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Fe, oxidative and nitrosative metabolism in the Antarctic limpet *Nacella concinna*☆

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ABSTRACT

The hypothesis of this work was that oxidative and nitrosative metabolism in the digestive gland (DG) of two limpet populations (intertidal and subtidal) of the Antarctic species *Nacella concinna* show different behavior when they were exposed to either intermittent (intertidal) or constant (subtidal) natural Fe. Total Fe content and labile Fe pool were higher in the DG of the subtidal compared to the intertidal population. However, no significant differences between populations were seen on the Fe atoms content of the isolated ferritin. Ascorbyl radical content was 2.0 ± 0.4 and 6.5 ± 0.8 pmol/mg FW in the DG of the intertidal and subtidal animals, respectively. Lipid damage, assessed as content of thiobarbituric reactive substances, was different between the tissues of intertidal and subtidal samples, 491 ± 102 and 1242 ± 367 pmol/mg FW, respectively. Catalase and superoxide dismutase activities showed no differences between the limpets. Nitric oxide (NO) content was 25 ± 3 and 22 ± 2 pmol/mg FW in DG from intertidal and subtidal animals, respectively. NO synthase-like (NOS-like) activity was evaluated supplementing the samples with the enzyme co-factors, and the inhibitory effect of Nω-nitro-L-arginine methyl ester hydrochloride was tested. NO generation rate was 3.4 ± 0.3 and 4.7 ± 0.6 pmol/min mg FW in DG from the intertidal and subtidal population, respectively. These results showed that the oxidative condition of the limpet population constantly covered by the Fe enriched water is more affected than the intertidal population. However, the nitrosative metabolism seems to be independent of the environmental high Fe content since similar NO steady state concentration and NOS-like activity were measured in both populations.

1. Introduction

The Antarctic marine environment is characterized by its physical extreme parameters, with low marine and atmospheric temperatures, an annual light extreme pattern, strong winds, and isolation, among others. King George Island has a volcanic origin with a sediment rich in Fe, Mn, Cu and Zn (5 and 7% Fe, Rey et al., 1995; Tatur et al., 1999). Specially in summer, surface erosion from glacial melting enriches seawater with Fe (Ahn et al., 1996; Dierssen et al., 2002). Fe is involved in a wide array of biological functions since it has access to different redox potentials and can participate in many electrons transfer reactions (Galatro et al., 2007) and biomineralization. Limpets incorporate Fe, as Fe_3O_4, in the mineralized deposits of the teeth of the radula to toughen them (Lu et al., 1995). The labile Fe pool (LIP) is a low-molecular-weight complex of weakly chelated Fe consisting of both forms of ionic Fe (Fe^{2+} and Fe^{3+}) associated with ligands with poor affinity for Fe ions. The LIP, representing a minor fraction of the total cellular Fe (3–5%, Kakhlon and Cabantchik, 2002), is catalytically active conducing to the generation of reactive oxygen species (ROS) by the partial reduction of O_2 by the Fenton and Haber–Weiss reactions. Several biomolecules are damaged by the effect of ROS causing protein oxidation, DNA strand breaks and lipid peroxidation (Harrison and Arosio, 1996). The extracellular transferrins and the intracellular ferritins (Ft) are two specialized Fe-binding proteins, which maintain Fe^{3+} inside them; helping the cells to avoid the damaging effects of Fe catalytic actions. Ft is a nanobox protein which may contain and maintain up to 4500 Fe atoms (Murgia et al., 2002), avoiding aggregation in toxic precipitates. Ft is present in archeobacteria, eubateria, plants, mammals (Arosio and Levi, 2002) and invertebrates (von Darl et al., 1994; Dunkov et al., 1995; Charlesworth et al., 1997). In some molluscs, such as in limpets, bivalves and snails, it was identified the capacity to produce a great quantity of Ft that may act as a transport agent (Bottke, 1982; Burford et al., 1986; Webb et al., 1986; Harrison and Arosio, 1996; Zhang et al., 2003). Ft was recognized in the snail *Littorina littorea* (Taylor, 1995), in the limpet *Cellana toreuma* (Lu et al., 1995), in oysters and lobsters (Durand et al., 2002) and in the bivalve *Laternula elliptica* (González and Puntarulo, 2011). In the chiton *Clavarizona*

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hirtosa, the Ft from the hemolymph functions as Fe transporter to help in the radula mineralization (Kim et al., 1986).

On the other hand, antioxidant capacity is a main issue in the oxidative metabolism in the cells. A complex antioxidant system, including superoxide dismutase (SOD) and catalase (CAT) enzymes, contributes to cell living and development in an environment rich in O_2 by keeping reactive species concentrations in a controlled and low steady state.

The nitric oxide (NO) may also produce reactive nitrogen species (RNS) characterized by causing cellular damage through nitrosative stress that affects enzymatic functions, DNA injury and/or lipid peroxidation (Sergent et al., 1997). Paradoxically, the NO can act as an antioxidant as well, ending lipid oxidation chain reactions (O'Donnell and Freeman, 2001). In animals, NO production is associated to an enzyme family known as NO synthases (NOS). NOS-like enzyme activity has been detected in marine, freshwater and terrestrial molluscs (Jacklet, 1997; Stefano and Ottaviani, 2002) and bivalves, such as *Mya arenaria* and *L. elliptica* (González et al., 2008a; González and Puntarulo, 2011), but no direct measurement of NOS-like activity was reported in any gastropod tissues. On the other hand, NO and superoxide anion (O_2^-) react generating the dangerous species peroxinitrite ($ONOO^-$) linking oxidative and nitrosative metabolisms.

Many species of molluscs are being used as alimentary resources, and contamination or functional stress indicators in shore areas. The limpet *Nacella concinna*, a stress resistant species in contrast with other stenothermal Antarctic organisms, has a wide geographic distribution, and is one of most abundant macro-invertebrates in the Southern Ocean (Peck, 2005). *N. concinna* has a high mobility rate (10 mm/s at 0 °C, Davenport, 1997) and can be found in rocky grounds without forming a defined home. According to Powell (1973), this species is found from the intertidal until 110 m depth, decreasing their densities at increasing depths (Picken, 1980; Cadée, 1999). In Antarctic locations, including King George Island, a migratory population of *N. concinna* lives from the intertidal zone, at the superior littoral limit, until 4 m depth during summer, but it mobilizes to the superficial sublittoral at the beginning of autumn (de Aranzamendi et al., 2008; Weihe and Abele, 2008). The animals describing this migratory behavior are the adult limpets. A non-migratory population inhabits the sublittoral zone at depths higher than 4 m (Davenport, 1988), and stays under the tide level all along the year.

The hypothesis of this work was that oxidative and nitrosative metabolism in the digestive gland (DG) of two limpet populations (intertidal and subtidal) of the Antarctic species *N. concinna* show different behavior between the intermittent (intertidal) or constant (subtidal) natural Fe exposure. The total Fe, the LIP and the Ft content in DG homogenates of *N. concinna* from the intertidal and subtidal populations were evaluated. Lipid peroxidation and the ascorbyl radical ($A^{\bullet}$) content, and antioxidant enzymatic activities were measured. NO was identified in the limpets DG tissues, and NOS-like activity was estimated by the measurement of the NO generation rate.

