Influence of season and pollution on the antioxidant defenses of the cichlid fish acará (*Geophagus brasiliensis*)

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Abstract

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The livers of *Geophagus brasiliensis* collected from both a non-polluted site and a polluted site were analyzed for different antioxidant defenses, O_2 consumption, thiobarbituric acid-reactive substance (TBARS) levels, and histological damage. Compared to controls (116.6 ± 26.1 nmol g^{-1}), TBARS levels were enhanced at the polluted site (284.2 ± 25.6 nmol g^{-1}), as also was oxygen consumption (86.6 ± 11.3 and 128.5 ± 9.8 μmol O_2 min^{-1} g^{-1}, respectively). With respect to enzymatic antioxidants, increased catalase activities (8.7 ± 1.3 and 29.2 ± 2.4 mmol min^{-1} g^{-1}, respectively), unchanged superoxide dismutase activities (767.2 ± 113.3 and 563.3 ± 70.2 U g^{-1}, respectively), and diminished glutathione S-transferase activities (29.0 ± 3.2 and 14.9 ± 3.2 μmol min^{-1} g^{-1}, respectively) were detected. Reduced glutathione (1.91 ± 0.17 and 1.37 ± 0.25 mM, respectively), oxidized glutathione (1.50 ± 0.20 and 0.73 ± 0.17 mM, respectively), and total glutathione (3.40 ± 0.26 and 2.07 ± 0.27 mM, respectively) concentrations were also below control values at the polluted site. Nevertheless, the observed ethoxyresorufin-*O*-deethylase activities (1.34 ± 0.11 and 16.7 ± 0.21 pmol min^{-1} mg^{-1}, respectively) showed enhanced values at the polluted site. The main histological damage observed in the hepatocytes from fish collected at the polluted site was characterized by heavy lipid infiltration. Fish collected at the end of spring showed higher O_2 consumption, higher superoxide dismutase and glutathione S-transferase activities, and higher total and oxidized glutathione concentrations compared to the beginning of autumn. No seasonal changes were observed in catalase activities, glutathione or TBARS levels. Fish chronically exposed to relatively high pollution levels seem to be unable to set up adequate antioxidant defenses, probably due to severe injury to their hepatocytes. The higher antioxidant defenses found at the end of spring are probably related to the enhanced activities during high temperature periods in thermoconforming organisms.

Introduction

Qualitative data related to the evaluation of enzymatic antioxidants in fish such as superoxide dismutase (SOD) and catalase show that they are structurally and functionally very similar to those of mammals [1,2]. Nevertheless, fish enzyme specific activities are quantitatively lower than those of mammals [1-4]. Industrial and agricultural activities depend on the production and utilization of a variety of chemical and physical agents that continuously damage the natural environment. Much evidence indicates that xenobiotics can generate reactive oxygen species, including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen (O_2^1), which in turn are responsible for cell and tissue damage associated with different pathologic processes, including mutagenesis and carcinogenesis [5]. Recently, oxidative stress and some parameters of fish antioxidant defenses and biotransformation enzymes (i.e., cytochrome P-450 1A1 and glutathione S-transferase, GST) of fish and molluscs have been used as biomarkers of water pollution [6-11]. Although the seasonal interference with the antioxidant defense system has not been well established in these organisms, the data available in the literature [12-14] suggest a general antioxidant defense enhancement during spring and summer when compared to low temperature periods. Since most rivers are a sink of numerous spills, urban residues, pesticides and trace metals, the present study was carried out to verify the effect of seasonality and pollution on the antioxidant defense system and the biotransformation system of the acará, *Geophagus brasiliensis*, caught at a polluted site (Benedito River) compared to fish caught at a non-polluted site (Ratones), southern Brazil.

