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Oxidative stress in limpets exposed to different environmental conditions in the Beagle Channel

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Abstract

The aim of this work was to study the oxidative profile of digestive glands of two limpets species (*Nacella* (*Patinigera*) *magellanica* and *Nacella* (*Patinigera*) *deaurata*) exposed to different environmental conditions. The intertidal population of *N.* (*P.*) *magellanica* is subjected to a wide variety of stresses not experienced by *N.* (*P.*) *deaurata*. Although a typical electron paramagnetic resonance (EPR) spectrum of ascorbyl radical in digestive gland from both limpets was observed, neither ascorbyl radical content nor the ascorbyl radical content/ascorbate content ratio was significantly different, suggesting that the difference in the environmental conditions did not appear to be responsible for developing alterations in the oxidative status of both organisms at the hydrophilic level (e.g. cytosol). Lipid peroxidation in the digestive glands was estimated, both as the content of thiobarbituric acid reactive substances (TBARS) and as the content of lipid radicals assessed by EPR, in both organisms. TBARS and lipid radical content were 34.8 and 36.5%, respectively, lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*. On the other hand, total iron content and the rate of generation of superoxide anion were 47.9 and 51.4%, respectively, lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*. The activity of catalase and superoxide dismutase (SOD) was 35.3 and 128.6% higher in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*, respectively. No significant differences were determined between the digestive glands of both molluscs regarding the content of total thiols. α-Tocopherol and β-carotene content were significantly lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*. A distinctive EPR signal for the adduct Fe–MGD–NO ($g = 2.03$ and $a_N = 12.5$ G) was detected in the homogenates of digestive glands of both limpets. A significant difference in the content of the Fe–MGD–NO adduct in digestive glands from *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata* (491 ± 137 and 839 ± 63 pmol/g FW, respectively) was observed. Taken as a whole, the data presented here indicated that coping with environmental stressing conditions requires a complex adjustment of the physiological metabolic pathways to ensure survival by minimizing intracellular damage. It is likely that *N.* (*P.*) *magellanica* has a particular evolutionary adaptation to extreme environmental conditions by keeping iron content low and antioxidant activities high.

Keywords: Antioxidants; Beagle Channel; Limpets; Nitric oxide adducts; Oxidative stress

1. Introduction

The generation of reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2)

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and hydroxyl radical ($^{\bullet}$OH), takes place continuously in living cells, mainly as by-product of respiration. Nitric oxide (NO) is an inorganic free-radical gaseous molecule, which has been shown to act as an intracellular signal, as a transcellular signal or as a cytotoxic host defense molecule (Moncada et al., 1991; Moncada and Higgs, 1995; Knowles and Moncada, 1994). NO can be generated in cells and tissues from L-arginine by the activity of the enzyme nitric oxide synthase (NOS, EC 1.14.13.39) (Knowles, 1997) and this activity has been found in marine, freshwater and terrestrial molluscs including three gastropod subclasses (prosobranchs, opisthobranchs and pulmonates) (Jacklet, 1997); however, little is known about the metabolism of nitrogen-active species in marine invertebrates and fish.

It has been suggested that in marine bivalves, both species-specific adaptations to euryoxic (intertidal) and stenoxic (subtidal) environments and metabolic requirements of different tissues should result in different profiles in the oxidative cellular status (Walker et al., 2000). The general scenario of pro-oxidant and antioxidant processes was usually assessed in molluscs and other marine invertebrates, according to data from mammalian systems (Livingstone et al., 1990; Livingstone, 1991; Winston and Di Giulio, 1991), lacking in many cases of specific information from these organisms. In this regard, peculiarities of membrane lipids in marine organisms, particularly high contents of unsaturated fatty acids (Joseph, 1982), suggest a special pattern for lipid peroxidation and a complex system of antioxidants (Halliwell and Gutteridge, 1984).

Limpets are very common archaeogastropod molluscs that inhabit intertidal rocky shores. *Nacella* (*Patinigera*) *magellanica* (Gmelin 1971) and *Nacella* (*Patinigera*) *deaurata* (Gmelin 1971) are the two most conspicuous limpet species in the Beagle Channel due to their abundance and their relatively large sizes. *N.* (*P.*) *magellanica* inhabits the middle and the upper intertidal zones, whereas *N.* (*P.*) *deaurata* lives in the lower intertidal zone and the sublittoral zone (Morriconi and Calvo, 1993; Morriconi, 1999). In spite of living in the same area, the difference in shore level location affects animal exposure to aerial or marine environmental conditions. Due to tidal characteristics of the Beagle Channel, *N.* (*P.*) *magellanica* limpets are daily exposed to air twice for 3–5 h each time, but *N.* (*P.*) *deaurata* limpets are daily exposed to air for 3 h, only during spring tides (Morriconi and Calvo, 1993; Morriconi, 1999). The different regime of exposition includes extreme temperatures under 0 °C during winter time and more than 20 °C in summer for *N.* (*P.*) *magellanica*; meanwhile, *N.* (*P.*) *deaurata* limpets are usually covered by more than 0.3 m water that reaches a temperature of 4 °C in winter and 11 °C in summer.

