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Main features of the oxidative metabolism in gills and liver of *Odontesthes nigricans* Richardson (Pisces, Atherinopsidae)

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ABSTRACT

The aim of this work was to study comparatively the oxidative metabolism in gills and liver of a silverside, *Odontesthes nigricans*, in their natural environment, the Beagle Channel. Oxidative damage to lipids was evaluated by assessing TBARS and lipid radical content, in gills and liver. Gills showed a significantly higher degree of damage than liver. The content of α-tocopherol, β-carotene and catalase activity showed significantly higher values in the liver than in the gills. The ascorbyl radical ($A^•$) content showed no significant differences between gills and liver. The ascorbate (AH^-) content was 12 ± 2 and 159 ± 28 nmol/mg FW in gills and liver, respectively. Oxidative metabolism at the hydrophilic level was assessed as the ratio $A^•/AH^-$. The ratio $A^•/AH^-$ was significantly different between organs, $(6 \pm 2)10^{-5}$ and $(5 \pm 2)10^{-6}$, for the gills and the liver, respectively. Both, lipid radical content/α-tocopherol content and lipid radical content/β-carotene content ratios were significantly higher in gills as compared to the values recorded for the liver, suggesting an increased situation of oxidative stress condition in the lipid phase of the gills. Taken as a whole, the *O. nigricans* liver exhibited a better control of oxidative damage than the gills, allowing minimization of intracellular damage when exposed to environmental stressing conditions.

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1. Introduction

The generation of reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^•OH$), takes place continuously in living cells, mainly as a by-product of respiration (Halliwell and Gutteridge, 1989). Once produced, ROS may damage cellular components and tissues particularly targeting proteins, lipids and nucleic acids, often leading to cumulative organ injury (Lushchak and Bagnyukova, 2006). In this regard, peculiarities of membrane lipids in marine organisms, such as high contents of unsaturated fatty acids (Joseph, 1982), suggest a special pattern for lipid peroxidation and a complex system of antioxidants (Halliwell and Gutteridge, 1989). The lipid peroxidation, assessed as the content of thiobarbituric acid reactive substances (TBARS) (Oakes et al., 2004; Almroth et al., 2005; Lushchak and Bagnyukova, 2006, 2007; Bagnyukova et al., 2007), has been used in fishes as a biomarker of biochemical perturbations resulting from oxidative stress. This process can be understood as a situation derived either from an enhanced rate of ROS generation or from a diminished level of antioxidant defences. There is an increasing interest in the use of ascorbyl radical ($A^•$) content in biological tissues as an informative, non-invasive and natural indicator of oxidative stress (Roginsky and Stegmann, 1994) in the hydrophilic medium. However, these authors also showed that the $A^•$ steady-state concentration in tissues may be considered as an objective but not a universal indicator characterizing oxidative stress intensity, since the ascorbate (AH^-) oxidation rate depends on AH^- concentration, pH and non-disproportionation mechanisms of $A^•$ decay, but pointed out that it is very unlikely that any universal indicator of such a kind could be found. Lately, data obtained from different systems suggest that when AH^- content is affected by oxidative conditions, this effect should be considered and, thus the ratio $A^•/AH^-$ content seems to be the best general indicator of oxidative stress in the hydrophilic medium (Kozak et al., 1997; Gonzalez et al., 2008). Moreover, this ratio was successfully used to assess oxidative stress in diabetic patients (Courderot-Masuyer et al., 2000), myocardial ischemia–reperfusion injury (Vergely et al., 1998), renal failure (Clermont et al., 2000) and iron overload-dependent oxidative stress (Galleano et al., 2002). On the other hand, the balance free radical damage/antioxidant protection in the lipid phase is appropriately described by the ratio lipid radical/α-tocopherol content, and lipid radical/β-carotene content, depending on the nature of the main lipid soluble antioxidant in the studied system (Galleano et al., 2002).

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The antioxidant defences in fishes are similar to those exhibited by other vertebrates (Wilhelm Filho et al., 2000). However, the antioxidant defences of 22 species of teleosts from the Central Amazon which were analyzed by Wilhelm Filho and Marcon (1996), appeared to be qualitative and quantitative different from that observed in marine teleosts (Wilhelm Filho et al., 1993). Ansaldo et al. (2000) also found differences in the distribution of antioxidant defences in various tissues of red-blooded (Nototheniidae) and white-blooded (Channichthyidae) Antarctic fishes, which were correlated to their distinct physiological adaptations to transport and consumption of oxygen, with and without hemoglobin. The differences found in these studies may be an indication that environmental and/or evolutionary factors could be responsible for the distinct adaptation of these groups.

