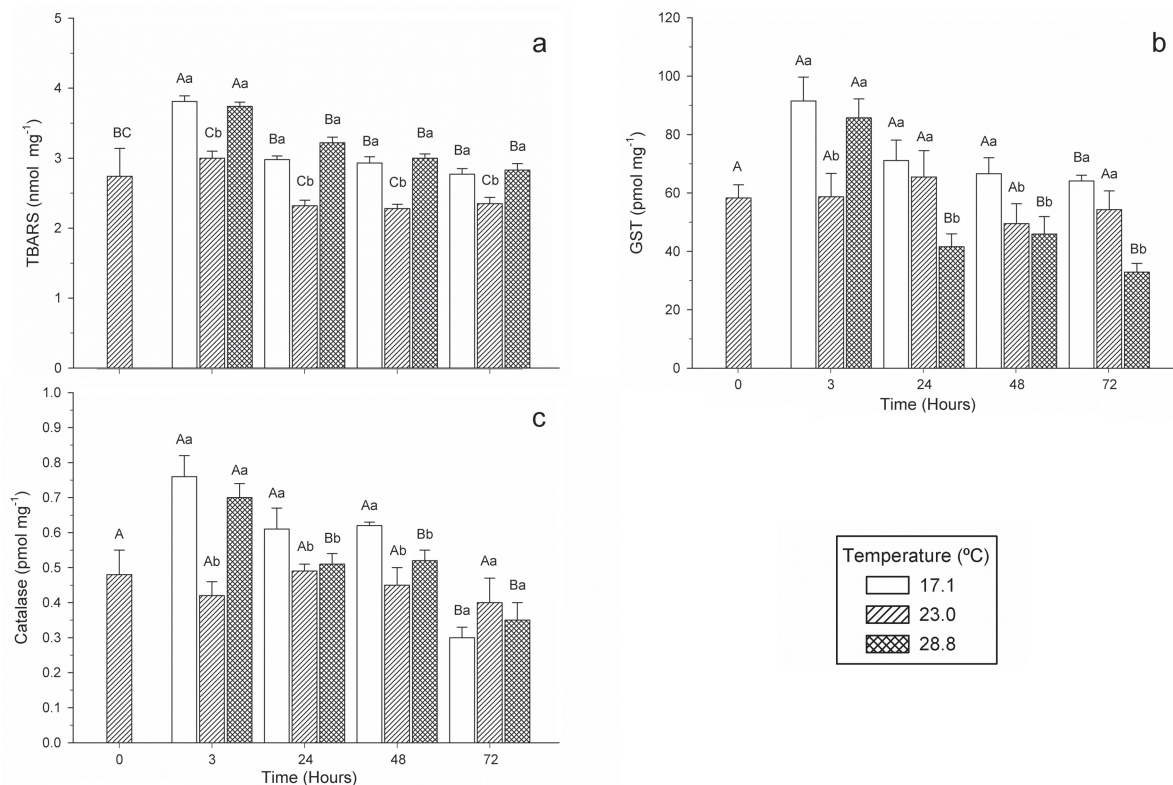


Fig. 2. (TBARS), (GST) and (CAT) activity in the gills of *Paralichthys orbignyanus* juveniles exposed to different temperatures (17.1, 23.0 and 28.8°C) as a function of time exposition (72 h). Values are expressed as means \pm SEM, N=5. ^aLower case letters indicate significantly different at the different temperatures and same time ($P < 0.05$), determined by two-way ANOVA and by Dunnett test. ^ACapital letters indicate significantly different at the same temperatures and different times ($P < 0.05$), determined by two-way ANOVA and by Dunnett test.

Discussion

Temperature is an important factor for aquatic animals which are dependents on this variable for their survival and development (Bhat & Desai, 1998), moreover great variations in this parameter cause alterations in the stress oxidative parameters (Malek *et al.*, 2004; Bagnyukova *et al.*, 2006, 2007a, 2007b). In our work, changes in LPO levels, CAT and GST activities in the liver and gill of flounder fish submitted to different temperatures were detected.

