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Martín Ansaldo · Carlos M. Luquet · Pablo A. Evelson
José M. Polo · Susana Llesuy

Antioxidant levels from different Antarctic fish caught around South Georgia Island and Shag Rocks

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Abstract Antarctic fish have been isolated for over several million years in an environment with a very low and constant temperature and high oxygen concentration. In such conditions the oxidative stress might be an important factor affecting their metabolic adaptive strategies. Activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), vitamin E levels and total antioxidant capacity (TRAP) were measured in liver, gill, heart and muscle homogenates of red-blooded (Nototheniidae) and white-blooded (Channichthyidae) Antarctic fish. SOD activity was also measured in blood samples. Gill SOD activity was threefold higher in channichthyids than in nototheniids while CAT and GPx were significantly higher in the gills of channichthyids. The increased SOD activity of channichthyids probably reflects the large PO_2 gradient across their gills. The H_2O_2 produced seems to be preferentially eliminated by diffusion, according to the low levels of CAT and GPx found in the gills of these species. In contrast, blood SOD was about fivefold higher in the latter group, which possesses erythrocytes and thus a much higher oxygen-carrying capacity. CAT activity was always higher in nototheniids except in muscle. However, vitamin E did not show clear differences between families except for the pattern observed in muscle. The higher content of vitamin E in this tissue shown in channichthyids is related to the higher volume density of mitochondria reported for this group, since vitamin E is responsible for preventing membrane lipid peroxidation. Accordingly, TRAP (representative of hydrosoluble antioxidant capacity) was also higher in muscle of channichthyids. This is probably related to the role of ascorbic (a hydrosoluble compound) acid in regenerating vitamin E.

M. Ansaldo (✉)
Instituto Antártico Argentino,
Cerrito 1248, (1010) Buenos Aires, Argentina
e-mail: tincho@bg.fcen.uba.ar

P.A. Evelson · J.M. Polo · S. Llesuy
Cátedra de Química General e Inorgánica,
Departamento de Química Analítica y Fisicoquímica,
Facultad de Farmacia y Bioquímica,
Universidad de Buenos Aires, Junín 956,
(1113) Buenos Aires, Argentina

C.M. Luquet
Departamento de Ciencias Biológicas,
Facultad de Ciencias Exactas y Naturales,
Universidad de Buenos Aires, Pabellón II,
Laboratorio 24, Ciudad Universitaria,
(1428) Buenos Aires, Argentina

Introduction

Temperature is a thermodynamic variable of wide and complex influence on biological systems; it encompasses molecular to organismic level, having an effect over all processes related to energy. Antarctic bottom waters possess a virtually constant temperature of about −1.8°C; thus it is the most common environmental parameter considered in studies dealing with the adaptation of marine species to extreme climatic conditions because of its influence on the metabolic rate (Macdonald et al. 1987; Eastman 1993; Clarke and Johnston 1996; Di Muro et al. 1996). Another characteristic related to the low temperature of Antarctic seawaters is its high oxygen concentration of 0.18–0.36 mmol/l (Hardy and Gunther 1935).

Increased oxygen concentration in seawater allows the loss of respiratory pigments in the Channichthyidae, a family of perciform fish (Eastman 1993). In the species of this group, oxygen reaches the tissues only in the dissolved form and the O_2-carrying capacity is equal to 0.67% by volume, about one-tenth that of red-blooded Antarctic species (Holeton 1970). The physiological adaptations, which enable icefish to live with no haemoglobin, have been discussed in several papers (Hemmingsen and Douglas 1970, 1977; Holeton 1970; Macdonald et al. 1987; Acierno et al. 1995; Clarke and Johnston 1996; di Prisco 1997). These adaptations

include: low metabolic rate, large and highly perfused gills, increased blood volume, a larger heart and stroke volume, larger capillary diameter and cutaneous respiration. In addition, low temperature increases oxygen solubility in plasma; thus more oxygen can be carried in physical solution, reducing the need for a transport protein (di Prisco 1997). Moreover, at a molecular level, evolutionary changes have occurred in several enzymatic and structural proteins allowing Antarctic fish to sustain metabolic activities comparable to those found in fish with higher cell temperature (Detrich 1998; Somero et al. 1998).