2. Materials and methods

2.1. Animal collection and maintenance

N. concinna limpets were collected over the summer of 2005–2006 in two locations outside the Potter Cove, in King George Island, Antarctic Peninsula, 62°15′S, 58°44′W, near Carlini scientific station. The intertidal animals were sampled by hand in a rocky shore called Peñón I (C2); and the subtidal ones by SCUBA divers from 10 m depth in an area named Peñón de Pesca (A1). The collected organisms were kept in the laboratory aquaria with fully aerated natural seawater from the cove, at 0 °C for 1 week prior to analyses. Animals were fed with macroalgae collected in the same intertidal zone. Specimens were dissected and DG were rapidly frozen and stored in liquid N_2.

Body parameters in both limpet populations were calculated according to Wallace (1972) and Lowell (1984). The age of the animals was estimated according to Brêthes et al. (1994) employing the von Bertalanffy growth model where Lt stands for the valve length, L∞ value is 71.4 mm; K value is 0.077 $years^{-1}$ and t0 is 0.58 years.

$$Lt = L\infty[1-e\text{-}K\ (t-t0)] \quad (1)$$

2.2. Total Fe content

Total Fe content was analyzed by enzymatic tissue digestion. Approximately 40 mg of DG were homogenized in 1 ml of 150 mM KCl in 0.1 N HCl, pH 2.5, prepared with Fe free water (IFW) according to Bralet et al. (1992) with modifications. The samples were incubated for 24 hat 37 °C in the presence of 9 mg/ml pepsin, 4.5 mM 2,2′ dipyridyl and 103 mM ascorbic acid, pH 7 prepared in IFW. For each sample both, a reactive blank and a sample blank without adding 2,2′ dipyridyl were prepared. Absorbance at $\lambda = 520$ nm was measured. A standard curve was prepared with Fe in a concentration range from 0 to 120 μM.

2.3. Ft isolation, purification and quantification

Ft from DG was isolated according to van Gelder et al. (1996) with modifications. Each extraction was performed using 30 to 40 pooled DG. The samples were homogenized in a blender in 7 volumes of extraction buffer (10 mM sodium phosphate buffer, 0.15 M NaCl, 1% (*w*/w) phenylmethanesulfonyl fluoride, pH 7.4) at 4 °C. The homogenate was centrifuged at 3000 *g* for 45 min. The supernatant was ultracentrifuged at 78,000 *g* for 1 h and the pellet was resuspended in the extraction buffer and centrifuged at 4000 *g* at 4 °C for 1 h. These two processes were repeated twice and the resuspended pellets were loaded on a Sephacryl S-300 (30 × 1.6 cm) column equilibrated in 10 mM Tris–HCl buffer, 0.15 M NaCl, pH 7.0. Fractions were collected and measurements of protein and Fe concentrations were assessed by absorbance. The Fe-rich fractions were pooled and centrifuged for 1 h at 78,000 *g* at 4 °C. The Ft pellets were dissolved in 10 mM Tris–HCl buffer, 0.15 M NaCl, pH 7.0 and stored at 4 °C until use. Protein content in the sample was measured according to Bradford (1976) using bovine albumin (Sigma) as standard. Fe content in Ft was assessed according to Brumby and Massey (1967). Quality of the Ft extraction procedure from DG was verified by electrophoresis in an SDS-page (Stacking gel: 4% T, 75 V, 20 min. Resolution gel: 14% T, 150 V, 50 min; on denaturizing conditions). Coomassie blue was used to stain the gels to reveal the Ft bands. Commercial Ft from horse spleen was used with comparison purposes.

2.4. LIP content determination

The LIP content was determined by employing both, electronic paramagnetic resonance (EPR) and a fluorescence technique.

2.4.1. LIP content by EPR

The LIP was determined by EPR using a Bruker (Karlsruhe, Germany) spectrometer ECS 106 with a cavity ER 4102ST; at 77 K, according to Woodmansee and Imlay (2002). DG was homogenized 5% (*w*/*v*) in 10 mM Tris–HCl buffer, 120 mM KCl pH 7.4, in IFW and 1 mM deferoxamine mesylate (DF). Homogenates were incubated at room temperature for 10 min and were freeze in liquid N_2 until use. The measurements were performed using the following instrument settings: 50 kHz modulation frequency, 20 mW microwave power, 9.44 GHz microwave frequency, 1600 G centered field, 81.92 ms time constant, 1×10^4 receiver gain, 4.753 G modulation amplitude and 800 G sweep width. A standard curve was prepared with a solution of 1 mM $FeCl_3 \cdot 6H_2O$ in a concentration range from 0 to 150 μM.

2.4.2. LIP content by calcein

The LIP was determined with the Fe sensor calcein according to Darbari et al. (2003) with modifications, as described by González et al. (2008a). DG from *N. concinna* intertidal was homogenized at 2.5% (*w*/*v*) and *N. concinna* subtidal at 0.2% (*w*/*v*) in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4.

2.5. $A^{\bullet}$ content determination

$A^{\bullet}$ content measurements were performed at room temperature (18 °C) employing an EPR technique. Homogenates from DG were prepared in pure dimethylsulfoxide (DMSO) with 1 mM DF at 4.3% (*w*/*v*) from *N. concinna* intertidal and at 2.3% (*w*/*v*) from *N. concinna* subtidal; and were immediately transferred to a Pasteur pipette for $A^{\bullet}$ detection. Instrument settings were as follows: 9.76 GHz microwave frequency, 10 mW microwave power, 50 kHz modulation frequency, 1 G modulation amplitude, 3487 G centered field, 327.68 ms time constant, 81.92 ms conversion time, 1×10^5 receiver gain and 15 G sweep width. Quantification of the spin adduct was performed using an aqueous solution of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) introduced into the same sample cell used for the samples. EPR spectra for both, sample and TEMPOL solutions, were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double integrated to obtain the area intensity, from which the concentration of the radical was calculated (Kotake et al., 1996).

2.6. Thiobarbituric acid reactive substances (TBARS) content determination

TBARS content in DG was measured according to Uchiyama and Mihara (1978), as described by Storch et al. (2001) (1:5 *w*/*v*), and absolute malondialdehyde (MDA) concentrations were calculated as described by Abele et al. (2002). MDA standards were prepared from 1,1,3,3-tetraethoxypropane.

2.7. Antioxidant enzymatic activity determinations

DG were homogenized in 30 mM potassium phosphate buffer, 120 mM KCl, pH 7.4 (1:9 *w*/*v*), and centrifuged at 600 *g* for 10 min at 4 °C. SOD activity was analyzed spectrophotometrically measuring the cytochrome *c* reduction by O_2^- generated by the xanthine/xanthine oxidase system (McCord and Fridovich, 1969). Measurements were performed in 50 mM potassium phosphate buffer, 100 μM EDTA, pH 7.8, adding 21 μM cytochrome *c* and 10 μM xanthine. Absorbance was recorded at $\lambda = 550$ nm at 20 °C. One unit of SOD was defined as the amount of the enzyme able to inhibit the cytochrome *c* reduction rate by 50%. CAT activity was determined according to Aebi (1984). The supernatant was added to 50 mM potassium phosphate buffer, pH 7, and 15 mM hydrogen peroxide (H_2O_2). The H_2O_2 consumption was measured spectrophotometrically at $\lambda = 240$ nm during 30 s ($\varepsilon = 40$/M cm) at 20 °C. Protein content was calculated according to Lowry et al. (1951).

