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THE MAGELLAN-ANTARCTIC CONNECTION: LINKS AND FRONTIERS AT HIGH SOUTHERN LATITUDES.
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Oxidative stress in gills of limpets from the Beagle Channel: comparison with limpets from the Antarctic*

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SUMMARY: The aim of this work was to study the oxidative profile of gills of two limpet species (*Nacella (Patinigera) magellanica* and *Nacella (Patinigera) deaurata*) (Gmelin, 1971) exposed to different environmental conditions. Due to the tidal characteristics of the Beagle Channel, *N. magellanica* are exposed to air twice daily for 3 to 5 hours each time, whereas *N. deaurata* are exposed to air for 3 hours only during spring tides. The different regime of exposure includes extreme temperatures under 0ºC during winter and more than 20°C in summer for *N. magellanica*, whereas *N. deaurata* are usually covered by more than 0.3 m of water at 4°C in winter and 11°C in summer. No significant differences were found between the two molluscs regarding the oxygen uptake, the content of α-tocopherol and β-carotene and the activities of the antioxidant enzymes catalase and superoxide dismutase. Lipid peroxidation in gills was estimated as the content of lipid radicals, assessed by electron paramagnetic resonance (EPR). Lipid radical content and total iron content were respectively 80.6 and 62% lower in *N. magellanica* than in *N. deaurata*. A typical EPR spectrum of ascorbyl radical in gills from both limpets was observed. Both the ascorbyl radical content and the ascorbyl radical content/ascorbate content ratio were significantly lower in *N. magellanica* than in *N. deaurata*. In the Antarctic *Nacella concinna* inhabits all levels of the littoral zone. Limpets at the highest level in the intertidal showed significantly increased activities of both catalase and superoxide dismutase as compared to their intertidal and subtidal relatives. Thus, it seems that Antarctic high intertidal conditions, involving regular exposure to air and presumably also thermal stress on sunny days during the Antarctic summer, cause a necessity for *N. concinna* to ward off higher oxygen radical species production by increasing its antioxidant defence. Taken as a whole, the data presented here indicate that coping with environmentally demanding conditions requires a complex adjustment of the physiological metabolic pathways to ensure survival by minimising intracellular damage.

Keywords: Antarctic region, antioxidant enzymes, ascorbyl radical, ascorbate, Subantarctic region

RESUMEN: ESTRÉS OXIDATIVO EN BRANQUIAS DE LAPAS DEL CANAL DEL BEAGLE: COMPARACIÓN CON LAPAS DE LA REGIÓN ANTÁRTICA.El objetivo del presente trabajo fue estudiar el perfil oxidativo en branquias de dos especies de lapas (*Nacella (Patinigera) magellanica* and *Nacella (Patinigera) deaurata*) (Gmelin, 1971) expuestas a diferentes condiciones ambientales. Debido a las características de las mareas en el Canal del Beagle, las lapas *N. magellanica* están expuestas diariamente al aire dos veces durante 3 a 5 h cada vez, pero las lapas *N. deaurata* están expuestas al aire durante 3 h, solamente en las mareas de primavera. El diferente régimen de exposición incluye temperaturas extremas debajo de 0°C durante el invierno y más de 20°C en verano para *N. magellanica*, mientras que las lapas *N. deaurata* están habitualmente cubiertas por más de 0.3 m de agua que alcanza una temperatura de 4°C en invierno y 11°C en verano. No se observaron cambios significativos en ambos moluscos con respecto al consumo de oxígeno, el contenido de α-tocoferol, β-caroteno y la actividad de las enzimas antioxidantes catalasa y superóxido dismutasa. La peroxidación lipídica fue estimada como el contenido de radicales lipídicos, determinados por resonancia paramagnética electrónica (EPR). El contenido de radicales lipídicos y de hierro total fue de 80,6 y 62% menor en *N. magellanica* en comparación con *N. deaurata*. Se observó un típico espectro de EPR del rad-

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ical ascorbilo en branquias de ambas lapas. Tanto el contenido de radical ascorbilo como el cociente contenido de radical ascorbilo/contenido de ascorbato fue significativamente menor en *N. magellanica* en comparación con *N. deaurata*. Estudios realizados en *Nacella concinna* (Antártida) indican que esta especie cuenta con mayor actividad de catalasa y superóxido dismutasa que sus congéneres de la región subantártica. Por lo tanto, condiciones de baja marea en la región antártica, con una exposición continua al aire y probablemente estrés térmico en días soleados durante el verano, podrían ser responsables de la necesidad de *N. concinna* de contar con una mayor protección antioxidante. Tomados en su conjunto, los datos presentados aquí indican que soportar condiciones ambientales demandantes requiere un complejo ajuste de las vías metabólicas fisiológicas para asegurar la supervivencia minimizando el daño intracelular.