In the presence of high concentrations of dissolved oxygen in cold seawater, the formation of free radicals could be easier (Dirks et al. 1982). Thus, one would expect to find distinctive adaptive responses in the antioxidant defence system among Antarctic fish. The rate of formation of products from the partial reduction of O_2 and reactive oxygen species usually increases in biological systems with the increase in oxygen concentration. Oxidative stress can be understood as a situation derived either from an enhanced rate of generation of oxygen radicals or from a diminished level of antioxidant (enzymatic or non-enzymatic) defences. Moreover, a biological situation associated with oxidative stress could be estimated by a physicochemical condition in which an increase in the steady-state levels of oxidative species (i.e. O_2^-, H_2O_2, $HO^{\cdot}$, $R^{\cdot}$ and $ROO^{\cdot}$) occurs. This increased steady-state level of oxidants may lead, in consequence, to reversible or irreversible cell damage and eventually to cell death. Oxygen free radicals are produced in a series of biochemical reactions that will normally occur within the cellular compartments (mitochondria and the endoplasmic reticulum are the most important sources of oxygen free radicals).

It is well known that different enzymes and non-enzymatic compounds participate in the antioxidant chain in biological systems. Among them, superoxide dismutase (SOD) converts superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), catalase (CAT) reduces H_2O_2 to water, and glutathione peroxidase (GPx) detoxifies both H_2O_2 and organic hydroperoxides (ROOH) using reduced glutathione as a cofactor. Total antioxidant capacity (TRAP) is a technique often used to evaluate the hydrosoluble antioxidant status (measuring altogether ascorbic acid, reduced glutathione, uric acid and bilirubin) of biological samples (Lissi et al. 1992). Vitamin E is a non-enzymatic liposoluble compound generally accepted as a chain-breaking antioxidant in biological systems. It is interesting to note that distribution of vitamin E in tissues and biological fluids shows a close correspondence with both the concentration of polyunsaturated fatty acids and the oxygen concentrations to which they are exposed (Kornbrust and Mavis 1980).

Some comparative studies on antioxidant compounds in various tissues of tropical and temperate fish species have been performed (Matkovics et al. 1977; Fitzgerald 1992; Wilhelm Filho et al. 1993), but only a few concern Antarctic fish (Witas et al. 1984; Cassini et al. 1993). In this study we performed a comparative analysis of SOD, CAT, GPx, vitamin E and TRAP. The activity and/or level of the different antioxidant compounds were measured for comparison in different tissues of fish belonging to two families of Antarctic fish: Nototheniidae (red-blooded) and Channichthyidae (white-blooded). Specific responses are expected for haemoglobinless fish, since they lack erythrocytes which are known to contain an important fraction of the total enzyme SOD, observed in the tissues of temperate fish (Wdziêczak et al. 1980; Scott and Harrington 1990).

Materials and methods

Preparation of samples

The samples used in this study were collected in surveys of the R/V *Dr. Eduardo L. Holmberg* in the Statistical Subarea 48.3 (CCAMLR), around the South Georgia Islands and Shag Rocks, at depths not greater than 500 m, in the late summer of 1996/1997.

Each catch was separated by species. Specimens of *Notothenia rossii*, *Dissostichus eleginoides* (Nototheniidae, red-blooded fish) and *Chaenocephalus aceratus*, *Champsocephalus gunnari* and *Pseudochaenichthys georgianus* (Channichthyidae, white-blooded fish) were sexed, and measured (total length) and weighted, in accordance with the protocol developed by Marschoff et al. (1994). Later, blood was drawn by cardiac punction and kept on ice. Samples of heart, gills, skeletal muscle and liver were dissected and immediately stored at −70°C.

Tissue homogenates

Tissue samples to be processed for the determination of antioxidant enzyme activities, vitamin E levels and total reactivity antioxidant power were homogenised in 20 mM sodium phosphate, pH 7.4, 0.15 M sodium chloride, 0.1% Triton X-100 and 0.1 mM phenylmethyl-sulfonylfluoride (PMSF) at 0–4°C in an Omni 2000 homogeniser. The suspension was centrifuged at 10000 *g* for 10 min at 0–4°C. The pellet was discarded and the supernatant was used as homogenate (Wilhelm-Filho et al. 1993, with minor modifications).

Blood extracts

After plasma removal, sediments were washed three times in saline solution. The hemolysates were obtained after addition of cold distilled water, and shaking and centrifugation at 3000 *g* for 10 min. Then a chloroform:ethanol (3:5) extraction was performed followed by a centrifugation at 5000 *g* (for 15 min). The aqueous supernatant was saved for SOD analysis.