Material and Methods

Adult male specimens weighing 15 to 40 g (N = 28) were collected from a non-polluted pond at Ratones, a private fish farm located far away from the influence of urban areas, on Santa Catarina Island (27° 34' 14"-27° 35' 31" S; 48° 30' 07"-48° 31' 33" W), and the same number (N = 28) of specimens was collected from a polluted area (Benedito River), located near the town of Indaial, in the northern part of the State of Santa Catarina, southern Brazil. For the investigation of seasonal variations, fish (N = 40) were collected only at the non-polluted site Ratones. Twenty adult males were sampled during the month of November, corresponding to the end of spring in the southern hemisphere, and 20 specimens were sampled during the month of April, corresponding to the beginning of autumn.

After biometry, the livers of the animals were carefully excised and rapidly weighed, and tissue oxygen consumption was measured in a thermostated (25 ± 1°C) Tucker chamber containing a Clark electrode, where minute tissue slices were kept in Ringer solution for teleosts, containing 15 mM glucose (final concentration). The homogenates were obtained in 20 mM Na-phosphate buffer, pH 7.4, containing 0.1% Triton X-100 and 0.15 M sodium chloride. Homogeneizations were carried out at 4°C using 15 strokes in a Potter-Elvehjem homogenizer, followed by centrifugation at 5000 *g* for 10 min at 4°C. The supernatants were used for evaluation of antioxidants and thiobarbituric acid-reactive substance (TBARS) contents. Aliquots of the corresponding extracts were stored in liquid nitrogen (-170°C) and examined separately for each enzyme.

The different parameters were analyzed spectrophotometrically according to the following procedures: SOD at 550 nm by the reduction of cytochrome *c* promoted by the superoxide anion generated by the xanthine/xanthine oxidase system [15], and catalase at 240 nm by the decay of hydrogen peroxide levels [16]. GST was measured at 340 nm using 1-chloro-2,4-dinitrobenzene as sub-

strate (17); reduced glutathione was determined by the DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)] method at 412 nm (18); total glutathione and oxidized glutathione were determined by the enzymatic method at 412 nm (19); lipid oxidation was measured at 535 nm by the TBARS method (20,21).

Ethoxyresorufin-*O*-deethylase (EROD) activity, which is associated with the expression of cytochrome P-450 1A, was estimated by direct fluorimetric detection of the 7-hydroxyresorufin metabolite, essentially as reported by Prough et al. (22). Microsomes were prepared by the calcium aggregation method and differential centrifugation (23), and protein content was determined by the method of Lowry et al. (24).

Histopathological analysis was carried out by light microscopy on liver slices fixed in 10% buffered Bouin and stained with hematoxylin-eosin.

Statistical analysis was performed using the Student *t*-test with a confidence interval of 5% (P<0.05).

Results and Discussion

Effect of pollution

Oxygen consumption and TBARS levels were significantly increased at the polluted site compared to control (Figure 1A,B). An increase in metabolic rate is typically associated with acute and chronic stress in fish (25), and a TBARS increase in liver of fish chronically exposed to pollutants has also been well documented in the literature (e.g., 7,26). The only antioxidant defense enhancement observed in fish from the polluted area was the increase in catalase activity (Figure 1C), the same response as that found in the mullet (27), but opposite to that found in the Nile tilapia (28). On the other hand, liver SOD activity of fish caught in the polluted area exhibited lower values than fish collected at the reference site (Figure 1D). A similar pattern was observed for GST activ-

