Trends in Comparative Biochem & Physiol
Vol. 17, 2013

Review

Oxidative stress and antioxidant capacity in different species of limpets

Paula Mariela González, Susana Puntarulo* and Gabriela Malanga

Physical Chemistry-Institute of Biochemistry and Molecular Medicine (IBIMOL), School of Pharmacy and Biochemistry, University of Buenos Aires-CONICET, Buenos Aires, Argentina

ABSTRACT

Limpets are gastropod molluscs that inhabit intertidal rocky shores. Due to the localization of these animals, they are exposed to a wide variety of environmental stressors: tidal immersion/ emersion cycles, sharp salinity variations, extreme temperature effects, solar and UV radiation and anthropogenic influences. It was suggested that antioxidant systems may be altered after exposure to these stressors. The aim of this work is to summarize oxidative disruption related to environmental stress in limpets from different geographical areas, comparing the available information on antioxidant defenses (enzymatic activities, content of water and lipid soluble antioxidants), as well as lipid damage content. To further characterize the complex interaction between oxygen and nitrogen reactive species, the more recent reports on the presence of NO in limpets will be shown and discussed.

KEYWORDS: oxidative stress, antioxidant capacity, lipid damage, limpets

1. INTRODUCTION

Marine coastal environments, especially intertidal zones, are high-stress areas and are further characterized by steep vertical gradients in abiotic conditions and stress levels during tidal immersion/ emersion cycles. Especially in the high intertidal zones, the marine fauna and flora are periodically exposed to extreme temperature, solar and UV radiation effects, desiccation, warming or freezing depending on latitude, to sharp salinity variations caused by precipitation and evaporation which lead to osmotic stress, and also to anthropogenic influences [1-5]. However, many marine animals living temporarily above the waterline have behavioral adaptations that help their survival by avoiding stressful conditions. Either they retreat to tidal pools during low tides, or they change their behavior to establish locally confined conditions under which they can minimize danger effects, e.g. some molluscs hermetically close their shells during aerial exposure [6].

Limpets are gastropod molluscs, which are one of the most conspicuous and large invertebrates inhabiting physically unstable intertidal rocky shores. They forage commonly in dense patches on rocky substrates feeding on the algal biofilm that develops on these surfaces. Limpets are present around the globe, making them good candidates for the assessment of environmental stress and water pollution [7]. Both thermal tolerance impact and anthropogenic pollution on the horizontal and vertical distribution of species, have received growing interest during the past decade, to look more intensively for observable effects of climate change and global overpopulation. Actual knowledge on the oxidative stress and metabolic responses associated to

*Corresponding author: Dr. Susana Puntarulo, Fisicoquímica-IBIMOL, Facultad de Farmacia y Bioquímica, Junín 956 (C1113AAD), Buenos Aires, Argentina.
susanap@ffyb.uba.ar

different stressors in limpets are summarized in this report to contribute to the deeper understanding of the complex life style of these species.

2. Oxidative stress indicators in natural environments

O_2 consumption with a tetravalent reduction to water is required by animals for the oxidation of food and the generation of energy. This partial reduction results in the formation of reactive oxygen species (ROS), including superoxide anion radical (O_2^-), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2). Right now it is understood that the intracellular production of ROS does not necessarily imply cellular toxicity, but oxidative stress will occur when ROS formation exceeds antioxidant defense capability or disrupt redox signaling, affecting cell functionality [8].

Oxidative condition of a tissue might be assessed by measuring damage/protection ratio as indicators [9, 10]. The indexes, lipid radical (LR•) content/ α-tocopherol (α-T) content and ascorbyl radical (A•) content/ascorbate (AH^-) content ratio, reflect the actual state of the oxidative defense system at the lipophilic and hydrophilic levels, respectively, providing an early and simple diagnosis of stress [9, 11, 12].

Assuming that LR• content could be understood as an indicator of radical-dependent damage to lipids and α-T content as the most efficient antioxidant protection in the lipid compartment, the LR•/α-T ratio was analyzed in limpets exposed to different conditions. Significantly higher ratios (approximately 22 folds) in digestive glands (DG) compared to gills in *Nacella deaurata* and *Nacella magellanica* were found [13]. However, no-significant differences of this ratio were seen in the same tissue between the two limpet species (Figure 1A). Under physiological control conditions, pro-oxidant and antioxidant processes seem well balanced in both of these molluscs, however some low-level oxidative damage might occur physiologically over their life span and alter this equilibrium.