Palabras clave: enzimas antioxidantes, radicales ascorbilos, ascorbato, región subantártica, región antártica.

INTRODUCTION

Since the discovery of the importance of radical reactions in normal biological processes, there has been an explosion of research into pro-oxidant and antioxidant processes, principally in mammalian systems (Halliwell and Gutteridge, 1984). The normal fate of most of the molecular oxygen consumed by animals is tetravalent reduction to water coupled with the oxidation of food and the production of energy. Partial reduction results in the formation of 'reactive oxygen species' (ROS), including superoxide anion radical (O_2^-), hydroxyl radical ($^\bullet OH$), peroxyl radical ($ROO^\bullet$), alkoxyl radical ($RO^\bullet$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$). It has been estimated that about 1–3% of O_2 consumed in animal systems is converted to ROS (Halliwell and Gutteridge, 1984). Moreover, iron can catalyze the conversion of H_2O_2 into $^\bullet OH$, via Fenton or Haber-Weiss reactions. Of more recent interest has been ROS production and resulting oxidative damage as a mechanism of toxicity in aquatic organisms (Di Giulio *et al*., 1989; Livingstone *et al*., 1990; Livingstone, 1991; Winston and Di Giulio, 1991). Relatively little information is available on the mechanistic aspects of redox cycling in aquatic organisms, but measurements of oxygen consumption and ROS have demonstrated the production of O_2^-, the dismutation of O_2^- to H_2O_2, and the involvement of O_2^- and/or H_2O_2 in the production of $^\bullet OH$ (Di Giulio *et al*., 1989; Livingstone, 1991; Estevez *et al*., 2002). ROS produced in biological systems are detoxified by antioxidant defences, which are ubiquitous in aerobic species and vary in different tissue types. They are widely found in aquatic organisms and their presence, properties and other characteristics have been extensively reviewed (Di Giulio *et al*., 1989; Livingstone, 1991; Viarengo *et al*., 1998).

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. *Nacella magellanica* inhabits the middle and the upper intertidal zones, whereas *N. deaurata* lives in the lower intertidal and the subtidal zone (Morriconi and Calvo, 1993; Morriconi, 1999). Though they live in the same area, the difference in shore level location affects the animal's exposure to aerial or marine environmental conditions. Due to the tidal characteristics of the Beagle Channel, *N. magellanica* are exposed to air twice daily for 3 to 5 hours each time, and *N. deaurata* are exposed to air daily for 3 hours only during spring tides (Morriconi and Calvo, 1993; Morriconi, 1999). The different regime of exposure includes extreme temperatures below 0ºC in winter and above 20 ºC in summer for *N. magellanica*, whereas *N. deaurata* are usually covered by more than 0.3 m of water at 4ºC in winter and 11ºC in summer. Peculiarities of membrane lipids in marine organisms, particularly high contents of unsaturated fatty acids (Joseph, 1982), suggest a special susceptibility to lipid peroxidation. These organisms also showed a specific response of their antioxidant system that reflects their adaptation to the highly variable environment (Abele-Oeschger and Oeschger, 1995; Abele *et al*., 1998 a, 1998b, 2002).

In molluscs, oxygen is mainly taken up through the gills, and gill tissue could be the main target for oxidative injury. Lipid radical content was assayed by EPR and antioxidant capacity was studied by assaying the activities of superoxide dismutase (SOD) and catalase. The content of non-enzymatic antioxidants (α-tocopherol, β-carotene, and ascorbate) and the ratio of ascorbyl radical/ascorbate content was studied in both animals. The aim of this work was to characterise the oxidative status of gills of two limpet species naturally exposed to different environmental conditions on an intertidal rocky shore in the Subantarctic Beagle Channel.