Superoxide dismutase activity

SOD activity was determined spectrophotometrically by measuring the inhibition of the rate of autocatalytic adrenochrome formation at 480 nm, in a reaction medium containing 1 mM epinephrine and 50 mM glycine-NaOH (pH 10.2). The enzymatic activity was expressed as SOD units/mg of protein. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adrenochrome formation (Misra and Fridovich 1972).

Catalase activity

CAT activity was evaluated by measuring the decrease in the absorption at 240 nm in reaction medium consisting of 50 mM phosphate buffer pH 7.4 and 2 mM H_2O_2, thereby determining the pseudo-first-order reaction constant (k') of the decrease in H_2O_2 absorption. Results were expressed as catalase content in pmol/mg of protein (Chance 1954).

Glutathione peroxidase activity

Glutathione peroxidase was measured following NADPH oxidation at 340 nm in a reaction medium containing 50 mM phosphate buffer (pH 7.2), 0.17 mM GSH, 0.2 U/mL glutathione reductase, 0.1 mM NADPH and 0.5 mM *tert*-butyl-hydroperoxide. Glutathione peroxidase activity was expressed as μmol NADPH/min · mg protein (Flohé and Gunzler 1984).

Vitamin E

Vitamin E was quantified by reverse-phase high-performance liquid chromatography with electrochemical detection using a Bioanalytical Systems (West Lafayatte, Ind.) amperometric detector with a glassy-carbon working electrode at an applied oxidation potential of 0.6 V (Buttriss and Diplock 1984). Tissue samples were extracted with 1 mL ethanol and 3 mL hexane. After centrifugation at 1500 *g* for 10 min, the upper phase was removed and evaporated to dryness under nitrogen. Samples were dissolved in 0.3 mL methanol:ethanol (1:1 v/v). Results were expressed as nmol/mg of protein.

Total reactivity antioxidant power

Total reactivity antioxidant power or total antioxidant capacity was measured by chemiluminescence. The reaction medium consisted of 20 mM 2,2′-azo-bis-(2-amidinopropane) (ABAP) and 40 μM luminol. ABAP is a source of free radicals which reacts with luminol yielding chemiluminescence. The addition of 50–100 μL of homogenate decreases chemiluminescence to basal levels, for a period proportional to the amount of antioxidants present in the homogenate, until luminol radicals are regenerated. The system was calibrated with Trolox (vitamin E hydrosoluble analogue). Results were expressed as μmol Trolox/g of organ (Lissi et al. 1992).

Protein measurement

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistics

The numbers in Tables 1 and 2 indicate mean values ± SEM. Data were analysed by factorial analysis of variance (ANOVA) followed by a Tukey-Kramer Multiple Comparisons Test.

Results

Comparisons between blood SOD activity of fish from the families Channichthyidae and Nototheniidae are shown in Fig. 1. Blood SOD activity of red-blooded fish