In molluscs, the digestive gland is a major site of uptake of natural (dietary) and organic xenobiotic pro-oxidant chemicals and as a consequence, the main target for oxidative disruption. The aim of this work was to characterize the oxidative status of digestive glands of two limpets species exposed to disparate environmental conditions on an intertidal rocky shore off the Patagonian coast, since it was suggested that antioxidant systems may be altered after exposure to pollutants and temperature stress (Abele et al., 1998, 2002). The ratio content of ascorbyl radical/content of ascorbate was used as an oxidative stress index in the hydrophilic medium (such as the cytosol), and lipid and protein oxidation was assessed as reactive species-dependent oxidative damage. Higher levels of lipid peroxidation may correlate, among other factors, with elevated accumulation of transition metals in animal tissues. Thus, to study the possible role of iron in the catalysis of lipid peroxidation, the iron content in the digestive gland of both molluscs was examined. Antioxidant defense was studied in both species by assaying the activity of antioxidant enzymes (SOD and catalase) and the content of non-enzymatic antioxidants (α-tocopherol, β-carotene, total thiols and ascorbate). Moreover, to further characterize the complex interaction between oxygen and nitrogen-reactive species, the presence of NO in digestive gland homogenates of both limpets was determined employing electron paramagnetic resonance technique.

2. Materials and methods

2.1. Collection of animals

The limpets *N.* (*P.*) *magellanica* (Gmelin 1971) and *N.* (*P.*) *deaurata* (Gmelin 1971) were collected from an intertidal area at Punta Occidental (54°50′S, 68°20′W) in the Beagle Channel, in July 2002 (Fig. 1). *N.* (*P.*)

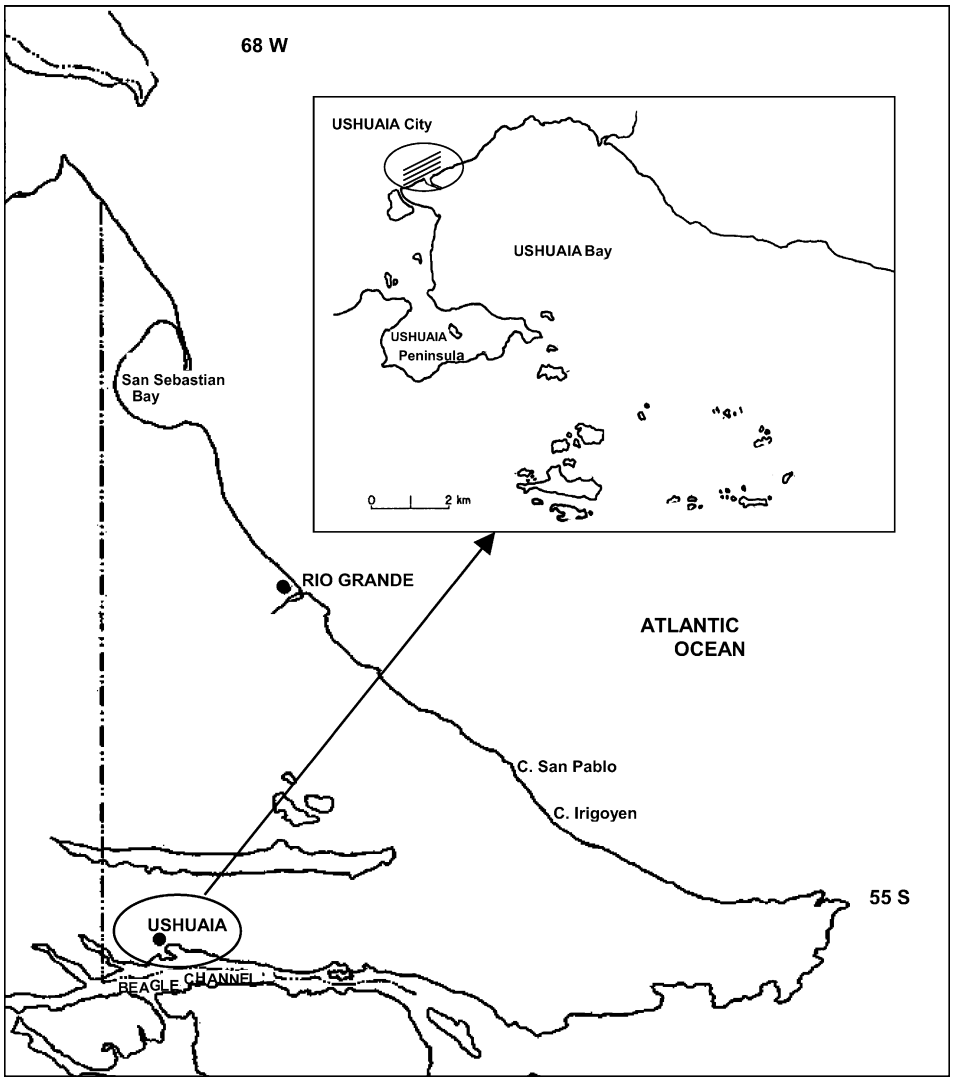

Fig. 1. Sampling area. Map of the south region of South America indicating the Beagle Channel. Insert: enlarged map showing the location of the sampling area.

deaurata limpets were sampled at 0.3–0.5 m water depth in shallow subtidal areas and they were scarcely exposed to air, whereas *N.* (*P.*) *magellanica* limpets are daily exposed to air for several hours. Animals had a mean shell length of 51 ± 1 and 51 ± 2 mm corresponding to a body fresh weight of 16 ± 1 and 12 ± 1 g, *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata*, respectively. No differentiation was made with respect to either sex or reproductive stage. Immediately after collection, digestive glands were separated and frozen at −40 °C until analyses.