MATERIALS AND METHODS

Collection of animals

In July 2002, the limpets *N. magellanica* and *N. deaurata* were collected in the intertidal of Punta Occidental (54°50´S, 68°20´W) in the Beagle Channel, at the southern tip of South America. *N. deaurata* limpets were sampled at 0.3-0.5 m water depth in shallow subtidal areas. The animals had a mean shell length of 51 ± 1 and 51 ± 2 mm corresponding to a body fresh weight (free of shell) of 16 ± 1 and 12 ± 1 g for *N. magellanica* and *N. deaurata* respectively. No differentiation was made with respect to either sex or reproductive stage. Immediately after the collection of the animals, the gills were dissected and frozen at -40ºC for later analysis. A total of 6 samples were used for each independent assay, with 2 replicates in each experiment.

Intertidal and subtidal specimens of the limpet *Nacella concinna* were sampled at King George Island, Antarctica. Intertidal specimens were collected from a rocky shore area near Jubany Base (62°14´S, 58°38´W), which at low ebb tides is air-exposed for between 2 and 4 hours. Here, N. *concinna* colonises shallow intertidal pools, which have a maximum depth of 0.25 m and remain water-covered throughout the whole ebb tide. The intertidal limpets were air-exposed in moist rocky niches for several hours during low tides. Shell length varied from 25 to 40 mm. Live animals were kept for 2 days at most in aquaria at the Dallmann Laboratory, Jubany Base, with seawater from the cove. Enzyme activities were measured directly after sacrificing the animals, because liquid nitrogen was not available for freeze clamping and storing samples at the base.

Oxygen consumption measurements

The oxygen uptake under laboratory conditions (temperature 4ºC and salinity 3.12 PSU) was determined in the whole animal according to Peck and Veal (2001) using an Rank Brothers oxymeter (High Street, Bottisham, Cambridge CB5 9DA, England).

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

The metabolism of ascorbate (AH^-) is an issue of particular importance since during its antioxidant action, AH^- undergoes two consecutive one-electron oxidations to dehydroascorbic acid (DHA) with intermediate formation of the ascorbyl radical (Hubel *et al*., 1997). $A^{\bullet}$ has a relatively long lifetime compared to other free radicals and is easily detectable by EPR even at room temperature in aqueous solution (Buettner and Jurkiewicz, 1993). In contrast to $A^{\bullet}$, AH^- and DHA are EPR silent (Hubel *et al*., 1997). Thus, there is an increasing interest in the use of $A^{\bullet}$ content in biological tissues as an informative, non-invasive and natural indicator of oxidative stress (Roginsky and Stegmann, 1994), and as an indicator of the phagocytic immune response (Halliwell and Gutteridge, 1984).

A Bruker ECS 106 spectrometer was used for $A^{\bullet}$ measurements. The homogenates were prepared in dimethylsulfoxide (DMSO) and the spectra were scanned under the following conditions: field modulation 50 kHz, room temperature, microwave power 20 mW, modulation amplitude 1 G, time constant 655 ms, receiver gain 1 x 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).

Ascorbate content (AH^-)

Ascorbate content was measured according to Foyer *et al*. (1983). The acid extracts were neutralised 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.

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

The homogenates were prepared in 50 mM α-(4-pyridyl 1-oxide)-N-t-butyl nitrone (POBN). EPR spectra were obtained at room temperature using an ECS 106 Bruker spectrometer operating at 9.81 GHz with a 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 x 10^4 (Jurkiewicz and Buettner, 1994). Quantification was performed according to Kotake *et al*. (1996).

Content of thiobarbituric acid reactive substances (TBARS)

The content of thiobarbituric acid reactive substances (TBARS) was measured according to Uchiyama and Mihara (1978) as an index of lipid peroxidation.

Iron content

Isolated gills 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).

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).

Content of lipid soluble antioxidants

The content of α-tocopherol and β-carotene in the gill homogenates supplemented with 100mM 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 methanol:hexane (1:4). 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.

Statistical analyses

Data are expressed as means ± SEM of 6 independent samples, with 2 replicates in each experiment. Statistical tests were carried out using Statview for Windows, ANOVA, SAS Institute Inc. version 5.0.