2.8. NO content determination

The NO content was determined by EPR in homogenized DG with 60 mM Tris–HCl buffer, 100 mM KCl, pH 7.0, supplemented with the spin trap solution 10 mM sodium-*N*-methyl-D-glucamine dithiocarbamate (MGD) and 1 mM $FeSO_4$ (1:1.5 *w*/*v*). The supernatant was transferred immediately to Pasteur pipettes for the EPR measurements. The spectra were recorded at room temperature (18 °C) in the EPR spectrometer described above, operating at 9.76 GHz microwave frequency, 20 mW microwave power, 50 kHz modulation frequency, 5.983 G modulation amplitude, 200 G field scan, 327.68 ms time constant, 83.886 s sweep time. Quantification of the spin adduct was performed using TEMPOL.

2.9. Determination of the activity of NOS-like

DG were homogenized with 60 mM Tris–HCl buffer, 100 mM KCl, pH 7.0, in the presence of the spin trap solution (10 mM MGD, 1 mM $FeSO_4$) (1:1.5 *w*/*v*). To assess NOS-like dependent NO generation, the homogenate was supplemented with 0.1 mM NADPH, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM L-Arginine (L-A) (complete system) (Jasid et al., 2006). The complete system was pre-incubated for 10 min at 10 °C before been measured. The L-A analog, Nω-Nitro-L-Arginine methyl ester hydrochloride (L-NAME) was used as a NOS-like inhibitor at a final concentration of 10 mM. The homogenates were pre-incubated with the inhibitor for 10 min at 10 °C before supplementation with the already described components of the complete system. NOS-like activity was assessed as the difference between the rates in the complete system (NOS-like + non-NOS-like activities) and the inhibited system (non-NOS-like activity).

2.10. Statistical analyses

Data in the text and tables are expressed as mean ± S.E.M. Statistical tests were carried out using Statview for Windows, SAS Institute Inc., version 5.0. The data was tested for normality distribution and variance homogeneity using the Komolgorov–Smirnov test and the F-test, respectively. When the data showed a normal distribution and homogeneity of the variances, a one factor ANOVA was applied. However, when the distribution was not normal and there was no homogeneity of variances, the Mann–Whitney non-parametric test was used as indicated in the legend of the figures.

3. Results

Data in Table 1 show the valve measurements from these animals and the age estimation. Except for slope and roundness, the rest of the measured parameters in the subtidal limpet were significantly higher than in the intertidal specimens. Data in Fig. 1 show that the size of subtidal limpets was significantly higher than for intertidal animals. The volume/circumference ratio (calculated for each animal) was 36 ± 1 and 24.6 ± 0.4 mm^2 ($p < 0.0001$, Mann–Whitney) for subtidal and intertidal animals, respectively.

The total Fe content showed significantly higher values in the DG of *N. concinna* subtidal than in the intertidal animals (Table 2). Ft, the main Fe storage protein, was isolated from DG homogenates from both limpet populations. Data shown in Fig. 2 indicated that absorbance and Fe content in the elution extracts followed a profile with two peaks. To identify the location of the Ft in the isolated aliquots, commercial Ft from horse spleen was run in the same column. Commercial Ft showed one peak coincident with the second peak observed in the DG samples from both limpet populations (Fig. 2A, showed for the intertidal animals). In two independent experiments, Ft content in the second

Table 1
Valval measurements and age estimation in *N. concinna* intertidal and subtidal populations.

	N. concinna	
	Intertidal	Subtidal
Length (mm)	31.3 ± 0.2	39.4 ± 0.8**
Width (mm)	21.9 ± 0.2	27.2 ± 0.6**
Height (mm)	11.6 ± 0.1	13.3 ± 0.2**
Volume (mm^3)	670 ± 15	1256 ± 78**
Circumference (mm)	27.0 ± 0.2	33.9 ± 0.6**
Slope	0.436 ± 0.004	0.402 ± 0.006**
Roundness	0.700 ± 0.003	0.688 ± 0.003*
Age estimation (years)	8.0 ± 0.6	11.0 ± 0.7

Measurements from one hundred (100) and sixty-one (61) individuals from intertidal and subtidal habits, respectively.
* Significantly different to *N. concinna* intertidal ($p < 0.001$), Mann–Whitney.
** Significantly different to *N. concinna* intertidal ($p < 0.0001$), Mann–Whitney.

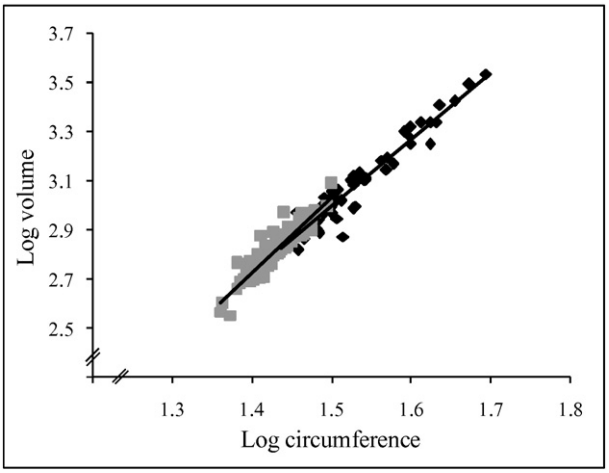

Fig. 1. Internal volume as a function of the circumference for *N. concinna* intertidal (■) and subtidal (■) populations. The function volume = f (circumference) for the intertidal population followed the lineal equation: log (volume) = 3.0882 (log circumference) − 1.6025, being R^2 = 0.8561. For the subtidal population the function was: log (volume) = 2.7133 (log circumference) − 1.0776, being R^2 = 0.9335.

peak represented 23 and 28% of the total protein content isolated from intertidal and subtidal *N. concinna*, respectively. Both peaks were independently pooled and transferred to an SDS gel. In both limpet populations, the first peak did not show any band corresponding to Ft (data not shown); however, the second peak showed a band near 21 kDa in coincidence with the Ft-band from the commercial Ft (Fig. 2B). Even more, in samples from DG isolated from both limpet populations a lighter band was found with a molecular weight of approximately 29 kDa. This extra band, not seen in the commercial horse spleen Ft, accounted for 27 and 17% of the stronger band detected in the DG from both, intertidal and subtidal limpet populations, respectively. Neither Ft content nor Fe content in Ft showed any significant difference between the intertidal and subtidal DG samples (Table 2).

The typical EPR signal of the DF-Fe^{3+} complex was obtained in DG samples (Fig. 3A b and c), in agreement with the signal detected in the presence of 15 μM $FeCl_3$ (Fig. 3A a). The quantification of the spectra recorded for the intertidal limpets DG was lower than in the DG from subtidal animals. The LIP content was confirmed by using the fluorescence technique with calcein (Espósito et al., 2002) (Fig. 3B) since significantly higher LIP content was measured in the DG from subtidal limpets than in the intertidal animals. The LIP content afforded for less than 5% of the total Fe content in both populations.

Typical EPR spectra of $A^{\bullet}$ were recorded in the DG of the limpets (Fig. 4A b and c). The EPR spectra showed the characteristic two lines at g = 2.005 and a_H = 1.8G, in agreement with the computer simulated signal obtained with the parameters mentioned above (Fig. 4A a). In this sense g is a quantity that characterizes the molecule in which the unpaired electrons are located (Borg, 1976). The parameter a_H refers to the hyperfine coupling constant with a hydrogen nucleus and measures the hyperfine intensity between the unpaired electron and the nucleus of the atom of hydrogen (Atkins and de Paula, 2006). Both parameters, g and a_H, allow the characterization, and eventually identification, of a free radical. DMSO was tested and no DMSO spin adduct was observed (Fig. 4A d). $A^{\bullet}$ content, assessed by the quantification of the EPR signals, was significantly higher in the subtidal group (3.3-fold) as compared to the observed value in the DG isolated from the intertidal limpets (Fig. 4B).