2.2. Ascorbyl radical content ($A^{\bullet}$)

A Bruker ECS 106 spectrometer was used for $A^{\bullet}$ measurements. Homogenates from digestive glands were prepared in pure dimethylsulfoxide (DMSO) (1:3) and the spectra were scanned in the following

conditions: 50 kHz filed modulation, room temperature, microwave power 20 mW, modulation amplitude 1 G, time constant 655 ms, receiver gain 1×10^5, microwave frequency 9.81 GHz, and scan rate 0.18 G/s (Giulivi and Cadenas, 1993). Quantification was performed according to Kotake et al. (1996).

2.3. Ascorbate content (AH^-)

The content of ascorbate was measured according to Foyer et al. (1983). The acid extracts were neutralized with 1.25 M K_2CO_3, and the amounts of ascorbate were determined by addition of 5 U/ml ascorbate oxidase. Ascorbate was used as standard.

2.4. Content of protein carbonyl groups

Homogenates of digestive gland were centrifuged at 68,000 × *g* for 15 min, and the supernatant was incubated for 1 h at room temperature in the presence of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M hydrochloric acid. The proteins were precipitated with 20% (w/v) trichloroacetic acid and the pellet was washed with ethanol/ethyl acetate (1:1) three times. The precipitates were dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). Carbonyl content was determined at 360 nm ($\varepsilon = 22{,}000\ M^{-1}\ cm^{-1}$) (Reznick and Packer, 1994). Proteins were measured according to Bradford (1976).

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

The homogenates 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 Fraga et al. (1987).

2.6. Content of lipid radical by electron paramagnetic resonance (EPR)-spin trapping

The homogenates were prepared in potassium phosphate buffer (pH 7.4) containing 50 mM α-(4-pyridyl 1-oxide)-*N*-*t*-butyl nitrone (POBN). 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; and receiver gain, 2×10^4 (Jurkiewicz and Buettner, 1994). Quantification was performed according to Kotake et al. (1996).

2.7. Iron content

Isolated digestive glands of the molluscs were digested with an HNO_3 solution. After heating to dryness, the digests were dissolved in 2 ml 5% (v/v) HCl (Lawrie et al., 1991). Concentrations of iron in the extracts were measured spectrophotometrically after reduction with thioglycolic acid, followed by the addition of bathophenanthroline (Brumby and Massey, 1967).

2.8. Iron reduction rates

The rate of reduction by digestive gland homogenates was spectrophotometrically determined at 510 nm ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$) using 10 mM 2-2′-bipyridyl, 50 μM Fe–EDTA and 100 μM NADPH in 100 mM Tris–HCl buffer (pH 7.4) (Végh et al., 1988).

2.9. Generation of superoxide anion (O_2^-)

Generation of O_2^- was determined by monitoring the superoxide dismutase-sensitive generation of adrenochrome using 1 mM epinephrine, 40 mM potassium phosphate buffer (pH 7.4) and 0.5 μM SOD (Boveris, 1984). Determinations were carried out at 30 °C, measuring the absorbance at 480 nm ($\varepsilon = 6200\ M^{-1}\ cm^{-1}$).

2.10. Enzyme assays

Total SOD activity (EC 1.15.1.1) was determined according to Misra and Fridovich (1972). Catalase activity (EC 1.11.1.6) was assayed spectrophotometrically by the decomposition of H_2O_2 at 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). Protein measurements were performed according to Lowry et al. (1951).

2.11. Total thiol content

Total thiol content was assayed according to Sedlak and Lindsay (1968), employing 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and measuring absorbance at 412 nm, using glutathione as standard.

2.12. Content of lipid-soluble antioxidants

The content of α-tocopherol and β-carotene in the digestive glands homogenates supplemented with 100 mM SDS 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 (Desai, 1984). 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 isocratic HPLC analysis (Desai, 1984). D,L-α-Tocopherol (Sigma) and β-carotene were used as standards.

2.13. Nitrite content

Aliquots from the homogenates were treated with ethanol 95% (w/v) (Miranda et al., 2001). After centrifugation at 2500 × *g* during 10 min, the content of NO_2^- was determined according to Andronik-Lion et al. (1992). A standard solution of $NaNO_2$ was used for quantification.

2.14. Nitric oxide content

The digestive glands were homogenized in 100 mM potassium phosphate buffer (pH 7.4) and 10 mM *N*-methyl-D-glucamine-dithiocarbamate iron (MGD-Fe) as spin trap. 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, 5.983 G; time constant, 20.48 ms; and receiver gain, 1×10^5 (Kalyanaraman, 1996). Quantification was performed as previously described by Kotake et al. (1996).

2.15. Statistical analyses

Data in the text and tables are expressed as mean ± S.E.M. of four to six independent experiments, with two replicates in each experiment. Statistical tests were carried out using Statview for Windows, ANOVA, SAS Institute Inc., version 5.0.