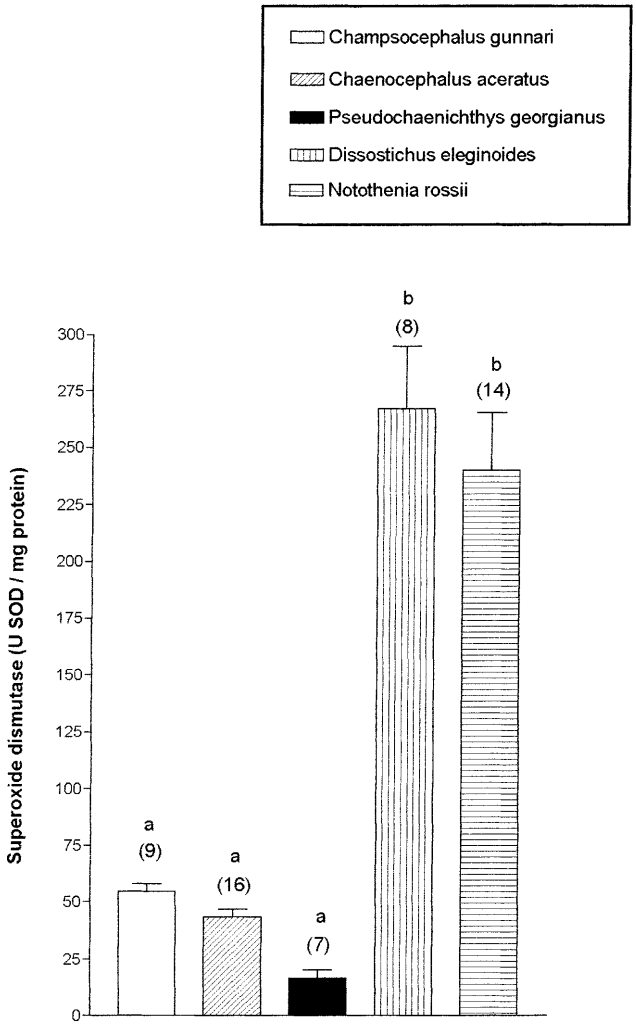

Fig. 1 Superoxide dismutase activity in blood of Antarctic fish. Enzyme activity is expressed in units/mg of protein. The *bars* indicate mean values ± SEM; the number of animals is indicated in *parentheses*. Multiple comparisons among species: values followed by different *letters* are significantly different at $P < 0.001$

was always significantly higher than that in white-blooded fish ($P < 0.001$).

Gill SOD activity was threefold higher in *Champsocephalus gunnari*, *Chaenocephalus aceratus* and *P. georgianus* than in *D. eleginoides* and *N. rossii* (Table 1). No significant difference was observed between species for the activity levels of SOD measured in muscle and liver. Heart of *N. rossii* showed a significantly higher value of activity than the other studied species (Table 1).

CAT activity was distributed similarly among the tissues examined in white-blooded and red-blooded fish; the highest level was measured in liver. However, the overall level of activity was markedly lower in the former group. In liver of red-blooded fish the level of CAT activity was about three to fourfold higher than in white-blooded fish. Gill CAT activity was about 3- to 12-fold higher in notothenids than in channicthyids. Similarly, in heart the difference was three to fourfold. Muscle was the tissue with the lowest CAT activity for all the studied

Table 1 Antioxidant enzyme activities in different tissues of Antarctic fish. Values shown represent the means ± SEM. Number of animals is indicated in *parentheses*. SOD activity is expressed in units/mg protein. CAT activity is expressed in pmol/mg protein. GPx activity is expressed in μmol NADPH/min·mg protein. Multiple comparisons among species: values followed by different *letters* are significantly different at $P < 0.05$

Enzyme	Tissue	Species				
		Champsocephalus gunnari	*Chaenocephalus aceratus*	*Pseudochaenichthys georgianus*	*Dissostichus eleginoides*	*Notothenia rossii*
Superoxide dismutase	Liver	0.75 ± 0.06 (14)	0.96 ± 0.10 (16)	0.69 ± 0.06 (11)	1.08 ± 0.16 (10)	0.91 ± 0.06 (16)
	Gills	0.87 ± 0.08 a (5)	0.88 ± 0.07 a (9)	0.86 ± 0.10 a (7)	0.27 ± 0.03 b (8)	0.19 ± 0.02 b (10)
	Muscle	0.87 ± 0.08 (10)	1.02 ± 0.12 (6)	0.78 ± 0.07 (8)	1.00 ± 0.19 (7)	0.70 ± 0.05 (13)
	Heart	0.85 ± 0.06 a (11)	1.05 ± 0.06 a (12)	1.05 ± 0.02 a (9)	0.89 ± 0.05 a (9)	1.39 ± 0.11 b (10)
Catalase	Liver	0.98 ± 0.13 a (15)	0.47 ± 0.08 a (15)	0.84 ± 0.08 a (14)	4.47 ± 0.45 b (9)	3.23 ± 0.31 c (12)
	Gills	0.065 ± 0.006 a (8)	0.048 ± 0.004 a (8)	0.058 ± 0.009 a (9)	0.276 ± 0.028 b (7)	0.697 ± 0.035 c (10)
	Muscle	0.039 ± 0.006 ab (8)	0.011 ± 0.001 a (4)	0.036 ± 0.004 ab (5)	0.031 ± 0.005 a (7)	0.060 ± 0.010 b (9)
	Heart	0.12 ± 0.02 a (11)	0.07 ± 0.01 a (14)	0.09 ± 0.01 a (8)	0.34 ± 0.03 b (9)	0.38 ± 0.03 b (13)
Glutathione peroxidase	Liver	4.84 ± 0.71 (13)	6.03 ± 0.48 (12)	3.15 ± 0.54 (6)	4.76 ± 0.64 (8)	7.16 ± 1.4 (15)
	Gills	0.94 ± 0.09 a (5)	1.06 ± 0.37 a (5)	1.15 ± 0.16 a (5)	3.04 ± 0.79 b (6)	3.05 ± 0.46 b (8)
	Muscle	0.94 ± 0.17 (9)	1.82 ± 0.27 (6)	1.75 ± 0.43 (6)	1.08 ± 0.18 (6)	0.99 ± 0.14 (12)
	Heart	6.88 ± 0.58 (6)	5.73 ± 0.53 (6)	6.38 ± 0.90 (5)	8.47 ± 0.69 (6)	6.94 ± 0.71 (10)

species. This was the only tissue that showed no difference between families (Table 1).