ity and reduced glutathione concentrations, with both parameters being significantly decreased in relation to controls (Figure 2A,B). The induction of liver catalase activity in the acará exposed to pollutants may reflect an increase in endogenous hydrogen peroxide levels, despite the fact that the tendency towards diminished SOD activity would yield less hydrogen peroxide. Since hydrogen peroxide elimination through the gills occurs in the acará (data not shown), probably consisting of a widespread mechanism in water-breathing organisms (29), and considering that this elimination accompanies the increase in oxygen consumption and therefore the oxyradical generation, the interpretation of this increase in liver catalase activity becomes rather difficult. Nevertheless, although reduced glutathione peroxidase (25,30) and glutathione reductase (25) activities were found in fish exposed to environmental pollution, both enzymes in general showed induced activities in the liver (7,28,31). These
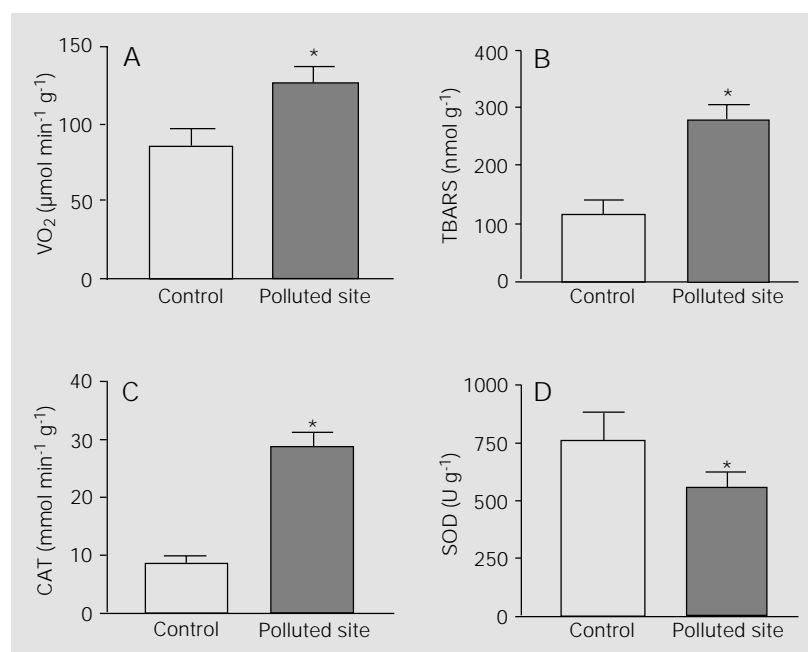

Figure 1. Comparison between different parameters measured in the liver of Geophagus brasiliensis collected at the polluted and at a reference site (control). A, Oxygen consumption (VO$_2$, µmol min^{-1} g^{-1}); B, cellular damage (TBARS, nmol g^{-1}); C, catalase activities (CAT, mmol min^{-1} g^{-1}); D, superoxide dismutase activities (SOD, U g^{-1}). *P<0.05 compared to control (Student t-test).

findings emphasize the need for healthy hepatocytes to detoxify peroxides, irrespective of the induction of catalase and/or glutathione peroxidase, and the importance of keeping constitutively high levels of reduced glutathione.

The concentrations of total and reduced glutathione found in the liver of *Geophagus* were similar to those usually found in other fish species exposed to pollutants (26-28,32). Reduced glutathione and TBARS/malondialdehyde concentrations in the liver of acará showed an inverse correlation, and the same relationship was found in other fish (32).

Acute and subchronic exposure to pollutants seems to increase reduced glutathione contents in fish cells (26,32-34) while fish submitted to heavy and extensive chronic exposure seem to be unable to maintain high constitutive glutathione and total glutathione levels (11,28; Wilhelm Filho D, Torres MA, Testa CP and Tribess TB, unpublished results; present study). The decrease observed both in reduced and oxidized glutathione concentrations may be attributable to the decrease in total glutathione.