TBARS content, a widely accepted index for lipid peroxidation in biological tissues, was 2.5-fold higher in the subtidal DG than in the DG from the intertidal limpets (Fig. 4B). However, SOD and CAT activities were similar in both studied groups of limpets (Fig. 5).

The NO content in the DG was evaluated by EPR spin trapping measurements using the NO trap MGD-Fe. The signal of the EPR is characterized by an isotropic triplet at g = 2.03 and a_N = 12.5 G, which its features are unique and enable a fingerprint-like identification of NO (Fig. 6A d). The a_N factor refers to the hyperfine coupling constant with a nitrogen nucleus. The EPR NO trap signal was neither detectable in the absence of homogenate nor in the presence of a boiled homogenate (data not shown). The content of NO in the DG, assessed by this technique, was similar in DG from both limpet populations (Fig. 6B). The extra peak observed in the DG, not visible in the computer-simulated signal, could be assigned to the formation of the complex Cu-MGD, as was previously reported (Gisone et al., 2003; González et al., 2010). The samples were incubated up to 10 min in the presence of the spin trap $(MGD)_2$-Fe^{2+} at 10 °C and the required cofactors, as previously described for the assessment of NOS-like activity in mammals and other molluscs (González et al., 2008a; González and Puntarulo, 2011). NO generation rate was measured by EPR recording the appearance of the NO signal during the incubation of the homogenate of limpet DG in the presence of L-A and NADPH either with or without the addition of L-NAME, a well known NOS-like inhibitor (Jasid et al., 2006). No NO generation was detectable when the spin trap was incubated only with the cofactors. Even more, the boiled samples incubated with all the NOS-like co-factors showed no detectable EPR signal in all the analyzed samples. The generation rate of NO was measured as the increase on the spectrum area in the samples incubated with the NOS-like co-factors (Fig. 6A b and B). When DG were pre-incubated with the known NOS-inhibitor L-NAME in the presence of all the required co-factors for NOS-like activity, the rate of NO generation was inhibited by 49% and 55% in samples from the intertidal and subtidal animals, respectively, as compared to the complete system (Fig. 6A c and B).

Table 2
Total Fe and Ft content in DG from populations of *N. concinna* collected from intertidal and subtidal habitats.

	N. concinna	
	Intertidal	Subtidal
Fe content (nmol/mg FW)	2.0 ± 0.1	3.4 ± 0.4*
Ft content (mg Ft/g FW)	24 ± 16	15 ± 2
Fe content in Ft (Fe atoms/Ft molecule)	1900 ± 529	1421 ± 344

Measurements of total Fe content were performed by duplicate using fifteen (15) and eighteen (18) individuals of intertidal and subtidal habits, respectively.
* Significantly different to N. concinna intertidal ($p < 0.05$), Mann–Whitney.

4. Discussion

N. concinna, that in nature can reach a life span of 14 years, is considered a long live species. According to Brêthes et al. (1994), the age of the animals can be estimated from the valve length. *N. concinna* may reach sexual maturity when the length range 20 mm (Picken, 1980; Kim, 2001; Weihe and Abele, 2008), which corresponds to an age of 4–5 years (Clarke et al., 2004). Thus, only adult limpets were used in this study. Moreover, the existence of two characteristic morphotypes with unique life habits, intertidal and subtidal, was proposed (Kim, 2001; Clarke et al., 2004). The body size differences might account for a phenotypic adaptation since lower size and higher slope and roundness in the intertidal, with respect to the subtidal animals, could help to avoid desiccation during low tide hours supported by intertidal molluscs.

Ahn et al. (1999, 2002) reported that metals, specially Cu, Mn and Fe, showed significantly higher concentration in tissues from *N. concinna* at King George Island as compared with other Antarctic populations. The elevated lithogenic contribution from this island to the sea during summer primarily affects the subtidal limpets since they fed projecting their radula out and graze over microalgae or macroalgae, bringing back the food particles and the sediments towards the mouth (Ahn et al., 2002; Weihe et al., 2010; Hickman et al., 1994). In opposition, intertidal limpets graze over rock surfaces and ingest, in proportion, a lower

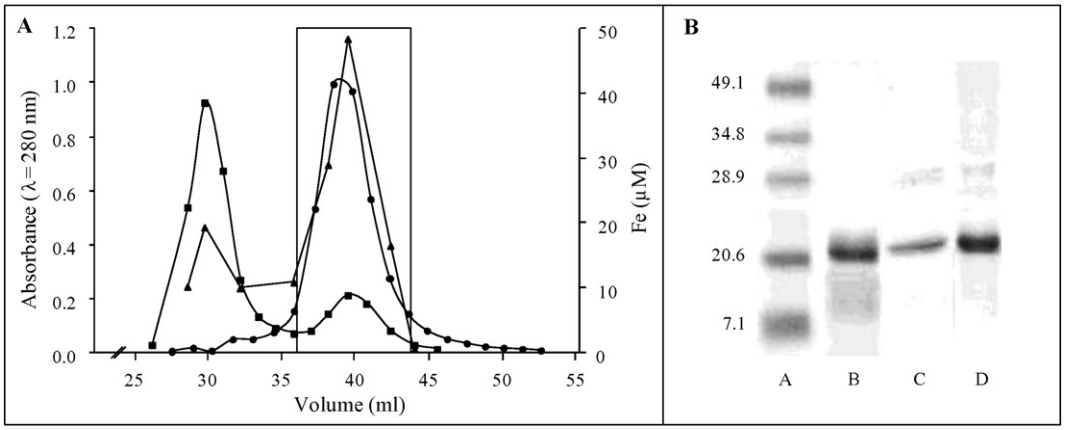

Fig. 2. Ft isolation from GD in *N. concinna*. A: Elution profile from the Ft of *N. concinna* intertidal population. Protein content (■), measured as absorbance at λ = 280 nm, and Fe content (▲) were estimated in the isolated Ft. Fe content was determined in horse spleen Ft (commercial) (●). B: SDS gel at 14% showing: street A: molecular weight markers, street B: horse spleen Ft (commercial), street C: Ft bands from DG of *N. concinna* intertidal and street D: Ft bands from DG of *N. concinna* subtidal.

quantity of inorganic matter (Weihe et al., 2010). Intertidal individuals migrate towards the marine littoral at the beginning of the spring season. Even though diverse aspects might be involved in relation to the reason for the displacement, higher Fe concentration in the subtidal population could suggest that the intertidal animals acquired this behavior to avoid the enrichment in metals (Brêthes et al., 1994; Stanwell-Smith and Clarke, 1998; Zacher et al., 2007; de Aranzamendi et al., 2008). The data reported here showed that the natural Fe concentration in the environment is reflected on the Fe content in the organisms since subtidal animals, been constantly exposed to water sediments, showed higher metal content than the intertidal limpets which may be avoiding such conditions.