RESULTS

Tidal level, and thus air exposure, differ substantially for *N. deaurata* and *N. magellanica*. However, gills isolated from *N. deaurata* and *N. magellanica* showed no statistically significant difference with respect to the oxygen uptake or activity of the antioxidant enzymes catalase and superoxide dismutase (Table 1). Moreover, no significant differences were determined between the gills of the two molluscs regarding the content of α-tocopherol and β-carotene (Table 1).

TABLE 1. – Oxygen uptake and antioxidant capacity in *N. deaurata* and *N. magellanica*. Data are expressed as means SEM of 6 independent samples.

	N. deaurata	*N. magellanica*
Oxygen uptake (10^{-2}) (μmol/h/g FW)	8 ± 1	10 ± 2
Catalase (U/mg prot)	4 ± 1	3 ± 1
SOD (U/mg prot)	3 ± 1	4 ± 1
α-Tocopherol (10^{-1}) (nmol/mg prot)	7 ± 2	6 ± 1
β-carotene (10^{-1}) (nmol/mg prot)	14 ± 4	9 ± 2

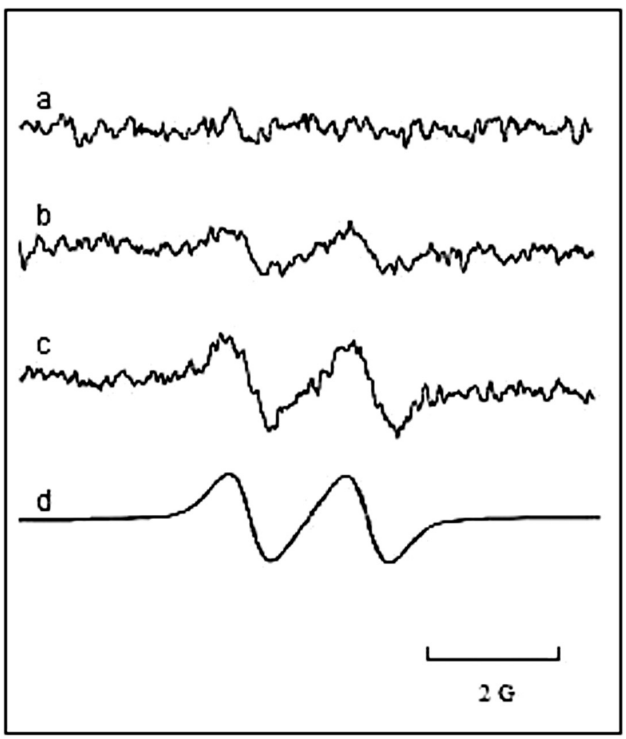

FIG. 1. – EPR detection of ascorbyl radicals. (a) EPR spectra of DMSO, (b) Typical EPR spectra of *Nacella magellanica* gills, (c) Typical EPR spectra of *Nacella deaurata* gills, and (d) computer simulated-spectra.

A typical EPR spectrum of ascorbyl radical ($A^{\bullet}$) was recorded in gills from both limpets. The EPR spectrum showed the characteristic two lines at g = 2.005 and a_H = 1.8 G (Fig. 1 b, c), in accordance with computer spectral simulated signals obtained using the parameters given in the Material and Methods section (Fig. 1 d). DMSO was examined and no DMSO spin adduct was observed (Fig. 1 a). $A^{\bullet}$ con-

TABLE 2. – Ascorbyl radical content/ascorbate content ratio in *N. deaurata* and *N. magellanica*. Data are expressed as means SEM of 6 independent samples.

	N. deaurata	*N. magellanica*
$A^{\bullet}$ (10^{-1}) (pmol/mg FW)	60 ± 20	36 ± 2*
Ascorbate (nmol/mg FW)	14 ± 2	17 ± 2
$A^{\bullet}/AH^{-}$ (10^{-5})	49 ± 6	25 ± 7*

* significantly different at $P < 0.05$.