Among the native fish fauna of Tierra del Fuego, the silverside *Odontesthes nigricans* (Richardson, 1848) is of particular interest to the local community, since both recreational and artisanal fisheries are being developed upon this species. *Odontesthes nigricans* has been reported in the Atlantic Ocean from Orense (39ºS, Buenos Aires) up to Cabo de Hornos (56ºS) (Dyer, 2000). They can be found following rising tides along coastal waters and estuaries of the Atlantic Ocean and the Beagle Channel. In such environments, the animals are consistently subjected to varying ambient parameters and must cope with these fluctuations (Bagnyukova et al., 2007), in addition to naturally generated toxic products, such as ROS (Wilhelm Filho et al., 2005).

In fishes, the aerobic tissues e.g. the liver, possess high potential for ROS generation, which seems to be efficiently counterbalanced by powerful protective mechanisms to detoxify and repair damaged lipid and proteins (Lushchak and Bagnyukova, 2006). Ahmad et al. (2008) found that the gills responded earlier than the liver in terms of lipid peroxidation and alterations in antioxidant content or activity. The fish gill is a multifunctional organ performing vital functions, such as respiration, osmoregulation, acid–base balance and nitrogenous waste excretion (Evans et al., 2005). The filaments and lamellae of gills are covered by epithelial, pavement, chloride and mucous cells supported by a complex system of blood vessels. Hence, the gill epithelium provides an extensive surface of contact with the environment. The aim of this work was to study comparatively oxidative metabolism in gills and liver of *O. nigricans*, in order to detect differential pathways to control intracellular damage when exposed to environmental conditions. Lipid peroxidation was assessed as reactive species-dependent oxidative damage in lipids, and antioxidant defence was studied by assaying the activity of antioxidant capacity (superoxide dismutase and catalase) and the content of non-enzymatic antioxidants (α-tocopherol, β-carotene and ascorbate), along with general indexes of stress in the aqueous and lipid phases.

2. Materials and methods

2.1. Fish collection and processing

Fish (*Odontesthes nigricans*, Actinopterygii, Atherinopsidae, Atherinopsinae) were collected with seine net at Cambaceres Bay (54°52′S, 67°16′W) in October 2007. The seine (60 m long, 1.5 m high and 12 mm stretched mesh) covered c. 200 m, from 1 m depth to the shallower littoral zone. Fish were carried alive to the laboratory, held in a 500 L tank under field conditions, and sacrificed by a blow to the head around 24 h after being captured. Both total length (TL) and standard length (SL) of each individual were measured with a digital calliper (±0.1 mm) and total body mass (BM) was recorded to the nearest 0.01 g. Only adult fish ($n=5$; 183–208 mm TL; 38–48 g BM) were used in the present study. No differentiation was made with respect to either sex or reproductive stage. Organs (gills and liver) were dissected, weighed ($\pm 10^{-5}$g) and stored at −80 °C. Particularly, these organs were chosen because gills are the first organs which come in contact with the environment hence being a potential target for oxidative disruption and the liver because it usually exhibits the highest antioxidant levels in fish (Aksnes and Njaa, 1981; Wilhelm Filho et al., 1993).

2.2. Biochemical measurements

2.2.1. Quantification of lipid radicals by electron paramagnetic resonance (EPR)-spin trapping

Lipid radicals were detected by a spin trapping technique using N-t-butyl-α-phenyl nitrone (PBN). A 40 mM PBN stock solution was prepared in dimethyl sulfoxide (DMSO) immediately prior to use. The homogenates were prepared by adding liquid nitrogen frozen tissue (10 to 20 mg) to DMSO-PBN (stock solution). EPR spectra were obtained at room temperature using a Bruker spectrometer ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. EPR instrument settings for the spin trapping experiments were: microwave power, 20 mW; modulation amplitude, 1.194 G; time constant, 81.92 ms; scan numbers, 5; center fields, 3480 G; modulation frequency, 50 kHz; and receiver gain, $2 \cdot 10^4$ (Lai et al., 1986). Quantification was performed according to Kotake et al. (1996). There was not a significant increase in the EPR recorded signal over a 30 min period, and the addition of 50 µM desferoxamine (DF) to the reaction medium did not affect the signal.