3. Results

Electron paramagnetic resonance is the method of choice for studying paramagnetic molecules, that is, molecules with unpaired electrons. Biologically important paramagnetic species include free radicals and many transition elements. An electron could be thought of as a bar magnet with two possible magnetic states, and in a magnetic field, each unpaired electron adopts a certain orientation. As with a conventional magnet, the two orientations have different energy. EPR exploits the existence of these two different energy states in a magnetic field to provide an absorption spectroscopy based on transitions between these states. The experimental technique is based on the application of a strong homogeneous external magnetic field across the sample simultaneously with the electromagnetic radiation of the correct resonance frequency. The *g* value represents the actual resonance position and for a symmetrical EPR, spectrum can be obtained from the field position of the center of the resonance. The hyperfine splitting reflects the interaction of the unpaired electron with the nuclear magnetic moment (a_H, a_N). A typical EPR spectrum of ascorbyl radical ($A^\bullet$) in digestive gland from both limpets, with the characteristic two lines at $g = 2.005$ and $a_H = 1.8$ G, was observed (Fig. 2A (b, c)). DMSO itself was examined and no DMSO spin adduct was observed (Fig. 2A (a)). Neither $A^\bullet$ content, assessed by quantification of EPR signals, nor the $A^\bullet$/ascorbate (AH^-) ratio was significantly different in the digestive gland of both limpets (Table 1).

Oxidative damage to proteins was assessed as the content of carbonyl groups in soluble proteins. Digestive glands from *N.* (*P.*) *deaurata* and *N.* (*P.*) *magellanica* did not show any statistically significant difference in the content of carbonyl groups in the soluble proteins (7.8 ± 0.8 and 6.8 ± 0.9 nmol/mg protein, respectively).

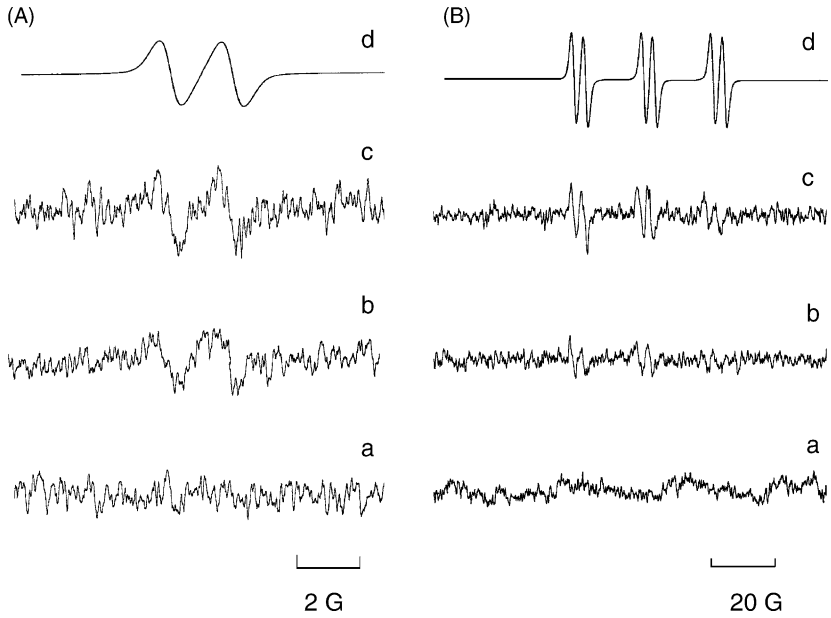

Fig. 2. EPR detection of ascorbyl and lipid radicals. (A) Detection of ascorbyl radical: (a) EPR spectra of dimethylsulfoxide, (b) typical EPR spectra of *N.* (*P.*) *magellanica* digestive gland, (c) typical EPR spectra of *N.* (*P.*) *deaurata* digestive gland, and (d) computer-simulated spectrum employing the following spectral parameters, $g = 2.005$ and $a_H = 1.8$ G, are shown; (B) lipid radical content in digestive glands from the molluscs: (a) EPR spectra of α-(4-pyridyl 1-oxide)-*N*-*t*-butyl nitrone (POBN) itself, (b) typical EPR spectra of *N.* (*P.*) *magellanica* digestive gland, (c) typical EPR spectra of *N.* (*P.*) *deaurata* digestive gland, and (d) computer-simulated EPR spectra exhibiting hyperfine splittings that are characteristic of POBN/lipid radicals, $a_N = 15.56$ G and $a_H = 2.79$ G (d), are shown.

Lipid peroxidation in the digestive glands from both organisms was estimated, both as the content of thiobarbituric acid-reactive substances and as the content of lipid radicals assessed by EPR. TBARS content was 34.8% lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* (Table 2). Lipid radicals in both digestive glands combined with the spin trap POBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of $a_N = 15.8$ G and $a_H = 2.6$ G (Fig. 2B (b, c)), in accordance with computer spectral-simulated signals obtained using those parameters (Fig. 2B (d)). POBN itself was examined and no POBN spin adduct was observed (Fig. 2B (a)). Lipid radical content was 36.5% lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* (Table 2).

Total iron content was 47.9% lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*, but the max-

Table 1
Ascorbyl radical content/ascorbate content ratio in *N.* (*P.*) *deaurata* and *N.* (*P.*) *magellanica*

	N. (*P.*) *deaurata*	*N.* (*P.*) *magellanica*
$A^{\bullet}$ (pmol/mg FW)	1.0 ± 0.4	1.1 ± 0.6
Ascorbate (nmol/mg FW)	26 ± 2	30 ± 3
$A^{\bullet}/AH^{-}$ (10^{-5})	3.8 ± 0.6	4.6 ± 0.9

Data are expressed as means ± S.E.M. of four to six independent experiments.