Ft, as a main Fe storage protein, plays a critical role in preventing Fe to catalyze ROS generation (Larade and Storey, 2004). Ft isolated from DG of both limpet populations showed a SDS band in the foreseen position as compared to commercial Ft, plus an additional higher subunit. These band profiles differ from that found in the Ft isolated from the DG of the Antarctic bivalve *L. elliptica* which only showed a band in a position similar to the commercial Ft (González and Puntarulo, 2011). The extra subunit could be understood as: i) another Ft sequences presented in the DG, as it was previously suggested by Harrison and Arosio (1996), ii) products of *in vivo* protein association before Ft degradation by proteinases acting on Ft molecules damaged by radicals during Fe exchange, or iii) products of association processes taking place during the isolation method. Even though, up to now there is not a clear explanation of the real meaning of these bands, they appeared constantly in all the extraction procedures performed and could represent a peculiar Ft feature in these species. More detailed analyzes are required to evaluate this aspect since no anti-body against Ft isolated from this species is currently available. Even more, González et al. (2008b) found the same extra subunit in the Ft of the limpets *N. magellanica* and *N. deaurata* from the Beagle Channel. Also, Lu et al. (1995) showed the presence of a Ft band at 21 kDa and other heavier band at 26 kDa from the radula of the limpet *C. toreuma*, but the biological meaning of this higher subunit is not known yet. Kim et al. (1986) found two Ft bands at 25.5 and 38 kDa in the hemolymph of the chiton *Acanthopleura hirtosa* with a content of Fe atoms similar to the value reported here for *N. concinna* DG (1500 to 2500 Fe atoms/Ft molecule).

It is remarkable that LIP content in the DG represented only a minor fraction of total cellular Fe (5%), and seems to be adequately controlled in both populations. LIP content was higher in the subtidal animals than in the intertidal population of *N. concinna*, following the same profile

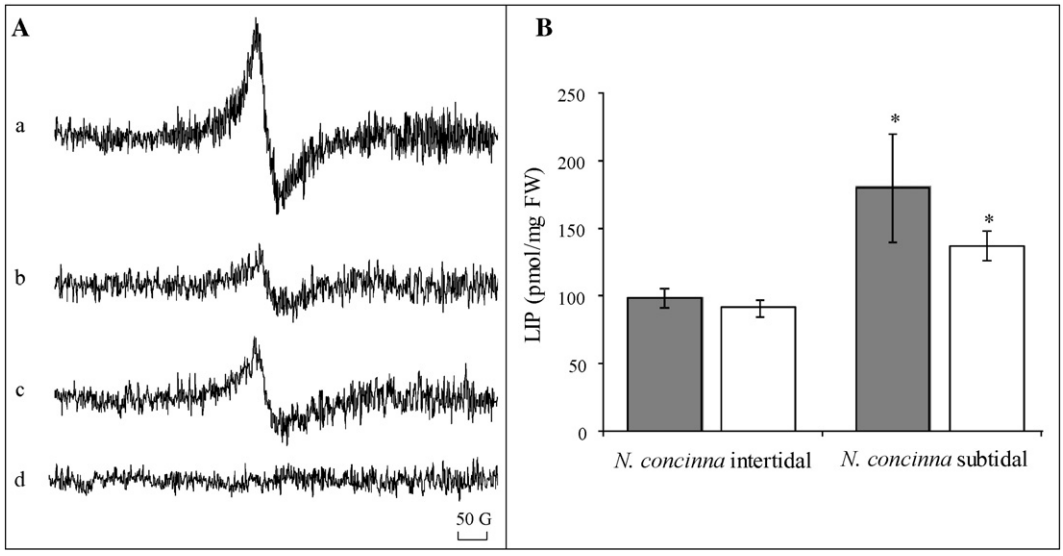

Fig. 3. LIP determination in DG of *N. concinna*. A: EPR, spectra from: a) 15 μM Cl_3Fe employed as standard, b) homogenates from DG of *N. concinna* intertidal population, c) homogenates from DG of *N. concinna* subtidal population, and d) homogenate medium (10 mM Tris–HCl buffer, 120 mM KCl pH 7.4 plus 1 mM DF) without tissue addition. B: Quantification of the LIP content from measurements performed by EPR (■) and by CA (□). *Significantly different to *N. concinna* intertidal ($p < 0.05$), Mann–Whitney and ANOVA for measurements performed for EPR and CA, respectively. Five (5) independent samples were used by duplicate for each population in both techniques.

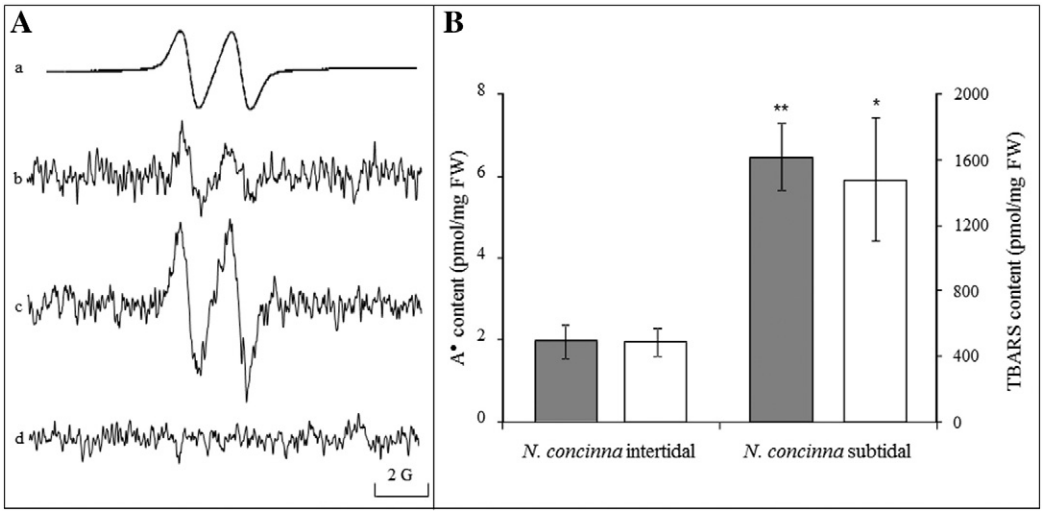

Fig. 4. $A^{\bullet}$ and TBARS content in GD form *N. concinna*. A: EPR spectra of $A^{\bullet}$ from: a) computer-simulated employing the following spectral parameters $g = 2.005$ and $a_H = 1.8$ G, b) DG homogenates from *N. concinna* intertidal population, c) DG homogenates from *N. concinna* subtidal population, and d) DMSO without tissue addition. Measurements from six (6) samples performed by duplicate in each population. B: $A^{\bullet}$ (▒) and TBARS (■) content. For TBARS analysis, five (5) samples were used by triplicate in each population. *Significantly different to *N. concinna* intertidal ($p < 0.05$), ANOVA. **Significantly different to *N. concinna* intertidal ($p < 0.01$), Mann–Whitney.

observed for the total Fe content. Thus, the subtidal condition enables the constant Fe exposure to influence the limpets from this area to increase the cellular content of catalytically active Fe. Even more, the rate of generation of $A^{\bullet}$ in DG collected from the subtidal limpets could be described by Eq. (2)

$$d[A^{\bullet}]/dt = k_1[LIP]\,[AH^-] \quad (2)$$

Both, AH^- and LIP content, are relevant factors to assess generation of $A^{\bullet}$ in the tissue. However, even though AH^- can reduce oxidized Fe^{3+} to Fe^{2+} generating catalytically active Fe that fuels hydroxyl radical ($^{\bullet}OH$) and $A^{\bullet}$ formation (Graziano and Lamattina, 2005) previous data suggested that changes in Fe content in DG from *M. arenaria* did not significantly affect the AH^- content in the tissue (González et al., 2010). Probably, molluscs contained an important content of AH^- and the fraction that is converted to $A^{\bullet}$ does not seem as significantly relevant to the total content of AH^-. On the other hand, the LIP content could be a key factor in the cellular radical generation of $A^{\bullet}$, which was increased in DG collected from the subtidal limpets.