2.2.2. Content of 2-thiobarbituric acid reactive substances (TBARS)

The homogenates of liver and gills, were treated with 30% (w/v) trichloroacetic acid and 50 mM potassium phosphate buffer (pH 7.0). After centrifugation, the content of TBARS was determined in the supernatant, according to Malanga et al. (2004).

2.2.3. Content of lipid soluble antioxidants

The content of α-tocopherol and β-carotene in homogenates from gills and liver was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glassy carbon working electrode at an applied oxidation potential of 0.6 V. Samples were extracted with 1 mL of ethanol and 4 mL of hexane. After centrifugation at 600*g* for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Extracts were dissolved in methanol/ethanol (1:1) and injected for HPLC analysis (Desai, 1984). D,L-α-tocopherol (Sigma) and β-carotene (Sigma) were used as standards.

2.2.4. Enzyme assays

Homogenates from gills and liver were prepared in 50 mM potassium phosphate–120 mM KCl (pH = 7.4). Total SOD activity (EC 1.15.1.1) was determined according to Misra and Fridovich (1972). Since KCN inhibits Cu, Zn-SOD, but not Mn-SOD activity, to differentiate between both forms of SOD, determinations were performed both in the presence and in the absence of 2 mM KCN solution in the assay mixture. Mn-SOD activity was measured in the presence of KCN, and Cu, Zn-SOD activity was calculated as the difference between measurements in the presence and absence of KCN. Catalase (CAT) activity (EC 1.11.1.6) was assayed spectrophotometrically by the decomposition of H_2O_2 at $\lambda = 240$ nm in a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) containing 1% Triton-X100, 1:9 (w/v) and 12.5 mM H_2O_2 (Aebi, 1984). Proteins were measured according to Lowry et al. (1951).

2.2.5. Ascorbyl radical content ($A^•$)

A Bruker ECS 106 spectrometer was used for $A^•$ measurements. Gills and liver homogenates were prepared by adding a piece of tissue (100 and 150 mg) to pure DMSO (1:3) (w/v). The spectra were scanned at room temperature under the following conditions: 50 kHz field modulation, microwave power 20 mW, modulation amplitude 1 G, time constant 655 ms, receiver gain $1 \cdot 10^5$, microwave frequency 9.81 GHz, and scan rate 0.18 G/s (Giulivi and Cadenas, 1993). The

amount of spin adduct was calibrated using an aqueous solution of TEMPO, introduced into the same cell used for spin trapping. EPR spectra of spin adduct solution and TEMPO solution were recorded at exactly the same spectrometer settings and the first-derivative EPR spectra were doubly integrated by a computer attached to the EPR spectrometer to obtain the area intensity, and then the concentration of spin adduct was calculated using the ratio of these areas. Quantification was performed according to Kotake et al. (1996).

2.2.6. Ascorbate content (AH^-)

The content of AH^- was measured by reverse-phase HPLC with electrochemical detection using a LC-4C amperometric detector with a carbon working electrode at an applied oxidation potential of 0.6 V. The samples were homogenized in metaphosphoric acid (10%, w/v) according to Kutnink et al. (1987). A Supelcosil LC-18 column was stabilized with metaphosphoric acid (0.8%, w/v) and a freshly prepared solution of ascorbic acid in metaphosphoric acid (10%, w/v) (1 μg/mL) was used as standard.

2.3. Statistical analyses

Data in the text and tables are expressed as mean ± S.E.M. of five independent experiments, with two replicates in each experiment. Statistical tests (Mann–Whitney) were carried out using GraphPad InStat, version 3.01.