Table 2
Lipid peroxidation, iron content and superoxide generation rate in *N.* (*P.*) *deaurata* and *N.* (*P.*) *magellanica*

	N. (*P.*) *deaurata*	*N.* (*P.*) *magellanica*
Lipid radicals (pmol/mg FW)	192 ± 32	122 ± 29[a]
TBARS (pmol/mg FW)	23 ± 4	15 ± 2[a]
Iron content (nmol/mg FW)	1.9 ± 0.3	0.99 ± 0.07[a]
Iron reduction rate (pmol/(min mg FW))	84 ± 3	89 ± 10
O_2^- generation rate (pmol/(min mg FW))	0.37 ± 0.03	0.18 ± 0.04[a]

Data are expressed as means ± S.E.M. of four to six independent experiments.

[a] Significantly different at $P \leq 0.05$ from the value in *N.* (*P.*) *deaurata*.

Table 3
Antioxidant capacity in *N.* (*P.*) *deaurata* and *N.* (*P.*) *magellanica*

	N. (*P.*) *deaurata*	*N.* (*P.*) *magellanica*
Catalase (U/mg protein)	1.7 ± 0.2	2.3 ± 0.2[a]
SOD (U/mg protein)	1.4 ± 0.2	3.2 ± 0.6[a]
Total thiols (nmol/mg protein)	8 ± 2	11 ± 3
α-Tocopherol (nmol/mg protein)	5.3 ± 0.8	2.7 ± 0.4[a]
β-Carotene (nmol/mg protein)	14 ± 2	5.2 ± 0.6[a]

Data are expressed as means ± S.E.M. of four to six independent experiments.

[a] Significantly different at $P \leq 0.05$ from the value in *N.* (*P.*) *deaurata*.

imum capacity for iron reduction rate was not significantly different between both limpets (Table 2). Moreover, the rate of generation of superoxide anion was 51.9% lower in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* (Table 2).

Due to the critical role of the antioxidants to maintain the steady-state concentration of reactive species in living cells, the activity of the antioxidant enzymes SOD and catalase was evaluated in both molluscs. The activity of catalase and SOD was 35.3 and 128.6% higher in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*, respectively (Table 3). However, no significant differences were determined between the digestive glands of both molluscs regarding the content of total thiols, whereas α-tocopherol and β-carotene contents were significantly lower (49 and 62.9%, respectively) in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* (Table 3).

The content of NO_2^- was 1.6 ± 0.2 and 1.8 ± 0.3 nmol/mg FW for *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata*, respectively. EPR analysis of digestive glands of *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata* was performed with homogenates supplemented with the spin trap MGD-Fe to assess the content of nitric oxide. A distinctive EPR signal for the adduct Fe–MGD–NO ($g = 2.03$ and $a_N = 12.5$ G) was detected in the homogenates (Fig. 3 (b, c)). 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 double integrated by a computer attached to the EPR spectrometer to obtain the area

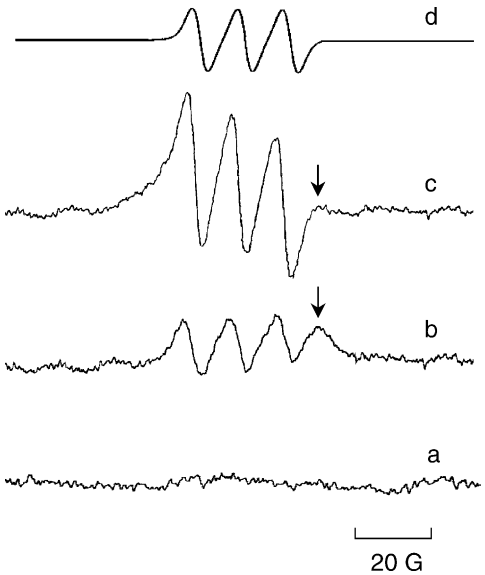

Fig. 3. Typical EPR spectra of the Fe–MGD–NO spin adduct: (a) spectra of *N*-methyl-D-glucamine-dithiocarbamate iron (MGD-Fe) itself, (b) typical EPR spectra of *N.* (*P.*) *magellanica* digestive gland, (c) typical EPR spectra of *N.* (*P.*) *deaurata* digestive gland, and (d) computer-simulated EPR spectra using the parameters, $g = 2.03$ and $a_N = 12.5$ G, are shown. The arrow (↓) indicates the spectrum of the [$(MGD)_2$/Cu] complex.

intensity, and then the concentration of spin adduct was calculated using the ratio of these areas. The spectrum obtained from *N.* (*P.*) *deaurata* homogenates showed a broad signal (Fig. 3 (b, c), indicated by an arrow) that could be part of the EPR spectrum of the [$(MGD)_2$/Cu] complex, resulting from the chelation of copper present in the mollusc. Quantification of the EPR signal showed a significant difference in the content of the Fe–MGD–NO adduct in digestive glands from *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata* (491 ± 137 and 839 ± 63 pmol/g FW, respectively).