Moreover, the higher lipid damage in the DG of the subtidal than in the intertidal animals might be due to both, the effects of the higher Fe exposure and the enhanced susceptibility of the membranes in the organisms from this region as compared to other animals. As a consequence, both the hydrophilic and lipophilic cellular compartments are affected in the DG isolated from subtidal animals. However, the antioxidant enzymatic system does not seem as a major contributor to the adaptive process to the different shore conditions since no significant differences were found between both limpet populations. The magnitude of the SOD activity observed here showed similarities to previous data from Weihe et al. (2010); but it was significantly higher to the values observed in the DG from the sub-Antarctic *N. magellanica* and *N. deaurata* (Malanga et al., 2004).

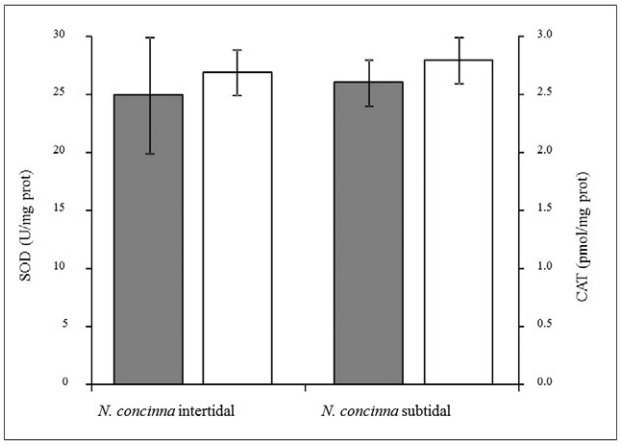

Fig. 5. Enzymatic antioxidant activity in *N. concinna*. SOD (▒) and CAT (■) activities were assessed in five (5) independent homogenates by duplicate for each population.

The NO generation rate detected by the EPR technique accounted mainly for the NO produced by the activity of the NOS-like enzyme. Boiling test was performed since the high temperature denaturalized the enzyme and thus, enzymatic generation is prevented. On the other hand, if some non-enzymatic production still persists, the minimum amounts of NO generated could diffuse to the atmosphere and escape to react with the trap due to the gaseous nature of the compound. A very complex regulatory interplay exists between NO and ROS with multiple effects of NO on the progression of lipid peroxidation and then the result will depend on the balance of competing factors. Direct EPR spectroscopy methodology, allowed us to show that NO is being generated in these animals by a NOS-like activity, as previously reported in other invertebrates. The formation of $ONOO^-$ could be considered as the main NO disappearance pathway (Reaction 1, Eq. (3)), and is a crucial link between oxidative and nitrosative metabolism in cells. It is likely, at least in a simple analysis, not to consider other NO reactions to evaluate its disappearance rate since this reaction proceeds at a rate close to the diffusional limit ($k_2 = 6.7 \times 10^9$/M s) (Huie and Padmaja, 1993).

$$NO + O_2^- \rightarrow ONOO^- \quad \text{(Reaction 1)}$$

$$-d[NO]/dt = d[ONOO^-]/dt = k_2[NO]\,[O_2^-] \quad (3)$$

On the other hand, O_2^- reacts with H_2O_2 in an the Fe-catalyzed Haber–Weiss reaction (Reaction 2) generating $^{\bullet}OH$ (Boveris, 1998).

$$O_2^- + H_2O_2 \xrightarrow{Fe} O_2 + HO^- + {}^{\bullet}OH \quad \text{(Reaction 2)}$$

The steady state O_2^- concentration in the DG of subtidal *N. concinna* was estimated as 3.4×10^{-14} M by González (2011), being in the same order of magnitude than in the DG of the bivalve *M. arenaria* collected

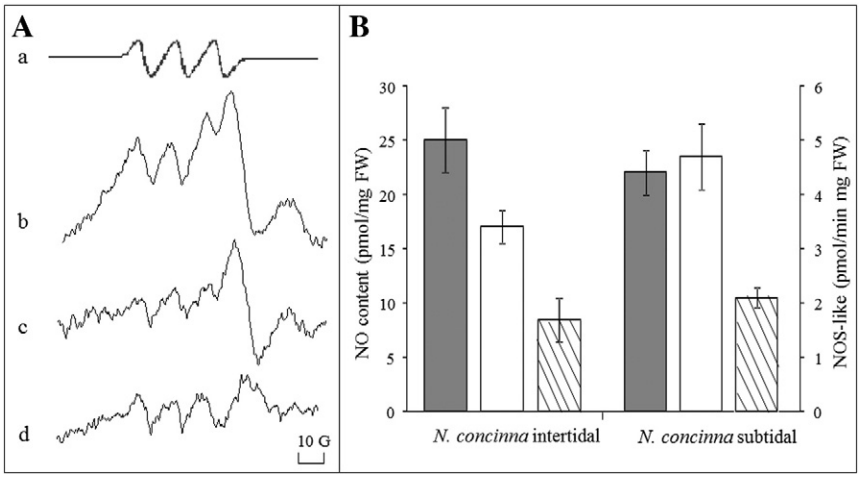

Fig. 6. NO content and generation rate measured in DG homogenates from *N. concinna*. A: Typical spectra of NO detected by EPR after 10 min of incubation: a) computer-simulated employing the following spectral parameters $g = 2.03$ y $a_N = 12.5$ G, b) DG homogenates of *N. concinna* subtidal supplemented with 0.1 mM NADPH, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM L-A (complete system), c) DG homogenates of *N. concinna* subtidal pre-incubated for 10 min with 10 mM L-NAME, and d) DG homogenates of *N. concinna* subtidal without any supplementation. Three (3) independent experiments were performed by duplicated for each population. B: NO content (■) and generation rate in the complete (■) and in the inhibited system (▧).

under physiological control conditions (González et al., 2012). However, no information is currently available for the steady state O_2^- concentration in the DG of the intertidal limpets. The data reported here showing that NO steady state concentration is not significantly different among subtidal and intertidal species, strongly suggest that the increase in the LIP measured in the subtidal population, as compared to the intertidal, is not able to modify O_2^- concentration in the magnitude required to decrease the rate of Reaction 1. Thus, neither NO nor $ONOO^-$ steady state concentration is expected to be affected. Further studies on the $ONOO^-$–dependent damage (e.g. generation of nitrotyrosines) should be performed to test this hypothesis.

Moreover, the three orders of magnitude lower of the O_2^- steady state concentration in invertebrates as compared to the reported value in mammalian cells (10^{-11} M; Cadenas and Davies, 2000) possibly reflects the lower metabolic activity and body temperature in invertebrates as compared to vertebrates; and the efficient antioxidant control mechanisms, which would contribute to the adaptation of these animals to their natural environmental conditions.

5. Conclusions

The limpet *N. concinna* can be considered as a model species for the adaptive response of a subtidal stenothermal and stenosmotic species. When these limpets colonized surfaced intertidal habitats, are increasingly exposed to the fast local climate changes that take place in the studied area (Weihe and Abele, 2008). Even more, since intertidal and subtidal populations seem to be almost genetically identical, the phenotypical differences of the valves might be induced by the environment (Walker, 1972; Nolan, 1991) and they must be considered as a unique species of limpets. The results showed here indicate that the Fe effect leads to a significant increase in the generation of active species, such as $A^{\bullet}$. However, the NOS-like activity, estimated as the activity measured in the complete system minus the activity in the inhibited system, was 1.7 ± 0.2 and 2.6 ± 0.4 pmol/min mg FW for the intertidal and subtidal populations, respectively. Thus, the nitrosative metabolism appears not to be affected by the increasing Fe content in the environment since neither NO steady state concentration nor NOS-like activity were significantly different between both limpet populations.