In channichthyids, GPx levels were four to sixfold higher in liver and heart than in gills and muscle (Table 1). In nototheniids GPx activity showed the lowest level in muscle. GPx activity was 2.5- to 3-fold higher only in gills in Nototheniidae as compared to Channichthyidae (Table 1).

Vitamin E showed great difference within families, especially within Nototheniidae. Contents of this vitamin were 8- and 33-fold higher in heart and liver of *N. rossii* compared to those in the same tissues of *D. eleginoides*. No difference was detected between muscle vitamin E levels of this species. No significant difference was found for this vitamin in liver and muscle of Channichthyidae. The only significant difference was found for *P. georgianus* heart, which showed less vitamin E than that measured in the hearts of the other related species.

Vitamin E tissue contents were higher in white-blooded fish than those in the same tissues of red-blooded fish in most of the comparisons done, with the exception of *N. rossii* liver, which showed the highest content of this vitamin (Table 2).

Total radical antioxidant potential was quite different between the two studied species of red-blooded fishes (Table 2). Although both species showed higher TRAP in liver than in heart, it was always significantly higher in *N. rossii*. This variable showed non-detectable values in muscle samples from these species. For white-blooded fish TRAP was much higher in liver than in the other tissues, being the lowest value measured in muscle.

This variable showed similar values among all the studied species excepting *N. rossii*, which showed significantly higher values of liver and heart TRAP.

Discussion

Icefish take advantage of the high oxygen content of Antarctic seawater in order to supply this gas to the

Table 2 Vitamin E and total antioxidant capacity (TRAP) in different tissues of Antarctic fish. Values shown represent the means ± SEM. Number of animals is indicated in *parentheses*. Vitamin E is expressed in nmol/mg protein. Total antioxidant capacity (*TRAP*) is expressed in μmol Trolox/g of organ. *ND* non-detectable values. Multiple comparisons among species: values followed by different *letters* are significantly different at $P < 0.05$

	Tissue	Species				
		Champsocephalus gunnari	*Chaenocephalus aceratus*	*Pseudochaenichthys georgianus*	*Dissostichus eleginoides*	*Notothenia rossii*
Vitamin E	Liver	126 ± 23 ab (8)	142 ± 21 ab (9)	242 ± 49 a (6)	29 ± 7 b (4)	969 ± 56 c (6)
	Muscle	94 ± 11 a (6)	107 ± 12 a (6)	138 ± 22 a (7)	45 ± 11 b (7)	32 ± 6 b (5)
	Heart	174 ± 22 a (4)	178 ± 27 a (6)	75 ± 14 b (6)	12 ± 2 c (6)	86 ± 19 b (6)
TRAP	Liver	234 ± 37 a (6)	180 ± 38 a (4)	252 ± 30 a (3)	193 ± 26 a (4)	498 ± 50 b (5)
	Muscle	17.4 ± 2.6 (6)	25.2 ± 2.5 (4)	29.1 ± 2.5 (3)	ND (4)	ND (4)
	Heart	49.5 ± 8.6 a (6)	42.3 ± 4.3 a (4)	49 ± 4 a (3)	48.8 ± 5.3 a (4)	120 ± 3.0 b (5)