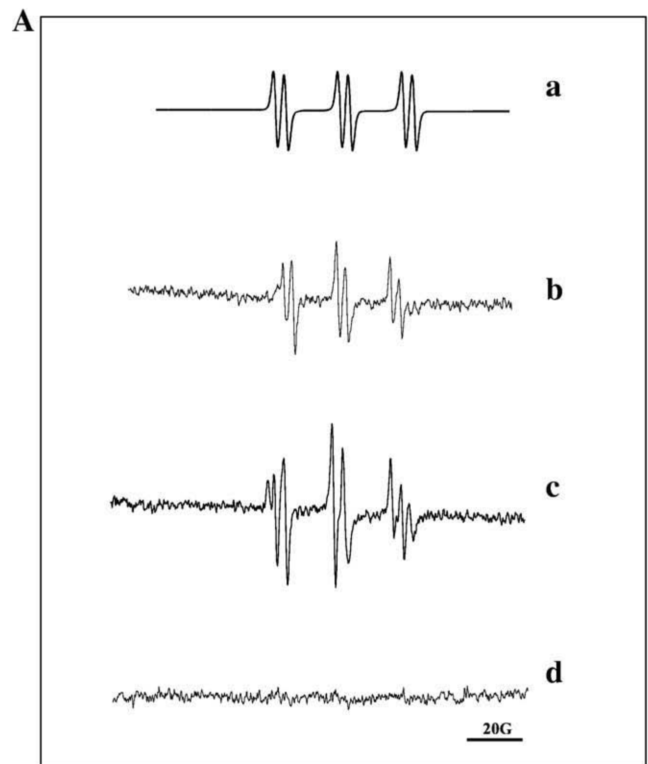
3. Results

Oxidative damage to lipid in the gills and the liver of silversides was estimated in the present study, both as the content of lipid radicals assessed by EPR and as the content of TBARS. Lipid radicals in the gills and the liver combined with the spin trap PBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of $a_N = 15.56$ G and $a_H = 2.79$ G, in concordance with computer spectral simulated signals obtained using the overall mentioned parameters (Fig. 1A). PBN itself was examined and no PBN spin adduct was observed (Fig. 1A (d)). Spectra from Fig. 1A, show a signal from a second radical species with hyperfine coupling constants $a_N = 15.91$ and $a_H = 3.19$, corresponding to a carbon-centered endogenous radical of unknown origin. However, even though the signal was not fully identified, it may reflect the formation of a PBN-$^{\bullet}CH_3$ adduct. Since hydroxyl radical formation *in vivo* may occur under these experimental conditions, its reaction with DMSO could result in the generation of methyl radical that could be trapped by PBN, to give the PBN-$^{\bullet}CH_3$ adduct detected by EPR (Mason et al., 1994; Dikolova et al., 2001). Lipid radical content showed significant differences between the organs (Figs. 1A (b, c) and B, Mann–Whitney, $n = 5$, $p < 0.05$), being 43% higher in the gills as compared to the liver.

TBARS content was significantly different between the gills and the liver of *O. nigricans* (Fig 1B, Mann–Whitney, $n = 5$, $p < 0.05$). Values for the gills were 84% higher than those measured in the liver.

Due to the critical role of the antioxidants in maintaining the steady-state concentration of reactive species in living cells, the activity of the enzymes catalase and SOD, and the content of non-enzymatic antioxidant were evaluated in the gills and the liver. Catalase activity showed significant differences between organs (Table 1, Mann–Whitney, $n = 5$, $p < 0.05$), since it was 88% higher in the liver as compared to the gills. However, no significant differences were determined between organs regarding the activity of both Cu, Zn-SOD and Mn-SOD (Table 1, Mann–Whitney, $n = 5$, $p > 0.05$). The content of non-enzymatic antioxidants (α-tocopherol and β-carotene), were both significantly lower in the gills than in the liver (Table 1, Mann–Whitney, $n = 5$, $p < 0.05$).

A typical EPR spectrum of $A^{\bullet}$ was observed in the gills and liver with the characteristic two lines at $g = 2.005$ and $a_H = 1.8$ G, in accordance with computer spectral simulated signals obtained using