4. Discussion

A number of biochemical alterations have been described and, in turn, proposed as basis for the irreversible injury that may follow exposure of cells to partially reduced oxygen species generated under stressful environmental conditions. The exposure of *N.* (*P.*) *magellanica* to diurnal immersion/emersion cycles, salinity variation and solar and UV radiation

does not appear to be responsible for developing alterations in the oxidative status at the hydrophilic cellular level, as compared to *N.* (*P.*) *deaurata*, suggesting the presence of antioxidant defense systems of different capacity. The data reported here compare oxidative status in two limpets species exposed to very different environmental conditions in their natural habitats. The $A^{\bullet}/AH^{-}$ ratio reflects the actual state of one part of the oxidative defense system mainly at the hydrophilic level and provides an early and simple diagnosis of stress (Kozak et al., 1997; Estevez et al., 2001; Galleano et al., 2002). Under the assay conditions, both molluscs showed no statistically significant differences either in the $A^{\bullet}/AH^{-}$ ratio or in the content of total thiols, suggesting that the exposure to more demanding environmental conditions did not affect oxidative stress at the level of the cytosolic medium.

However, a very different scenario was observed in the context of the lipophilic cellular medium, since significant decreases in the content of TBARS and lipid radicals were detected in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* (Table 2). Even though EPR detection of lipid radicals could be considered a fingerprint of radical presence, spin trapping studies cannot really distinguish among peroxyl ($ROO^{\bullet}$), alcohoxyl ($RO^{\bullet}$) and alkyl ($R^{\bullet}$) adducts owing to the similarity of the corresponding coupling constants (Jurkiewicz and Buettner, 1994).

Besides playing a key role in major intracellular processes and being a strict requirement for growth, iron is an effective catalyst for lipid peroxidation (Puntarulo and Cederbaum, 1988). The initiation reaction of lipid peroxidation is indicated by Reaction 1, where one proton is abstracted.

$$\text{Lipid} \xrightarrow[-H^{+}]{Fe^{2+}} \text{Lipid radical } (LR^{\bullet}) \quad (1)$$

The role of iron and superoxide anion in the initiation step of lipid peroxidation has been extensively discussed (Aust et al., 1985; Ursini et al., 1989; Puntarulo and Cederbaum, 1988). To study the possible role of iron in the catalysis of radical generation, the iron content in the digestive gland of both molluscs was examined. Lower levels of lipid peroxidation in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* correlate with a lower content of transition metals, in agreement with previous observations by comparing lipid peroxidation and iron content in *Laternula elliptica* and *Mya arenaria* (Estevez et al., 2002). On the other hand, the lower lipid radical formation in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* could also be ascribed to the lower concentration of catalytic active iron in *N.* (*P.*) *magellanica* or to a lower conversion rate of bound-to-bioavailable forms of iron. In an aerobic environment, iron is only available in the form of insoluble ferric iron (Fe^{3+}). Mechanisms of iron mobilization are still unclear in animal tissues; however, recent data suggest that reduction of bound iron might be the key primary event (Fontecave and Pierre, 1993). Fe^{3+} is reduced to Fe^{2+} to be incorporated into ferritin where it is stored as Fe^{3+} (inert form). By redox reaction with other cellular components (i.e. superoxide anion), Fe^{3+} from ferritin can be re-reduced to Fe^{2+}, released to the cytoplasm and become a catalyst in Fenton-type reactions. However, since Fe^{3+} reduction rates were not significantly different in both homogenates of digestive glands of the two molluscs, this does not seem to be the cause of the differential content of lipid radicals. Future studies will be required to assess the real magnitude of the catalytic active iron available in both organisms and to explore the possible role of lipid content and lipid saturation as a cause of the differential lipid peroxidation in both limpets.

Even though superoxide radical (O_2^{-}) is not particularly reactive, a reduced rate of generation of O_2^{-} radical in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata* could limit the formation of H_2O_2 and the ensuing production of highly reactive and noxious hydroperoxyl radicals deriving from H_2O_2. Reducing the formation of H_2O_2 would also reduce the formation of $^{\bullet}OH$ generated by Fenton (1894) or Haber and Weiss (1934) reactions, which can initiate lipid radical chain reactions. Moreover, the results presented here showed that the activity of both SOD (Reaction 2) and catalase (Reaction 3) are significantly higher in *N.* (*P.*) *magellanica* as compared to *N.* (*P.*) *deaurata*, contributing to decreased lipid peroxidation by scavenging of superoxide radical and hydrogen peroxide.

$$2O_2^{-} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2 \quad (2)$$

$k = 2 \times 10^9\ M^{-1}\ s^{-1}$ (Hassan and Scandalios, 1990).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (3)$$

$k = 10^7\,M^{-1}\,s^{-1}$ (Aebi, 1984).

The cellular reactive species include oxygen and nitrogen-derived radicals. Several considerations should be taken to estimate intracellular radical steady-state concentrations. According to Moroz and Gillette (1995), there are at least three NO inactivation mechanisms: (i) Interaction with various target biological molecules (e.g. free thiols, proteins with thiols or heme groups, other metal-containing proteins), but unfortunately there are not enough available data to estimate these values in molluscan tissues. (ii) Oxidation; in oxygenated solutions, NO is oxidized to NO_2, which rapidly hydrolyzes to produce nitrite (NO_2^-) and NO. $t_{1/2}$ may be only several seconds (Gilbert, 1994). However, because of the relatively low oxygen tension in molluscan blood, the lifetime of the released NO could be quite long and it is unlikely that pO_2 could be the limiting factor for NO inactivation in most molluscs (Moroz and Gillette, 1995). (iii) Interaction with different oxygen species, such as O_2^-.

$$O_2^- + NO \rightarrow ONOO^- \quad (4)$$

$k = 6.7 \times 10^9\,M^{-1}\,s^{-1}$ (Radi et al., 1995).