The apparent absence of SOD and GST induction and the low total glutathione levels (Figures 1D and 2A,C), together with the histological damage, may be attributable to the extension of the lesions found in the liver. In this regard, fish collected at the polluted site showed drastic lipid infiltration characterized by foamy structures in the adipocytes (Figure 3B). Nevertheless, no significant differences in micronucleus incidence were detected in *Geophagus* sampled at the polluted site in comparison to fish sampled in pristine upstream waters of the same river (35). Although some studies have shown induction of SOD (27,28) and other antioxidants (27) or biotransformation enzymes (27,28,31) under pollution exposure, unaltered or lowered SOD activities have been also found in other fish species (7,32). GST, together with EROD activity, is usually enhanced after exposure to different types of organic pollutants, both in laboratory studies (e.g., 31,34) and in field studies (27,31). They represent a complex system related to xenobiotic biotransformation (phase I) and conjugation and excretion (phase II), consisting of prospective biochemical markers of environmental contamination by organic compounds such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls (36). Surprisingly, EROD activities showed enhanced values at the polluted site (16.7 ± 0.21 pmol min^{-1} mg^{-1}) in comparison to controls (1.34 ± 0.11 pmol min^{-1} mg^{-1}), while GST activities were lower
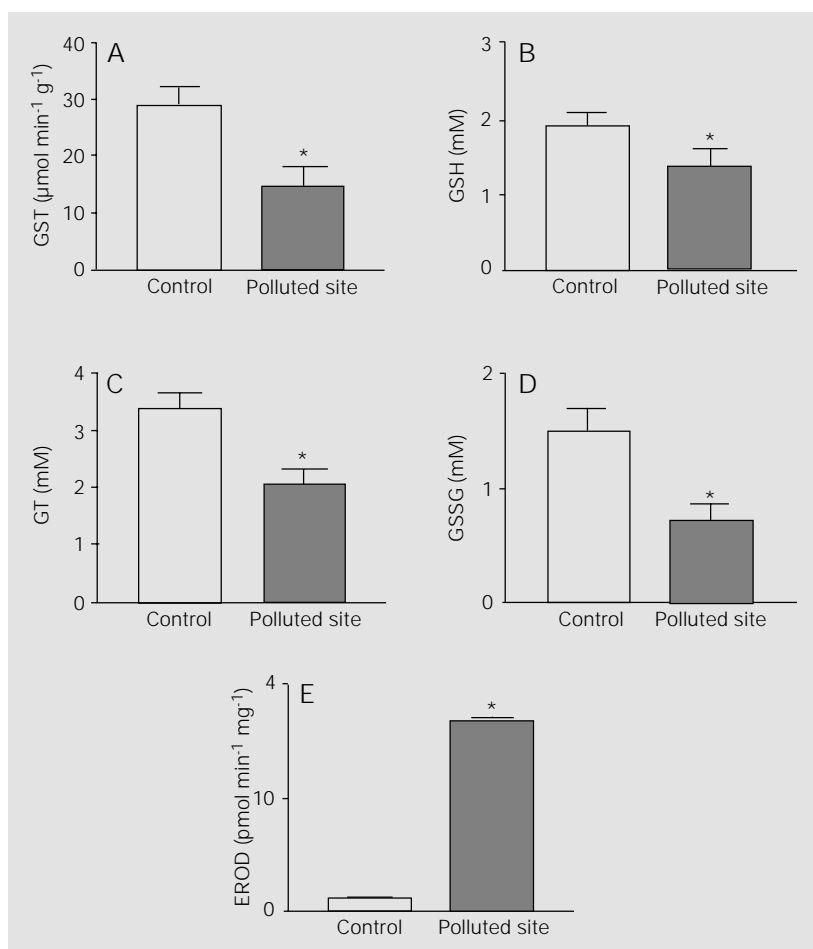

Figure 2. Comparison between different parameters measured in the liver of Geophagus brasiliensis collected at a reference site (control) and at a polluted site. A, Glutathione S-transferase activities (GST, μmol min^{-1} g^{-1}); B, reduced glutathione (GSH, mM); C, total glutathione (GT, mM); D, oxidized glutathione (GSSG, mM); E, ethoxyresorufin-O-deethylase activities (EROD, pmol min^{-1} mg^{-1}). *P<0.05 compared to control (Student t-test).