Acknowledgments

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References

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.
Ahn, I.-Y., Lee, S.H., Kim, K.T., Shim, J.H., Kim, D.Y., 1996. Baseline heavy metal concentrations in the Antarctic clam, *Laternula elliptica* in Maxwell Bay, King George Island Antarctica. Mar. Pollut. Bull. 32, 592–598.
Ahn, I.-Y., Kang, J., Kim, D.-Y., 1999. A preliminary study on heavy metals in the Antarctic limpet, *Nacella concinna* (Strebel, 1908) (Gastropoda: Patellidae) in an intertidal habitat on King George Island. Korean J. Polar Res. 10, 1–8.
Ahn, I.-Y., Kim, K.-W., Choi, H.J., 2002. A baseline study on metal concentrations in the Antarctic limpet *Nacella concinna* (Gastropoda: Patellidae) on King George Island: variations with sex and body parts. Baseline/Mar. Pollut. Bull. 44, 421–431.
Arosio, P., Levi, S., 2002. Ferritin, iron homeostasis, and oxidative damage. Free Radic. Biol. Med. 33, 457–463.
Atkins, P., de Paula, J., 2006. Química Física. Editorial Medica Panamericana, Buenos Aires, p. 551.
Borg, D.C., 1976. Applications of Electron Spin Resonance in Biology. In: Pryor, W.A. (Ed.), Free Radicals in Biology. Academic Press Inc., New York, pp. 69–147.
Bottke, W., 1982. Isolation and properties of vitellogenic ferritin from snails. J. Cell Sci. 58 (1), 225–240.
Boveris, A., 1998. Biochemistry of free radicals: from electrons to tissues. Medicina 54, 350–356.
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.
Bralet, J., Schreiber, L., Bouvier, C., 1992. Effect of acidosis and anoxia on iron delocalization from brain homogenates. Biochem. Pharmacol. 43 (5), 971–979.
Brêthes, J.-C., Ferreyra, G., de la Vega, S., 1994. Distribution, growth and reproduction of the limpet *Nacella* (*Patinigera*) *concinna* (Strebel 1908) in relation to potential food availability, in Esperanza Bay (Antarctic Peninsula). Polar Biol. 14, 161–170.
Brumby, P.E., Massey, V., 1967. Determination of nonheme iron, total iron, and copper. Methods Enzymol. 10, 463–472.
Burford, M.A., Macey, D.J., Webb, J., 1986. Hemolymph ferritin and radula structure in the limpets *Patelloida alticostata* and *Patella peronii* (Mollusca: Gastropoda). Comp. Biochem. Physiol. A 83 (2), 353–358.
Cadée, G.C., 1999. Shell damage and shell repair in the Antarctic limpet *Nacella concinna* from King George Island. J. Sea Res. 41, 149–161.
Cadenas, E., Davies, K.J., 2000. Mitochondrial free radical generation oxidative stress and aging. Free Radic. Biol. Med. 29, 222–230.
Charlesworth, A., Giorgieva, T., Gospodov, I., Law, J., Dunkov, B., Ralcheva, N., Barillas-Mury, C., Ralchev, K., Kafatos, F., 1997. Isolation and properties of *Drosophila melanogaster* ferritin. Eur. J. Biochem. 247, 470–485.
Clarke, A., Prothero-Thomas, E., Beaumont, J.C., Chapman, A.L., Brey, T., 2004. Growth in the limpet *Nacella concinna* from contrasting sites in Antarctica. Polar Biol. 28, 62–71.
Darbari, D., Loyevsky, M., Gordeuk, V., Kark, J.A., Castro, O., Rana, S., Apprey, V., Kurantsin-Mills, J., 2003. Fluorescence measurements of the labile iron pool of sickle erythrocytes. Blood 102, 357–364.
Davenport, J., 1988. Tenacity of the Antarctic limpet *Nacella concinna*. J. Molluscan Stud. 54, 355–356.