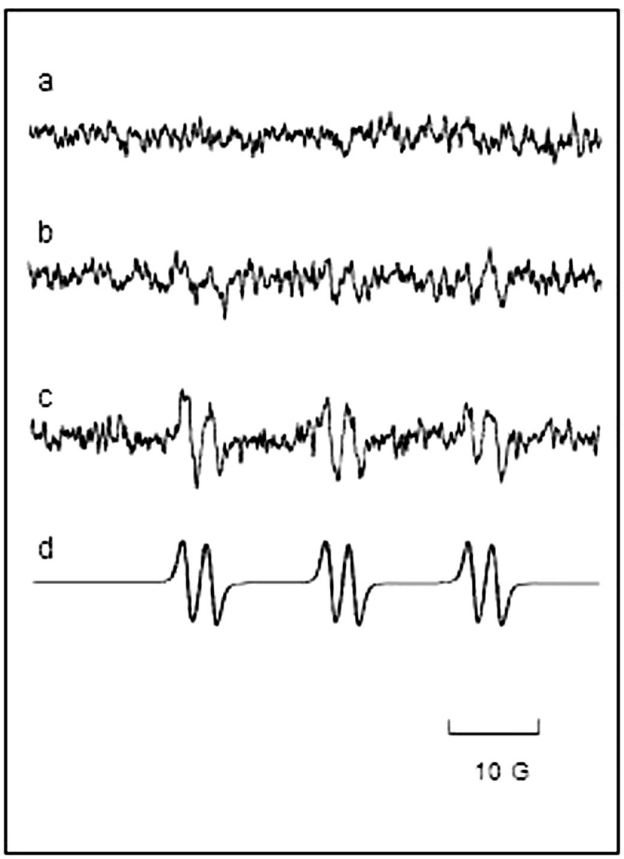

FIG. 2. – Typical EPR spectra of the POBN spin adduct of lipid radicals. (a) Spectra of POBN, (b) Typical EPR spectra of *Nacella magellanica* gills, (c) Typical EPR spectra of *Nacella deaurata* gills, and (d) computer simulated EPR spectra.

tent, assessed by quantification of EPR signals, was significantly lower (40%) in *N. magellanica* than in *N. deaurata* (Table 2). Ascorbate plays a central metabolic role since it can act as an antioxidant and a pro-oxidant and its oxidation leads to $A^{\bullet}$ generation (Sadrzadeh and Eaton, 1988; Arrigoni, 1994). Ascorbate pro-oxidant activity is a result of its ability to reduce transition metals (especially iron) causing them to react with oxygen and initiators of lipid radical reactions (Wills, 1966). Ascorbate antioxidant activity consists in the ability to reduce various types of radicals, including peroxyl radicals that propagate lipid peroxidation, and to regenerate the

TABLE 3. – Lipid peroxidation, and iron content in gills from *N. deaurata* and *N. magellanica*. Data are expressed as means SEM of 6 independent samples.

	N. deaurata	*N. magellanica*
Lipid radicals (pmol/mg FW)	412 ± 98	80 ± 36*
Iron content (pmol/mg FW)	4453 ± 154	1930 ± 414*

* significantly different at $P < 0.05$.

antioxidant α-tocopherol from the oxidised form (Doba *et al*., 1985). The $A^{\bullet}/AH^{-}$ ratio, which serves as an appropriate and accurate indicator of oxidative stress (Galleano *et al*., 2002), was significantly lower in the gills of the intertidal *N. magellanica* than in the subtidal *N. deaurata* (Table 2). The exposure of high intertidal *N. magellanica* to variable environmental conditions did not cause an increased oxidative status at the hydrophilic cellular level, as compared to subtidal *N. deaurata*. The $A^{\bullet}/AH^{-}$ ratio indicates that tissue oxidation might be decreased by 49% in the gills of the intertidal *N. magellanica* as compared to the subtidal *N. deaurata*.