at the polluted site (14.9 ± 3.2 µmol min^{-1} g^{-1}) in comparison with the control group (29.0 ± 3.2 µmol min^{-1} g^{-1}). This suggests an uncoupled process between the phase I and phase II systems, reinforcing the above inference that fish from the polluted site are unable to set up adequate compensation, because the reactive metabolites generated in phase I will not be properly neutralized in phase II. If fish are not able to make further investments due to hepatic failure in such a system, at least considering the decreased GST activities, they probably also cannot adequately cope with an oxidative challenge. The Benedito River is characterized as a sink of a variety of pollutants over many years, and receives a complex mixture of domestic residues, different industrial effluents, and agricultural chemicals (37). Therefore, the fish sampled in this freshwater system were considered to have been long-term exposed to a heavily polluted environment. In animals experiencing intense chronic stress the stress response may lose its adaptive value and become dysfunctional (38). The above results suggest that protein synthesis in liver was probably generally affected in fish collected at the polluted site, and the observed lipid accumulation in the hepatocytes (Figure 3B) may be linked to the lack of sufficient protein transport of lipids in plasma (38). Accordingly, the histological damage was already evident when fish were dissected for liver excision. In this regard, some specimens revealed a high degree of organ fragility, and tissues were sometimes disrupted even if carefully manipulated, indicating severe tissue injury. Furthermore, the increase of the somatic index (percent organ weight compared to body weight) of liver from fish collected at the polluted site ($2.70 \pm 0.44\%$) compared to the index of fish collected at the reference site ($1.11 \pm 0.35\%$) also confirms histological damage provoked by pollution in fish (38). A study carried out in our laboratory on an estuarine catfish species also chronically exposed to heavy

anthropogenic influence revealed similar results (Wilhelm Filho D, Torres MA, Testa CP, Tribess TB, Pedrosa RC and Hostim-Silva M, unpublished results), also suggesting that highly polluted environments can jeopardize an adequate compensatory response of the liver.

Effect of season

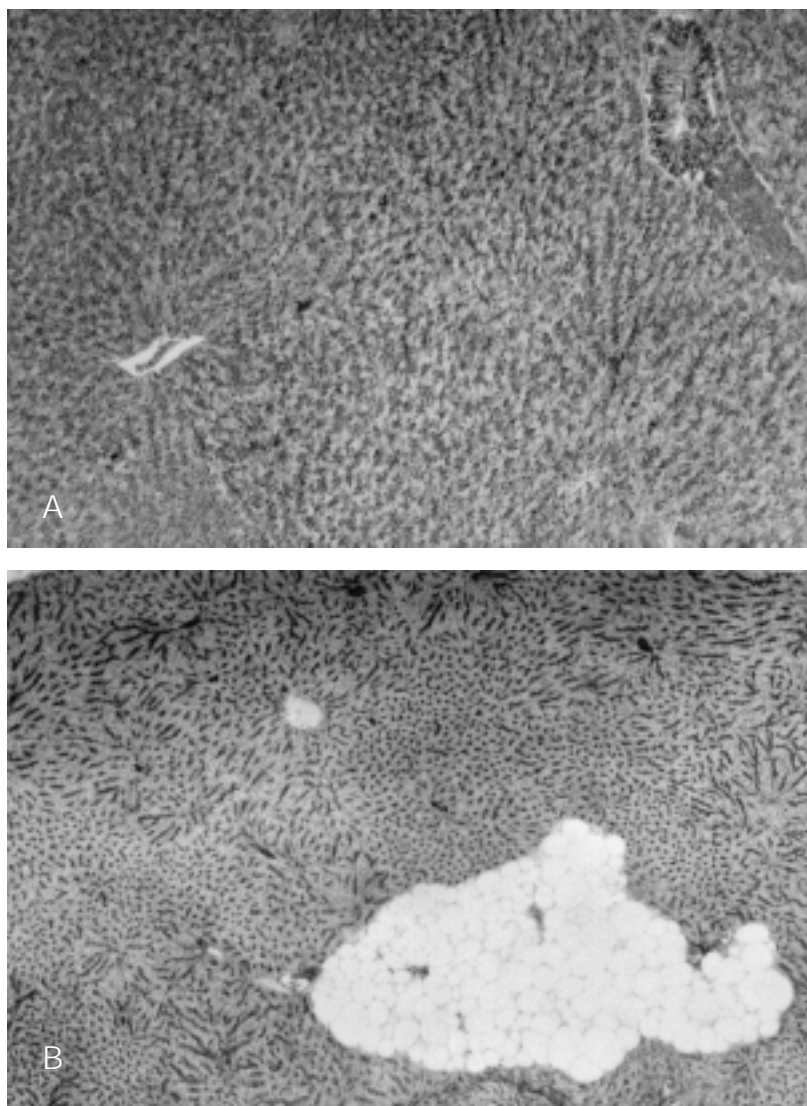Healthy fish collected at the non-polluted site during the high temperature period