tissues without the aid of any respiratory pigment. Holeton (1970) measured a high PO_2 gradient across the gills of the icefish *Chaenocephalus aceratus*, together with high PO_2 in the expired water and low percentage of O_2 extraction. This finding indicates that PO_2 inside the gill cells of haemoglobinless fish should be higher than that in their red-blooded counterparts. Thus, a large amount of superoxide anion (O_2^-) would be expected to be produced inside the gill tissue of icefish. Superoxide anion as a charged species does not diffuse through the gills. As a consequence, this active O_2 species must be converted to H_2O_2 inside the gill cells by the enzyme superoxide dismutase. Our results agree by showing that gill SOD activity is threefold higher in icefish than in red-blooded fish. Wilhelm-Filho et al. (1994) reported that most of the produced H_2O_2 is eliminated by simple diffusion to the water. Thus, the diffusion through gills seems to provide a mechanism by which to keep a low steady-state concentration of this reactive oxygen species. This should hold particularly true for icefish, since they are known to possess high ventilation rates (see Macdonald et al. 1987 for a review). Moreover, our results show that the studied channichthyids possess very low gill CAT activity compared to Antarctic red-blooded fish. In this way, the theory of co-ordination of the expression of antioxidant enzymes reported in previous papers, meaning that the higher the levels of SOD, the higher the levels of enzymes converting H_2O_2 (Harris 1992; Cassini et al. 1993), seems not to fit to the gill enzymes of the icefish species considered in the present paper. Also, while gill SOD activity is significantly higher, gill CAT and GPx activities are several-fold lower than in red-blooded fish. As already mentioned, the high concentration of H_2O_2 produced by SOD within the gills of icefish should be eliminated by diffusion to the water and not by enzymatic conversion.

However, SOD was higher in the blood of nototheniids. This result agrees with the fact that these fishes possess about 9–10 times the O_2-carrying capacity of channichthyids (Holeton 1970), due to accumulation of high O_2 concentrations bound to haemoglobin. Thus, red-blood cells must cope with the oxidative stress posed by superoxide anion formation.

The other tissues studied (liver, heart, muscle) do not show any difference in SOD activity within or between families. These observations differ from those reported by Cassini et al. (1993), who measured higher SOD activities in the same three organs in an Antarctic red-blooded species, *Pagothenia bernacchii*, compared to the icefish, *Chionodraco hamatus*.

The activity of antioxidant enzymes that take H_2O_2 as a substrate should also be lower in channichthyids than in nototheniids, considering the possible diffusion of H_2O_2 from erythrocytes in the latter group. Accordingly, CAT activity is much lower in icefish than in red-blooded fish in all the studied tissues, excepting muscle. However, GPx activity shows pronounced variations among tissues but not between families, except for gills.

Most of the Antarctic fish possess substantial body lipid accumulations, considered to be involved in buoyancy and aerobic metabolism (Eastman and De Vries 1982; Eastman 1993). This accumulation has led to a prevailing view that lipid is the primary fuel for energy metabolism of these species. This idea has received direct experimental support from Sidell (1991) and Crockett and Sidell (1993), who have shown that aerobic energy metabolism is predominantly dependent upon the oxidation of unsaturated lipids in heart, red pectoral muscle and liver of nototheniids. The fact that unsaturated fatty acids are susceptible to oxygen radical attack, which can produce lipid peroxidation through a chain of oxidative reactions (Roberfroid and Buc Calderon 1995), could be related to the relatively high levels of GPx activity of heart and liver compared to gills and muscle in all the fishes studied.

Vitamin E levels do not show clear tendencies between families or among tissues. However, muscle levels of this vitamin are clearly higher in channichthyids compared to nototheniids. This could be correlated with the higher volume density of muscle mitochondria of channichthyids compared to other fishes from similar habitats reported by Johnston et al. (1998). Biological membranes, especially those of microsomes and mitochondria, contain high amounts of polyunsaturated fatty acids, which are susceptible to peroxidation (Kornbrust and Mavis 1980). Vitamin E prevents these processes by scavenging the lipoperoxide radicals involved in peroxidation chains (Roberfroid and Buc Calderon 1995).

As already mentioned, the TRAP procedure has been developed mainly for hydrosoluble antioxidants associated with hydrosoluble molecules. The hydrosoluble antioxidant contents could be related to the vitamin E levels measured in this species. It is well known that a hydrosoluble compound like ascorbic acid (vitamin C) participates in the regeneration of the antioxidant power of vitamin E. A co-ordination in the levels of both antioxidant compounds should then be expected. Our results show higher values of both vitamin E and TRAP levels in liver and heart of *N. rossii* than in the other studied species. Hydrosoluble antioxidants could be detected in muscle of white-blooded fish but not in the same tissue of red-blooded fish. This is associated with the higher level of vitamin E found in channichthyid muscle and is probably related to the increased volume density of mitochondria referred to above.

In summary, we observed differences in the distribution of antioxidant defences between the studied families, which could be correlated to their distinct physiological adaptations to transport and consumption of oxygen with and without haemoglobin.

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