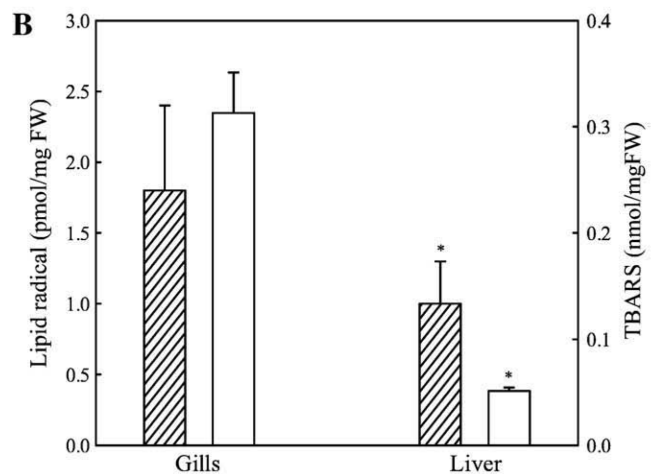


Fig. 1. A: EPR detection of lipid radicals in *O. nigricans* liver and gills: (a) computer-simulated EPR spectra exhibiting hyperfine splittings that are characteristic of PBN/lipid radicals, $a_N = 15.56$ G and $a_H = 2.79$ G, (b) typical EPR spectra of gills, (c) typical EPR spectra of the liver, and (d) EPR spectra of N-t-butil-α-phenyl nitrone (PBN) itself, are shown. B: TBARS (▭) and lipid radicals (▨) content in *O. nigricans* gills and liver. Data are expressed as means ± S.E.M. of five independent experiments. *Significantly different at $p < 0.05$ between organs.

the parameters stated in the Materials and methods section (Fig. 2A). DMSO itself was examined and no DMSO spin adduct was observed (Fig. 2A (d)). $A^{\bullet}$ content, assessed by quantification of EPR signals, was not significantly different in both organs (Figs. 2A (b, c) and B, Mann–Whitney, $n = 5$, $p > 0.05$).

At the hydrophilic level, the $A^{\bullet}/AH^-$ ratio was significantly higher in the gills (91%) as compared to the liver (Fig. 2B, Mann–Whitney, $n = 5$, $p < 0.05$) due to a higher AH^- content in the liver (Fig. 2B, Mann–Whitney, $n = 5$, $p < 0.05$). To further evaluate the oxidative stress in the lipid medium, the ratio content of lipid radical/lipid soluble antioxidants was used. Free radical-dependent damage was estimated as the lipid radical content shown in Fig. 1, and antioxidant protection was assessed in terms of α-tocopherol or β-carotene

Table 1
Antioxidant capacity in *O. nigricans*.

	Gills	Liver
α-Tocopherol (nmol/mg FW)	6 ± 2	1135 ± 240*
β-Carotene (nmol/mg FW)	0.09 ± 0.06	4.0 ± 0.9*
Catalase (U/mg prot)	0.013 ± 0.004	0.11 ± 0.01*
Total SOD (U/mg prot)	2.4 ± 0.4	3.5 ± 0.3
Mn-SOD (U/mg prot)	1.1 ± 0.1	2.0 ± 0.4
Cu, Zn-SOD (U/mg prot)	1.3 ± 0.6	1.5 ± 1.2

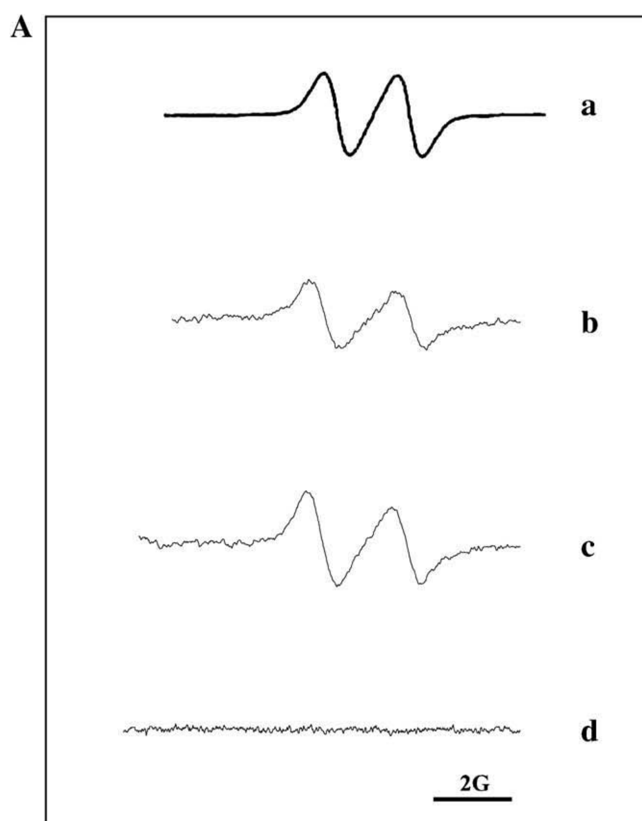
Data are expressed as means ± S.E.M. of five independent experiments.
* Significant differences at $p < 0.05$ between organs.

content (taken from Table 1) since these are the main lipid soluble antioxidants in the cell. The calculated ratios were significantly different between the tested organs (Table 2, Mann–Whitney, $n = 5$, $p < 0.05$).