Thus, it could be postulated as an estimation that the rate of disappearance of O_2^- will mainly depend on the rate of reactions (2) and (4), according to Eq. (5).

$$\frac{-d[O_2^-]}{dt} = k_2[O_2^-][SOD] + k_4[O_2^-][NO] \quad (5)$$

Since in steady-state condition, the rate of disappearance of O_2^- equals the rate of generation of O_2^- (Eq. (6))

$$\frac{-d[O_2^-]}{dt} = \frac{d[O_2^-]}{dt} \quad (6)$$

From Eqs. (5) and (6), the concentration of O_2^- in steady-state concentration could be estimated by Eq. (7).

$$[O_2^-] = \frac{d[O_2^-]/dt}{(k_2[SOD] + k_4[NO])} \quad (7)$$

Assuming that 1 U SOD = 11.5 pmol SOD (Puntarulo et al., 1988), 90% of total fresh weight is water, and $\delta_{H_2O} = \delta_{\text{internal medium}} = 1\,g/ml$ and according to the data presented here for *N.* (*P.*) *magellanica*, $d[O_2^-]/dt$, [SOD] and [NO] would be $3.3 \times 10^{-12}\,M\,s^{-1}$, $3.3 \times 10^{-6}\,M$ and $6 \times 10^{-7}\,M$, respectively. For *N.* (*P.*) *deaurata*, the $d[O_2^-]/dt$, [SOD] and [NO] are calculated as $6.8 \times 10^{-12}\,M\,s^{-1}$, $2.5 \times 10^{-6}\,M$ and $10 \times 10^{-7}\,M$, respectively. O_2^- steady-state concentration calculated according to Eq. (7) was 3.2×10^{-16} and $5.9 \times 10^{-16}\,M$ for *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata*, respectively. Thus, it is likely that *N.* (*P.*) *magellanica* has a particular evolutionary adaptation to cope with extreme environmental conditions, by keeping tissue iron content low (either by decreasing its uptake or increasing its excretion) and antioxidant activities high. On the other hand, the content of lipid soluble antioxidants, α-tocopherol and β-carotene, in the digestive glands of *N.* (*P.*) *magellanica* were significantly lower than in *N.* (*P.*) *deaurata*, suggesting that the overall systems of defense include not only prevention of formation, but also active scavenging of the already formed active species, leading to a significant consumption of lipid-soluble antioxidants.

It is likely that *N.* (*P.*) *magellanica* has a particular evolutionary adaptation to extreme environmental conditions by keeping iron content low and antioxidant activities high. Taken as a whole, the data presented here indicated that coping with demanding external oxidative conditions requires a complex adjustment of the physiological pathways to ensure survival by minimizing intracellular damage. Thus, alteration of the habitat (e.g. by contamination with metals) could have a different degree of importance on the species regarding their complex adaptive responses to oxidative conditions. However, further studies are required to explore if other factors, besides environmental conditions, could play a role in the detected interpopulation differences in the oxidative and antioxidant parameters in the limpets.

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References

Abele, D., Burlando, B., Viarengo, A., Pörtner, H.O., 1998. Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the intertidal limpet *Nacella concinna*. Comp. Biochem. Physiol. 120B, 425–435.

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. J. Exp. Biol. 205, 1831–1841.

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Andronik-Lion, V., Boucher, J.L., Delaforge, M., Henry, Y., Mansuy, D., 1992. Formation of nitric oxide by cytochrome *P*450-catalyzed oxidation of aromatic amidoximes. Biochem. Biophys. Res. Commun. 185, 452–458.

Aust, S.D., Morehouse, L.A., Thomas, C.E., 1985. Role of metals in oxygen radical reactions. J. Free Radic. Med. 1, 3–25.

Boveris, A., 1984. Oxygen radicals in biological systems. Methods Enzymol. 105, 429–435.

Brumby, P.E., Massey, V., 1967. Determination of nonheme iron, total iron, and copper. Methods Enzymol. 10, 463–472.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Desai, I., 1984. Vitamin E analysis methods for animal tissues. Methods Enzymol. 105, 138–146.

Estevez, M.S., Malanga, G., Puntarulo, S., 2001. UV-B effect on Antarctic *Chlorella* sp. cells. J. Photochem. Photobiol. B: Biol. 62, 19–25.

Estevez, M.S., Abele, D., Puntarulo, S., 2002. Lipid radical generation in polar (*Laternula elliptica*) and temperate (*Mya arenaria*) bivalves. Comp. Biochem. Physiol. 132B, 729–737.

Fenton, H.J.H., 1894. Oxidation of tartaric acid in presence of iron. J. Chem. Soc. 65, 899–910.

Fontecave, M., Pierre, J.L., 1993. Iron: metabolism, toxicity and therapy. Biochimie 75, 767–773.

Foyer, C., Rowell, J., Walker, D., 1983. Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta 157, 239–244.

Fraga, C.G., Leibovitz, B.E., Tappel, A.L., 1987. Halogenated compounds as initiators of lipid peroxidation in tissue slices. Free Radic. Biol. Med. 3, 119–123.