The lipid peroxidation in both organisms was estimated as the content of lipid radicals assessed by EPR. The lipid radicals combined with the spin trap POBN 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 (Fig. 2 b, c), in accordance with computer spectral simulated signals obtained using the overall mentioned parameters (Fig. 2 d). POBN was examined and no POBN spin adduct was observed (Fig. 2 a). Even though these constants could be assigned to lipid radicals, spin trapping studies cannot distinguish between peroxyl ($ROO^{\bullet}$), alcohoxyl ($RO^{\bullet}$) and alkyl ($R^{\bullet}$) adducts, owing to the similarity of the corresponding coupling constants (Jurkiewicz and Buettner, 1994). Bulk lipid radical content was significantly lower (80.6%) in the high intertidal *N. magellanica* than in the subtidal *N. deaurata* (Table 3). As no differences of tissue oxygen consumption and antioxidant enzyme activities were found, the higher levels of lipid peroxide formation may result, among other factors, from an elevated accumulation of transition metals in the tissues of the subtidal animals. To study the possible role of iron in the catalysis of lipid peroxidation, the gills iron content in both molluscs was examined. The overall iron content was found to be 62% lower in *N. magellanica* than in subtidal *N. deaurata* (Table 3).

The activity of catalase in the gills of the Antarctic mollusc *N. concinna* was 44 ± 22 U/mg

prot in animals from the subtidal areas, 140 ± 40 U/mg prot in animals from the intertidal areas, and 213 ± 57 U/mg prot in animals temporarily air-exposed on intertidal rocks. Superoxide dismutase activity was 4 ± 2 U/mg prot in subtidal limpets, 4 ± 3 U/mg prot in intertidal limpets, and 13 ± 2 U/mg prot in the high intertidal limpets. TBARS concentrations were 42 ± 7 nmol g fresh weight in subtidal limpets and 33 ± 9 in intertidal limpet gills, but were not measured in the high intertidal limpets.

DISCUSSION

A number of biochemical alterations have been described and, in turn, proposed as a basis for the injury that may follow exposure of cells to partially reduced oxygen species generated under stressful environmental conditions. The data reported in the present work compare the gill oxidative status of three limpet species from Subantarctic and Antarctic environments exposed to different abiotic conditions in their natural habitats. Limpets from the high intertidal, such as *N. magellanica* and *N. concinna* from King George Island, could both undergo transient metabolic depression during low tides, a behaviour common to intertidal molluscs during air exposure (Pannunzio and Storey, 1998). This shell closure strategy prevents desiccation and predation during low tides and triggers a hypoxic response in the enclosed animal, consisting in metabolic reduction and a switch of anaerobic metabolism (Ortmann and Grieshaber, 2003). Thus, adaptation to high shore environments involves extended periods of metabolic reduction which may reduce the overall rate of metabolically produced oxygen radicals compared to subtidal limpet species. Moreover, oxidative stress may be enhanced by a frequent shift between low oxygen and normoxic conditions, comparable to ischemia-reperfusion insult.

The $A^{\bullet}/AH^{-}$ ratio reflects the actual state of the oxidative defence system mainly in the hydrophilic phase and provides an early and simple means of diagnosing oxidative stress (Kozak *et al.*, 1997; Estevez *et al.*, 2001; Galleano *et al.*, 2002). In the case of the two species from the Beagle Channel, a lower $A^{\bullet}/AH^{-}$ ratio is indicative of lower oxidative stress levels in the gills of the intertidal species *N. magellanica* as compared to the subtidal *N. deaurata*. The same tendency is found in the lipophilic cellular phase. A significantly lower lipid radical content was detected by EPR in the high intertidal *N. magellanica* as compared to the subtidal *N. deaurata* (Table 3). Besides playing a key role in the functioning of important metabolic enzymes and redox compounds, iron is a strict requirement for growth, but also in its reduced Fe^{2+} form it is an effective catalyst for lipid peroxidation (Puntarulo and Cederbaum, 1988). The initiation reaction of lipid peroxidation is indicated by reaction 1, in which one proton is abstracted.