As shown in Figure 1B, the $A•/AH^-$ ratio was significantly different between GD and gills in *N. magellanica* and *N. deaurata*. Even more, the $A•/AH^-$ ratio was significantly lower (49%) in the gills of *N. magellanica* than in *N. deaurata* (Figure 1B) [13]. Gills oxidative condition may be related to the continuous contact of these tissues with the surrounding media. Even though *N. magellanica* is exposed to more demanding and variable conditions the oxidative status at the level of the cytosolic medium in the gills was lower than in gills from the subtidal *N. deaurata*. However, both limpets showed no significant differences in the GD $A•/AH^-$ ratio (Figure 1B) [14]. Besides, Malanga *et al.* [15] studied the seasonal variation on the response of the $A•/AH^-$ ratio in the DG of *N. magellanica*. Lower index of oxidative stress observed in winter, as compared to the other analyzed seasons, was in agreement with lower winter O_2 uptake rates, indicating that ROS production in the DG follows the overall animal O_2 turnover (Figure 2).

To define a cellular redox state, the ratio of interconvertible reduced/oxidized forms of molecules such as NAD^+/NADH, $NADP^+$/NADPH, and reduced glutathione (GSH)/oxidized glutathione (GSSG) were employed. The GSH/GSSG ratio is the most important and commonly measured redox couple to estimate the cellular redox state [16]. Under oxidative stress conditions GSH (reducing power) is decreased and GSSG is increased. In Antarctic intertidal and subtidal *Nacella concinna* limpet gills and foot tissues this ratio was in the same range [17]. Nevertheless, when these limpets were exposed to hypoxia conditions the GSSG/GSH ratio was significantly higher in subtidal than intertidal foot muscle at 24 h. Even more, an increase of the redox ratio in intertidal limpet foot muscle after 12 h of exposure to air was reported [17] suggesting that this tissue becomes more oxidized before the limpets eventually could contract their shells tightly to minimize water loss and in time become anaerobic. Intertidal limpets obviously avoid early onset of anaerobic energy production seen in their subtidal congeners when exposed to air and are still able to maintain tissue redox ratio balance. On the other hand, in the limpet *Cymbula nigra* the ratio was not modified in GD on comparing the animals from low and high-polluted areas from Southern Spain [7].

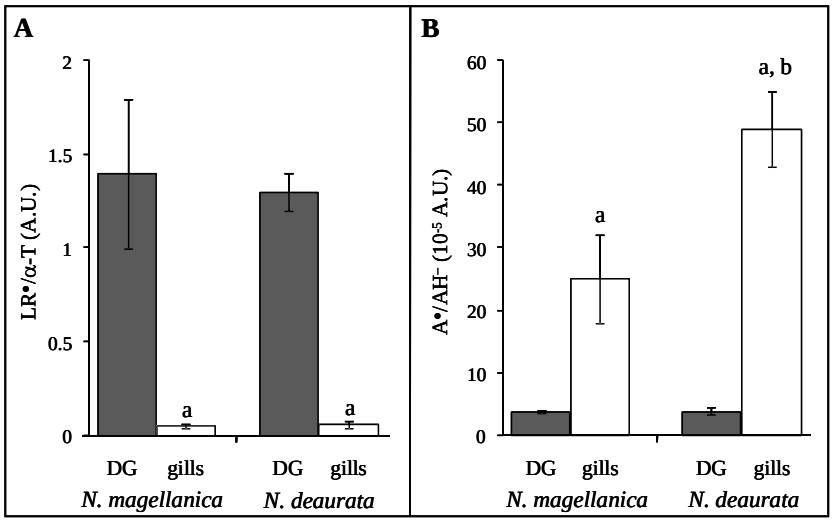

Figure 1. Oxidative condition ratios in tissues from Subantarctic limpets. **A.** LR•/α-T ratio in DG and gills from *N. magellanica* and *N. deaurata*. **B.** A•/AH⁻ ratio in DG and gills from *N. magellanica* and *N. deaurata*. Data are expressed as means ± SEM of 4-6 independent experiments. Taken and modified from Malanga *et al.* [13, 14]. [a]significantly different from DG ($p < 0.05$), [b]significantly different from *N. magellanica* gills ($p < 0.05$), ANOVA.