Galleano, M., Aimo, L., Puntarulo, S., 2002. Ascorbyl radical/ascorbate ratio in plasma from iron overloaded rats as oxidative stress indicator. Toxicol. Lett. 133, 193–201.

Gilbert, D.L., 1994. Keeping reactive oxygen species (ROS) in their proper place. Ann. NY Acad. Sci. 738, 1–7.

Giulivi, C., Cadenas, E., 1993. The reaction of ascorbic acid with different heme iron redox states of myoglobin. FEBS Lett. 332, 287–290.

Haber, F., Weiss, J., 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Lond. Ser. 147A, 332–351.

Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219, 1–14.

Hassan, H.M., Scandalios, J.G., 1990. Superoxide dismutase in aerobic organisms. In: Alscher, R.G., Cumming, J.R. (Eds.), Stress Responses in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss Inc., New York, pp. 175–199.

Jacklet, J.W., 1997. Nitric oxide signaling in invertebrates. Invertebr. Neurosci. 3, 1–14.

Joseph, J.D., 1982. Lipid composition of marine and estuarine invertebrates. Part II. Mollusca. Progr. Lipid Res. 21, 109–153.

Jurkiewicz, B.A., Buettner, G.R., 1994. Ultraviolet light-induced free radical formation in skin: an electron paramagnetic resonance study. Photochem. Photobiol. 59, 1–4.

Kalyanaraman, B., 1996. Detection of nitric oxide by electron spin resonance in chemical, photochemical, cellular, physiological, and pathophysiological systems. Methods Enzymol. 268, 168–187.

Knowles, R.G., 1997. Nitric oxide, mitochondria and metabolism. Biochem. Soc. Trans. 25, 895–901.

Knowles, R.G., Moncada, S., 1994. Nitric oxide synthases in mammals. Biochem. J. 298, 249–258.

Kotake, Y., Tanigawa, T., Tanigawa, M., Ueno, I., Allen, D.R., Lai, C., 1996. Continuous monitoring of cellular nitric oxide generation by spin trapping with an iron–dithiocarbamate complex. Biochim. Biophys. Acta 1289, 362–368.

Kozak, R.G., Malanga, G., Caro, A., Puntarulo, S., 1997. Ascorbate free radical content in photosynthetic organisms after exposure to ultraviolet-B. Recent Res. Dev. Plant Physiol. 1, 233–239.

Lawrie, S., Tancock, N., Mc Growth, S., Roger, J., 1991. Influence of complexation on the uptake by plants of iron, manganese, copper and zinc. I. Effect of EDTA in a multimetal and computer simulation study. J. Exp. Bot. 42, 509–515.

Livingstone, D.R., Garcia Martinez, P., Michel, X., Narbonne, J.F., O'Hara, S.C.M., Ribera, D., Winston, G., 1990. Oxyradical generation as a pollution-mediated mechanism of toxicity. Funct. Ecol. 4, 415–424.

Livingstone, D.R., 1991. Organic xenobiotic metabolism in marine invertebrates. Adv. Comp. Environ. Physiol. 7, 45–185.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide: Biol. Chem. 5, 62–71.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43, 109–142.

Moncada, S., Higgs, E.A., 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J. 9, 1319–1330.

Moroz, L.L., Gillette, R., 1995. From Polyplacophora to Cephalopoda: comparative analysis of nitric oxide signalling in mollusca. Acta Biologica Hungarica 46, 169–182.

Morriconi, E., Calvo, J., 1993. Influencia ambiental en el crecimiento alométrico de la valva de *Nacella* (*P.*) *deaurata* (Gmelin 1971) en el Canal Beagle. Malacologia 35, 1–6.

Morriconi, E., 1999. Reproductive biology of limpet *Nacella* (*P.*) *deaurata* (Gmelin 1971) in Bahía Lapataia (Beagle Channel). Sci. Mar. 63, 417–426.

Puntarulo, S., Cederbaum, A.I., 1988. Comparison of the ability of the ferric complexes to catalyze microsomal chemiluminescence, lipid peroxidation and hydroxyl radical generation. Arch. Biochem. Biophys. 264, 482–491.

Puntarulo, S., Sanchez, R.A., Boveris, A., 1988. Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol. 86, 626–630.

Radi, R., Rubbo, H., Freeman, B.A., 1995. The double-edged action of nitric oxide on free radical-mediated oxidations. Ciencia Cultura (J. Braz. Ass. Advanc. Sci) 47, 288–296.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.

Sedlak, J., Lindsay, R.H., 1968. Estimation of total, protein bound, and non-protein sulfhydryl groups in tissue with Ellman's reagent. Anal. Biochem. 25, 192–205.

Ursini, F., Maiorano, M., Hochtein, P., Ernster, L., 1989. Microsomal lipid peroxidation: mechanisms of initiation. The role of iron and iron chelators. Free Radic. Biol. Med. 6, 31–36.

Végh, M., Marton, A., Horváth, I., 1988. Reduction of Fe (III) ADP complex by liver microsomes. Biochim. Biophys. Acta 964, 146–150.

Walker, S.T., Mantle, D., Bythell, J.C., Thomason, J.C., 2000. Oxidative-stress: comparison of species specific and tissue specific effects in the marine bivalves *Mytilus edulis* (L.) and *Dosinia lupinus* (L.). Comp. Biochem. Physiol. 127B, 347–355.

Winston, G.W., Di Giulio, R.T., 1991. Pro-oxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxic. 19, 137–167.