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

The role of iron and superoxide anion in the initiation step of lipid peroxidation has been extensively discussed (Aust *et al.*, 1985; Puntarulo and Cederbaum, 1988; Ursini *et al.*, 1989). Lower levels of lipid peroxidation in *N. magellanica* as compared to *N. deaurata* correlate with a lower iron content. This agrees with previous observations in which we found a correlation of lipid peroxidation rates and iron content in digestive gland material of the mud clams *Laternula elliptica* and *Mya arenaria* (Estevez *et al.*, 2002). Additionally, the lower lipid radical formation in *N. magellanica* as compared to *N. deaurata* could be ascribed to the lower conversion rate of bound to bio-available forms of iron in *N. magellanica*. In this regard, gastropods are known to accumulate iron also in non-bioactive forms, while there are no reported data that these species possess regulatory mechanisms for iron uptake. The higher lipid radical formation in *N. deaurata* compared to *N. magellanica* could be mainly ascribed to the higher concentration of catalytically active iron in *N. deaurata* or to a higher conversion rate of bound to bioavailable iron. In an aerobic environment, iron is only available in the form of insoluble ferric iron (Fe^{3+}). Mechanisms of iron mobilisation are still unclear. 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). However, by redox reaction with other cellular components (i.e. superoxide anion), Fe^{3+} from ferritin could be reduced to Fe^{2+}, be released to the cytoplasm and become a catalyst in Fenton-type reactions. Future studies are required to assess the amount of the catalytically active iron in gills from both organisms and to explain what intrinsic (ctenidial intracellular haemoglobins, Terwilliger and

TABLE 4. – Ratio of lipid radical content to α-tocopherol content in Subantarctic molluscs.

	N. deaurata	*N. magellanica*
Gills (10^{-2})	6 ± 2*, **	5 ± 1*, **
Digestive glands (10^{-1})	13 ± 1**	14 ± 4**

* significantly different from digestive gland values at P< 0.05.
** Taken from Malanga *et al*. (2004).

Terwilliger, 1985) or extrinsic factors cause the higher iron content of the subtidal limpets. Moreover, different food algae might cause differences of lipid content and lipid saturation levels, which may be the basis of the disparate rates of lipid peroxidation in the two limpet species.

The content of the antioxidants α-tocopherol and β-carotene in the gills of *N. magellanica* was not significantly different from that in *N. deaurata*. Hence, we hypothesise that the basic defence strategy in these animals is aimed at preventing the formation of the active species by controlling the iron uptake.

Lipid radical content could be understood as an indicator of radical-dependent damage to lipids, and α-tocopherol content as the most efficient antioxidant protection in the lipid compartment. The ratio of lipid radical content to α-tocopherol content (damage/protection) can be considered as an index of oxidative stress levels in the lipid phase (Galleano *et al*., 2002). Significantly lower ratios in gills than in digestive glands were measured in both species (Malanga *et al*., 2004). Table 4 shows non-significant differences of this ratio in gills or digestive glands between the two limpets. Under physiological control conditions, pro-oxidant and antioxidant processes seem well balanced in the digestive glands and gills of molluscs, but this balance is not perfect and some low-level oxidative damage to key molecules such as DNA, protein and lipid occurs physiologically over the life span.

Oxidative damage associated with an imbalance of pro- and antioxidant functions occurs when cellular or whole animal metabolic functions are severely disturbed under severe physiological stress. The intertidal *N. magellanica* may have developed a particular evolutionary strategy to cope with the extreme fluctuations in its high shore habitat, for example by controlling the tissue iron content to minimise oxidative tissue damage and thus render the animals less susceptible to environmental stress.

Catalase and superoxide dismutase activity in gill tissues from the Subantarctic limpets can be compared to data from the Antarctic congener *N. concinna*. While SOD activities of sub- and intertidal specimens were in the same range in Subantarctic (Beagle Channel) and Antarctic animals, catalase activities were tremendously higher in the Antarctic species. This agrees with a general view that Antarctic species may be especially prone to suffering oxidative stress in their low-temperature, high-oxygen environment, and many species therefore acquire higher antioxidant levels (for a review see Abele and Puntarulo, 2004). However, as the data were obtained with slightly different protocols, caution must be taken in comparing the absolute values of catalase activity, until further confirmation is obtained. With respect to a "stress gradient" between high and low shore levels, our Antarctic data show that high intertidal limpets clearly up-stage their intertidal and subtidal relatives with respect to activities of both antioxidant enzymes analysed. Thus, without knowing the iron levels in *N. concinna* specimens, we conjecture that Antarctic high shore conditions, involving regular exposure to air and presumably also thermal stress on sunny days during the Antarctic summer season, lead to the necessity for *N. concinna* to ward off higher oxygen radical species production by increasing its antioxidant defence. However, these data provide only an initial hint and open up the field for more comprehensive comparative investigations.

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