4. Discussion

A battery of biomarkers, including both molecular and enzymatic parameters, is used to evaluate oxidative metabolism in marine organisms (Abele and Puntarulo, 2004). The data reported here studied comparatively the oxidative status in the gills and liver of the silverside *O. nigricans* in their natural environment. The degree of oxidative damage naturally imposed on the fish, was assessed through the estimation of lipid peroxidation products. Although the measurement of TBARS is a rather unspecific test, it can be used along with other methods to unequivocally characterize the lipid peroxidation process (Rice-Evans et al., 1991). Basal levels for TBARS content were established in the gills and liver of silversides, showing significantly different levels between organs. The mean content of liver TBARS was low as compared to the values reported by Wilhelm Filho et al. (1993, 2000) for marine teleosts and elasmobranchs. Hepatic TBARS content in *O. nigricans* was in the same range as those reported for the eelpout *Zoarces viviparous* (Linnaeus, 1758) (Almroth et al., 2005) and for the rotan *Perccottus glenii* Dybowski, 1877 (Lushchak and Bagnyukova, 2007). The content of TBARS in the gills was significantly increased as compared to the liver and quite similar to those values reported by Parihar and Dubay (1995) for the catfish *Heteropneustes fossilis* (Bloch, 1794). Since endogenous lipid radicals show short half-lives and are present in low concentrations, detection is a difficult task. Spin trapping-EPR analysis overcomes the limit of sensitivity of endogenous radical content in biological systems, and it has been proved to be the best method available to detect short-lived reactive free radicals generated in low concentrations in biological systems (Luo et al., 2006). Even though EPR detection of lipid radicals could be considered a fingerprint of radical presence, spin trapping studies cannot really distinguish among peroxyl (ROO•), alcohoxyl (RO•) and alkyl (R•) adducts owing to the similarity of the corresponding coupling constants (Jurkiewicz and Buettner, 1994). In this regard, previous data from Luo et al. (2006), used EPR technology to detect ROS generation in the fish *Carassius auratus* (Linnaeus, 1758) induced by 2-chlorophenol.

Under physiological conditions, a balance exists between the generation of free radicals and the antioxidant defence mechanisms (Halliwell, 1987). The effectiveness of the antioxidant defences in relation to the lipid peroxidation process is of particular interest in the case of aquatic animals, which shows metabolic variations related to fluctuation of environmental factors and the physiological status of the animals (Parihar and Dubay, 1995). In *O. nigricans*, the content of α-tocopherol and β-carotene was higher in the liver as compared to the gills. α-Tocopherol appears to be the most efficient inhibitor of lipid peroxidation due to its hydrophobic nature and therefore the solubility in lipid membranes and lipoproteins (Gieseg et al., 2000). A wide range of α-tocopherol content was found in the liver and plasma of some fishes. Wilhelm Filho and Marcon (1996) found a relatively low α-tocopherol content (0.7 ± 0.4 nmol g^{-1}) in the liver of Amazonian species in the dry season. Gieseg et al. (2000) found that two Antarctic fishes, *Pagothenia borchgrevinki* (Boulenger, 1902) and *Trematomus bernacchii* (Boulenger, 1902) had on average seven



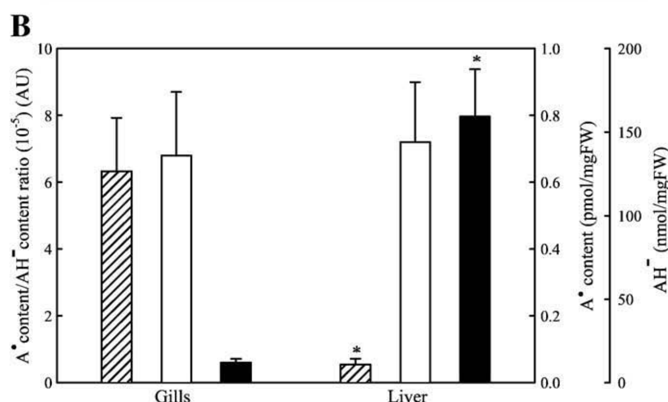


Fig. 2. A: EPR detection of A• in *O. nigricans* liver and gills: (a) computer-simulated EPR spectra employing the following spectral parameters $g = 2.005$ and $a_H = 1.8$ G, (b) typical EPR spectra of gills, (c) typical EPR spectra of the liver, and (d) EPR spectra of DMSO itself, are shown. B: A• (□) and AH− (■) content and A•/AH− ratio (▨) in *O. nigricans* gills and liver. Data are expressed as means ± S.E.M. of five independent experiments. *Significantly different at $p < 0.05$ between organs.