Figure 3. Microscopic view of the liver of Geophagus brasiliensis stained with hematoxylin-eosin, collected at a reference site (A; magnification, 100X) and at the polluted site (Benedito River) (B; magnification, 40X). Lipid infiltration can be observed in B.
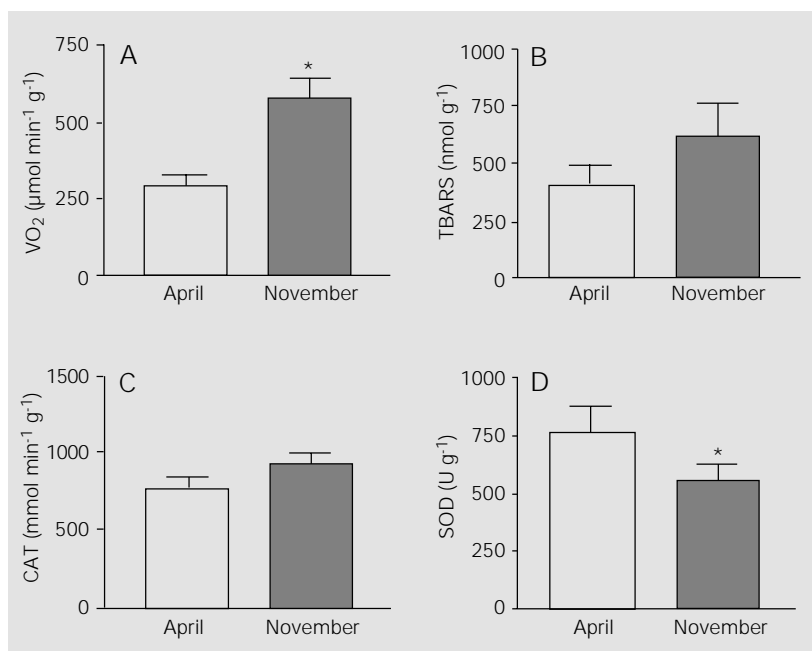
Figure 4. Seasonal pattern of different parameters measured in the liver of Geophagus brasiliensis. A, Oxygen consumption (VO$_2$, µmol min^{-1} g^{-1}); B, cellular damage (TBARS, nmol/g); C, catalase activities (CAT, mmol min^{-1} g^{-1}); D, superoxide dismutase activities (SOD, U g^{-1}). *P<0.05 compared to samples collected in April (Student t-test).
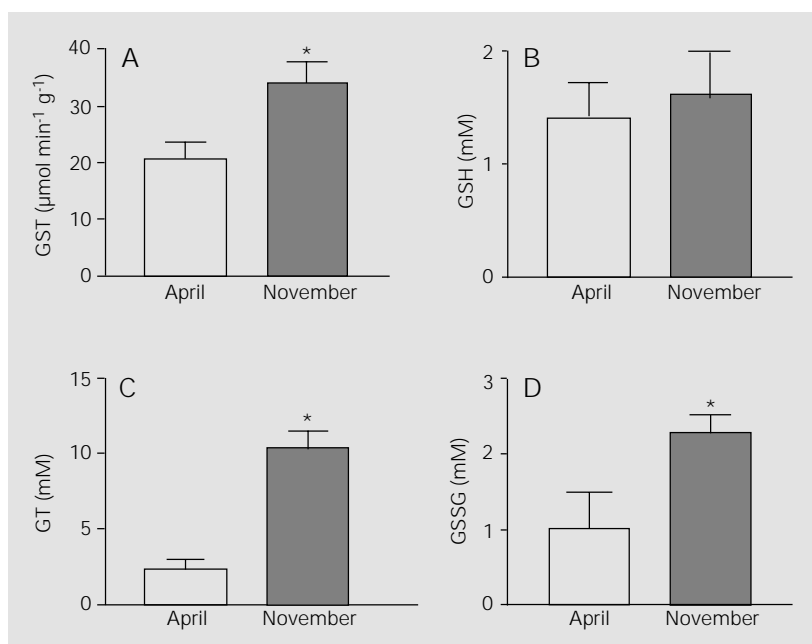