Davenport, J., 1997. Comparison of the biology of the intertidal Subantarctic limpets *Nacella concinna* and *Kerguelenella lateralis*. J. Molluscan Stud. 63, 39–48.
de Aranzamendi, M.C., Sahade, R., Tatián, M., Chiappero, M.B., 2008. Genetic differentiation between morphotypes in the Antarctic limpet *Nacella concinna* as revealed by inter-simple sequence repeat markers. Mar. Biol. 154, 875–885.
Dierssen, H.M., Smith, R.C., Vernet, M., 2002. Glacial meltwater dynamics in coastal waters west of Antarctic Peninsula. Proc. Natl. Acad. Sci. 99, 1790–1795.
Dunkov, B., Zhang, D., Choumarov, K., Winzerling, J., Law, J., 1995. Isolation and characterization of mosquito ferritin and cloning of a cDNA that encodes one subunit. Arch. Insect Biochem. Physiol. 29, 293–302.
Durand, J.P., Goudard, F., Barbot, C., Pieri, J., Fowler, S.W., Cotret, O., 2002. Ferritin and hemocyanin: 210Po molecular traps in marine fish, oyster and lobster. Mar. Ecol. Prog. Ser. 233, 199–205.
Espósito, B.P., Epsztein, S., Breuer, W., Cabantchik, Z.I., 2002. A review of fluorescente methods for assessing labile iron in cells and biological fluids. Anal. Biochem. 304, 1–18.
Galatro, A., Rousseau, L., Puntarulo, S., 2007. Ferritin role in iron toxicity in animals and plants. Curr. Top. Toxicol. 4, 65–76.
Gisone, P., Boveris, A.D., Dubner, D., Perez, M.R., Robello, E., Puntarulo, S., 2003. Early neuroprotective effect of nitric oxide in developing rat brain irradiated in utero. Neurotoxicology 24, 245–253.
González, P.M., 2011. Estrés oxidativo y nitrosativo asociado al metabolismo del hierro en moluscos marinos (Ph.D. Thesis) University of Buenos Aires, p. 234.
González, P.M., Puntarulo, S., 2011. Iron and nitrosative metabolism in the Antarctic mollusc *Laternula elliptica*. Comp. Biochem. Physiol. C 153, 243–250.
González, P.M., Abele, D., Puntarulo, S., 2008a. Iron and radical content in *Mya arenaria*. Possible sources of NO generation. Aquat. Toxicol. 89, 122–128.
González, P.M., Malanga, G., Puntarulo, S., 2008b. Ferritin and Labile Iron Pool in Limpets from the Beagle Channel. In: Svensson, E.P. (Ed.), Aquatic Toxicology Research Focus. Nova Science Publishers Inc., New York, pp. 177–188.
González, P.M., Abele, D., Puntarulo, S., 2010. Exposure to excess of iron in vivo affects oxidative status in the bivalve *Mya arenaria*. Comp. Biochem. Physiol. C 152, 167–174.
González, P.M., Abele, D., Puntarulo, S., 2012. A kinetic approach to assess oxidative metabolism related features in the bivalve *Mya arenaria*. Theory Biosci. 131 (4), 253–264.
Graziano, M., Lamattina, L., 2005. Nitric oxide and iron in plants: an emerging and converging story. Trends Plant Sci. 10 (1), 4–8.
Harrison, P.M., Arosio, P., 1996. The ferritins: molecular properties, iron storage function and cellular regulation. Biochim. Biophys. Acta 1275, 161–203.
Hickman Jr., C.P., Roberts, L.S., Larson, A., 1994. In: Pardos Martinez, F., Alvarado Ballester, R. (Eds.), Zoología. Principios Integrales. Interamericana-Mc Graw-Hill, Madrid, p. 1074.
Huie, R.E., Padmaja, S., 1993. The reaction of NO with superoxide. Free Radic. Res. Commun. 18 (4), 195–199.
Jacklet, J.W., 1997. Nitric oxide signaling in invertebrates. Invertebr. Neurosci. 3, 1–14.
Jasid, S., Simontacchi, M., Bartoli, C.G., Puntarulo, S., 2006. Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. Plant Physiol. 142, 1246–1255.
Kakhlon, O., Cabantchik, Z.I., 2002. The labile iron pool: characterization, measurement, and participation in cellular processes. Free Radic. Biol. Med. 33, 1037–1046.
Kim, D., 2001. Seasonality of marine algae and grazers of an Antarctic rocky intertidal, with emphasis on the role of the limpet *Nacella concinna* Strebel (Gastropoda: Patellidae). Ber. Polarforsch. Meeresforsch. 397, 136.
Kim, K.-S., Webb, J., Macey, D.J., 1986. Properties and role of ferritin in the hemolymph of the chiton *Clavarizona hirtosa*. Biochim. Biophys. Acta 884, 387–394.
Kotake, Y., Tanigawa, T., Tanigawa, M., Ueno, I., Randel Allen, D., Lai, C.-S., 1996. Continuous monitoring of cellular nitric oxide generation by spin trapping with an iron-dithiocarbamate complex. Biochim. Biophys. Acta 1289, 362–368.
Larade, K., Storey, K.B., 2004. Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate. J. Exp. Biol. 207, 1353–1360.
Lowell, R.B., 1984. Desiccation of intertidal limpets: effects of shell size, fit to substratum, and shape. J. Exp. Mar. Biol. Ecol. 77, 197–207.
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.
Lu, H.–.K., Huang, C.–.M., Li, C.–.M., 1995. Translocation of ferritin and biomineralization of goethite in the radula of the limpet *Cellana toreuma* Reeve. Exp. Cell Res. 219, 137–145.
Malanga, G., Estevez, M.S., Calvo, J., Puntarulo, S., 2004. Oxidative stress in limpets exposed to different environmental conditions in the Beagle Channel. Aquat. Toxicol. 69, 299–309.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.
Murgia, I., Delledonne, M., Soave, C., 2002. Nitric oxide mediates iron-induced ferritin accumulation in Arabidopsis. Plant J. 30, 521–528.
Nolan, C.P., 1991. Size, shape and shell morphology in the Antarctic limpet *Nacella concinna* at Signy Island, South Orkney Islands. J. Molluscan Stud. 57, 225–238.
O'Donnell, V.B., Freeman, B.A., 2001. Interactions between nitric oxide and lipid oxidation pathways: Implications for vascular disease. Circ. Res. 88, 12–21.
Peck, L., 2005. Prospects for surviving climate change in Antarctic aquatic species. Front. Zool. 2, 9.
Picken, G.B., 1980. The distribution, growth and reproduction of the Antarctic limpet *Nacella (Patinigera) concinna* (Strebel 1908). J. Exp. Mar. Biol. Ecol. 42, 71–85.
Powell, A.W.B., 1973. The patellid limpets of the world. Indo-Pac. Mollusca 3, 75–206.
Rey, J., Somoza, L., Martinez Frias, J., 1995. Tectonic, volcanic, and hydrothermal event sequence on Deception Island (Antarctica). Geo-Mar. Lett. 15, 1–8.
Sergent, O., Griffon, B., Morel, I., Chevanne, M., Dubos, M.-P., Cillard, P., Cillard, J., 1997. Effect of nitric oxide on iron-mediated oxidative stress in primary rat hepatocyte culture. Hepatology 25 (1), 122–127.
Stanwell-Smith, D., Clarke, A., 1998. The timing of reproduction in the Antarctic limpet *Nacella concinna* (Strebel, 1908) (Patellidae) at Signy Island, in relation to environmental variables. J. Molluscan Stud. 64, 123–127.
Stefano, G.B., Ottaviani, E., 2002. The biochemical substrate of nitric oxide signaling is present in primitive non-cognitive organisms. Brain Res. 924, 82–89.
Storch, D., Abele, D., Pörtner, H.O., 2001. The effect of hydrogen peroxide on isolated body wall of the lugworm *Arenicola marina* at different extracellular pH levels. Comp. Biochem. Physiol. C 128, 391–399.
Tatur, A., Valle, R., Barczuk, A., 1999. Discussion on the Uniform Pattern of Holocene Tephrochronology in South Shetland Islands, Antarctica. Polish Polar Studies, Proceedings of XXVI Polar Symposium, pp. 305–321.
Taylor, M.G., 1995. Mechanisms of Metal Immobilization and Transport in Cells. In: Cajaraville, M.P. (Ed.), Cell Biology in Environmental Toxicology. University of the Basque Country Press Country Press, Bilbao, pp. 155–170.
Uchiyama, M., Mihara, M., 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86, 271–278.
van Gelder, W., Huijskes-Heis, M.I.E., Klepper, D., Van Noort, W.L., Cleton-Soeteman, M.I., Van Eijk, H.G., 1996. Isolation and partial characterization of two porcine spleen ferritin fractions with different electrophoretic mobility. Comp. Biochem. Physiol. B 115, 191–196.
von Darl, M., Harrison, P., Bottke, W., 1994. cDNA cloning and deduced amino acid secuence of two ferritin: soma ferritin and yolk ferritin from the snail *Lymnaea stagnalis* L. Eur. J. Biochem. 222, 353–361.
Walker, A.J.M., 1972. Introduction to the ecology of the Antarctic limpet *Patinigera polaris* (Hombron and Jacquinot) at Signy Island, South Orkney Islands. Br. Antarct. Surv. Bull. 28, 49–71.
Wallace, L.R., 1972. Some factors affecting vertical distribution and resistance to desiccation in the limpet, *Acmaea testudinalis* (Muller). Biol. Bull. 142, 186.
Webb, J., Mann, S., Bannister, J.V., Williams, R.J.P., 1986. Biomineralization of iron: isolation of ferritin from the hemolymph of the limpet *Patella vulgata*. Inorg. Chim. Acta 124 (1), 37–40.
Weihe, E., Abele, D., 2008. Differences in the physiological response of inter- and subtidal Antarctic limpets *Nacella concinna* to aerial exposure. Aquat. Biol. 4, 155–166.
Weihe, E., Kriews, M., Abele, D., 2010. Differences in heavy metal concentrations and in the response of the antioxidant system to hypoxia and air exposure in the Antarctic limpet *Nacella concinna*. Mar. Environ. Res. 69 (3), 127–135.
Woodmansee, A.N., Imlay, J.A., 2002. Quantification of intracellular free iron by electron paramagnetic resonance spectroscopy. Methods Enzymol. 349, 3–9.
Zacher, K., Hanelt, D., Wiencke, C., Wulff, A., 2007. Grazer and UV radiation effects on an Antarctic intertidal microalgal assemblage: a long-term field study. Polar Biol. 30, 1203–1212.
Zhang, Y., Meng, Q., Jiang, T., Wang, H., Xie, L., Zhang, R., 2003. A novel ferritin subunit involved in shell formation from the pearl oyster (*Pinctada fucata*). Comp. Biochem. Physiol. B 135 (1), 43–54.