Table 2
Lipid radical/lipid soluble antioxidants in *O. nigricans*.

	Gills	Liver
Lipid radical/α-tocopherol (10^{-6})	275 ± 41	1.4 ± 0.7*
Lipid radical/β-carotene (10^{-4})	727 ± 542	2.4 ± 0.5*

Data are expressed as means ± S.E.M. of five independent experiments.
* Significant differences at $p < 0.05$ between organs.

times more α-tocopherol circulating in their plasma (116 μM and 106 μM, respectively), than temperate water fishes from New Zealand, *Parapercis colias* (Forster, 1801) and *Notolabrus fucicola* (Richardson, 1840) (14 μM and 18.9 μM, respectively). On the other hand, the content of β-carotene was drastically lower as compared to that of α-tocopherol. This result agrees with those previously reported by Wilhelm Filho et al. (1993) and Wilhelm Filho and Marcon (1996), who failed to show the presence of this antioxidant in significant amounts in the liver and erythrocytes of marine and freshwater fishes. This observation suggested that these substances are restrictively used as antioxidants in fish.

Moreover, the results presented here showed that the activity of total SOD, Cu, Zn-SOD and Zn-SOD were not significantly different between organs. On the other hand, the activity of catalase was significantly higher in the liver as compared to the gills in *O. nigricans*. The data presented here are in agreement with those reported by Ansaldo et al. (2000) in the gills and liver of red-blooded (Nototheniidae). Catalase activity seems as a main contributing factor to limit lipid peroxidation in the liver by scavenging of H_2O_2, that is continuously generated in living tissue. Moreover, elimination of H_2O_2 by gills seems as an important antioxidant mechanism in fish. Lower catalase activity in gills with respect to the liver, might reflect lower H_2O_2 concentration in the gills because of diffusion to the surrounding water. Therefore, low catalase in gills in comparison with the liver, might reflect the existence of an alternative antioxidant mechanism based on the free diffusion of H_2O_2 outside the organ to prevent H_2O_2-dependent damage.

Previous data from several mammalian species showed a clear correlation between ROS production and metabolic rate. Species with higher metabolic rate produce higher concentrations of O_2^- and H_2O_2 in mitochondria (Zielinski and Pörtner, 2000; Abele et al., 1998). Mitochondria are a center of energy metabolism utilizing oxygen, where ROS are produced under physiological conditions. Recently, new information on the formation of ROS by mitochondria in aquatic organisms was reported (Heise et al., 2003; Philipp et al., 2005). Reactive species produced in biological systems are detoxified by antioxidant defences, which are broadly investigated in aquatic organisms (Abele and Puntarulo, 2004; Abele et al., 2007). In fish, comparative studies of their antioxidant defences and mitochondrial ROS production rate revealed a linear relationship with oxygen consumption rate (Wilhelm Filho, 2007). Taking this in account, it could also be expected that single organs with different mitochondrial structures, such as the liver and gills, could have also differential radical production and, therewith oxidative damage in the presence of an alteration of the natural environment. In this way, Kamunde (2009) found that gills could act as a barrier to waterborne metals, limiting their internalization in the rainbow trout *Oncorhynchus mykiss* Walbaum. However, this way of limiting toxicity to internal organs (liver), increased the risk of injury by ROS in the gills after metal exposure.

Under natural conditions, different organs showed statistically significant differences, suggesting that exposure to natural conditions affect oxidative stress in the gills, at the level of the cytosolic medium. For the lipophilic compartment, the ratio lipid radical content/lipid soluble antioxidants can be understood as an indicator of the balance between free radical-dependent damage and antioxidant protection. This index was significantly higher in the gills as compared to the value recorded for the liver, suggesting an increased situation of oxidative stress in the lipid phase of the gills. Taken as a whole, the *O. nigricans* liver exhibited a better control of oxidative damage than the gills, allowing minimization of intracellular damage when exposed to environmental stressing conditions.

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