Activity of LPO parameters in Brazilian flounder exposed to water temperature of 17.1°C presented slight increase in the liver at 3 and 48 h when compared to 28.8 and 23.0°C (control), respectively. These levels also are demonstrated to 28.8°C in the period of 24 and 48 h in relation to the 17.1 and 23.0°C, respectively. This increase probably occurs due to heat shock in the first hours PE and to the end the levels decreased to near control values, demonstrating perhaps that an adaptation occurs over exposure time (72 h). In adult zebra fish (*Danio rerio*) and sea bass (*Dicentrarchus labrax*), submitted to temperature of 28 to 18°C and 28 or 18°C, respectively, also occur alterations in LPO of skeletal muscle causing oxidative stress (Malek *et al.*, 2004; Vinagre *et al.*, 2012). LPO levels also increase after heat shock (transfer of 19 to 32°C) exposure during 1 h in liver, muscle and brain of *Perccottus glenii*, but reverse to the control levels in lower temperatures (19°C) (Bagnyukova *et al.*, 2007a). Similar results were obtained with goldfish submitted to heat shocks (liver, kidney and brain), and in other work acclimated in 3°C and exposed to 23°C (liver) which LPO levels increase (Bagnyukova *et al.*, 2006, 2007b).

Brazilian flounder exposed to 17.1 and 28.8°C showed an increase in CAT activity in the gill and liver, respectively, in relation to exposed to 23.0°C (control), probably due to higher H₂O₂ production in this organ with result of cold and heat stress. This increase in the activity suggests that a scavenging ROS occurs by enzyme in cells and tissues helping to clear the peroxides accumulated under stress conditions. These alterations are also observed in white muscle of sea bass juveniles (*D. labrax*) which had an increase in the CAT levels when exposed to 18 and 28°C, outside thermal optimum range (Vinagre *et al.*, 2012).

In other study, goldfish submitted to acute transfer 3 to 23°C showed CAT levels slightly increased in the brain at period of 12 to 24 h (Bagnyukova *et al.*, 2007b). This increase in CAT activity generated by thermal stress can cause an enhancement of antioxidant potential including primary and associated antioxidant enzyme activities (Lushchak & Bagnyukova, 2006; Vinagre *et al.*, 2012).

This situation also occurs in animals with higher swimming activity also showed higher CAT activity values in the liver of rays and sharks compared to rested specimens, corresponding to the higher levels of oxygen consumption (Wilhelm Filho & Boveris, 1993). According to our and other studies (Wilhelm Filho & Boveris, 1993;

Lushchak & Bagnyukova, 2006; Wilhelm Filho *et al.*, 2001; Martínez-Álvarez *et al.*, 2005), the optimum level of water temperature is dependent for each species, age and life stage; moreover the temperatures outside the optimal range may induce the oxidative stress. These physiological variations were observed in CAT activity which occur due to hydrogen peroxide concentrations increase in tissues in attempt to reach homeostasis by degradation of this H₂O₂ in oxygen and water. Induction of this enzyme induces protection systems after exposure to ROS being an important adaptive response to non-lethal effects of H₂O₂ (Moslen, 1992; Tort *et al.*, 2005).

Here, the lower and higher temperature (17.1 and 28.8 °C) demonstrated increased GST activity in the gill and liver of juveniles in first 3 h in relation to the control. Higher GST activity may be explained by their anti-oxidant action which may promote a protective mechanism in different tissues (Zhang *et al.*, 2003; Lushchak & Bagnyukova, 2006). In other work can also be observed increase in GST activity in liver and white muscle of Nile tilapia juveniles exposed to 5 and 10 mg L⁻¹ total ammonia concentrations. Differently of our study in goldfish the GST activity was not affected with heat shock in brain and muscle, however, in liver occurred an increase after 24 h (Lushchak & Bagnyukova, 2006). The GST levels can be significantly increased by exposure to different environmental pollutants (chemicals or water quality parameters), suggesting that this increase is part of an adaptive response to stress and their action avoids damage to the biological systems.

These evidences allow us to conclude that *P. orbignianus* when submitted to cold and heat shocks present alterations in LPO levels and enzymatic activity in liver and gill, in response to ROS production by thermal shocks. This condition causes a more energy expenditure to attenuate ROS production (Souza *et al.*, 2014) and, as a result, lower animal growth in fish culture (Wei-Na *et al.*, 2006).

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