Figure 5. Seasonal pattern of different parameters measured in the liver of Geophagus brasiliensis. A, Glutathione S-transferase activities (GST, µmol min^{-1} g^{-1}); B, reduced glutathione (GSH, mM); C, total glutathione (GT, mM); D, oxidized glutathione (GSSG, mM). *P<0.05 compared to samples collected in April (Student t-test).

(November, spring) showed higher oxygen consumption, higher SOD and GST activity, and also higher total and oxidized glutathione concentrations in the liver (Figures 4D and 5A) compared to fish collected during the month of April (autumn). Although catalase activities and TBARS contents did not show any significant differences, the values of both parameters tended to increase in the warmer period (Figure 4B,C). Similar seasonal patterns, i.e., enhancement of antioxidants coinciding with high water temperatures, were verified in other fish (12-14; Wilhelm Filho D, Fraga CG and Boveris A, unpublished results), and also in a bivalve mollusc (39).

Total and oxidized glutathione were higher during the warm period compared to the cold period (Figure 5C,D). The enhancement of oxidized glutathione levels shown by fish collected during the warm period coincided with the increase in oxygen consumption and might represent a situation of oxidative stress. This increase is probably related to the higher ambient water temperature and therefore to the oxygen consumption and reactive oxygen species generation during the warm period. Seasonal adjustments in the antioxidant defense of thermoconformers like most fish and invertebrates suggest that this mechanism is a common adaptation in thermoconformer vertebrates and invertebrates. In a recent study (40), such a correlation between antioxidant defenses and water temperature was not found in the eelpout Zoarces viviparus. Nevertheless, viviparity implies extensive anabolic investments, and the eelpout seems to be unable to sustain a high antioxidant status from spawning to parturition. With the exception of glutathione reductase, all the other antioxidants and biotransformation enzymes showed decreased activities during this period, the gonadosomatic index was inversely related, and the liver somatic index was directly related to the antioxidants (40).

The seasonal variation observed here in

phase II conjugation-excretion activities of GST was also observed in a mussel species (39), but contrasts with the seasonal independence of this biomarker in another mussel species (41). Conversely, phase I activities associated with cytochrome P-450 1A displayed enhanced values in estuarine catfish sampled in the winter in comparison with animals sampled during summer (Geremias R, Curi-Pedrosa R, Wilhelm Filho D, Hostim-Silva M, Figna V and Locatelli C, unpublished results). The persistence of a xenobiotic compound depends on many features, including seasonality and reproductive cycle, its chemical properties, specific rates of bioconcentration and biotransformation, and also specific excretory mechanisms (42).

Specific tissues and organs can display different antioxidant defense status (4) depending on the organism and the circumstances involved (habit, habitat, age, thermoregulatory capacity, metabolic rate and nutritional status, among others). These assumptions are determinant if we consider that, even though the different antioxidant defenses are unevenly distributed in tissues and organs in quantitative terms and are located in subcellular compartments, they often act in a concerted way by synergism (5).

In conclusion, among other aspects, the seasonal influences need to be better understood in order to make further inferences regarding antioxidant defenses and biotransformation enzymes, especially in thermoconformers such as most fish and aquatic invertebrates living in non-polluted or polluted environments. Time of exposure and pollution levels seem to determine quantitatively the kind of response regarding biotransformation enzymes and antioxidants in fish, and this response depends therefore on the functional capacity of the organ and tissues involved. The use of biochemical indicators in environmental pollution studies at a lower organizational level such as in the present study is of high toxicological relevance (6), and oxidant-mediated responses are useful indices of environmental quality (25).

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