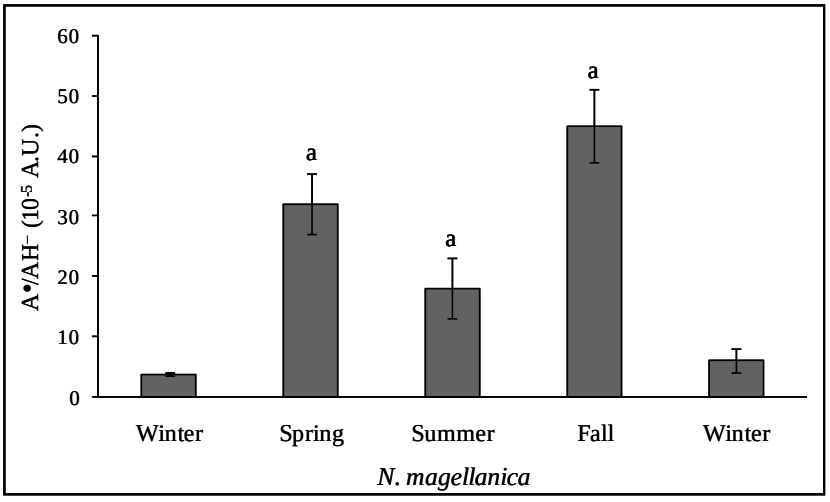

Figure 2. A•/AH⁻ ratio in DG from *N. magellanica* over the year. Data are expressed as means ± SEM of 4-6 independent experiments. Taken and modified from Malanga *et al.* [15]. [a]significantly different from values in winter samples ($p < 0.05$), ANOVA.

3. Antioxidant defense system

Living organisms have the ability to synthesize and control specific systems which can be used for repair and removal of the damaged proteins, lipids and DNA [18]. Also, since oxidative stress levels may diverge from time to time, organisms are able to adapt to such fluctuating stresses by inducing the additional synthesis of antioxidant enzymes to regulate oxidative damage [19, 20].

3.1. Content of non-enzymatic antioxidants

Non-enzymatic antioxidants are generally small molecules, such as AH^- and GSH acting in the aqueous phase, whereas the lipophilic antioxidants, such as α-T and β-carotene (β-C) are active in the

membrane environment. The hydrophilic AH^- and the lipophilic tocopherols might be effective in modulating tissue injury by predominantly reacting chemically with free radicals (e.g., alcohoxyl, RO• and peroxyl, ROO• radicals) whereas the β-C might be the compound scavenging excited species, such as singlet oxygen ($^1O_2$) and triplet states. Thus, the antioxidant action of these compounds *in vivo* is different qualitatively and quantitatively and depends on the type of stress imposed [21]. Weihe *et al.* [17] showed that there are no differences in the content of GSH in gills from *N. concinna* from different tidal conditions (Table 1). Moreover, neither GSH nor AH^- content was affected by tidal or air exposure during 24 h in the foot of these species.

AH^- content in DG from *N. magellanica* was significantly higher in winter than in the rest of the seasons and significantly higher than in gills measured in winter (Table 1) [13]. The α-T content in the DG was significantly higher during summer and fall than in winter and than in gills during winter (Table 1). The authors observed in the DG that the pronounced increase of metabolic activity in spring is accompanied by an increase of around three-times in the β-C level than in winter (Table 1). β-C is a reliable marker of the onset of feeding activity, because carotenoids cannot be synthesized by animals and, therefore, have to be sequestered from algal food. Campbell [22] observed carotenoid peaks in the *Mytilus edulis* in spring and fall, corresponding to phytoplankton blooms and the mussels reproductive cycle. Assimilation of dietary carotenoids into marine animal tissues and especially into eggs is found in many taxa [23]. It is important to ensure antioxidant- and photo-protection of eggs and pelagic larvae. As seen with the AH^- and α-T, the β-C content was significantly higher in the DG than in gills. The content of AH^-, α-T and β-C in *N. deaurata* gills were significantly lower than in DG (Table 1). On the other hand, the content of α-T and β-C in the DG of *N. magellanica* were significantly lower than in *N. deaurata* (Table 1), suggesting that these 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 [14].

The GSH content in the DG of *C. nigra* isolated from low- and high-polluted areas showed no differences, but the content in gills was significantly lower in high polluted areas than in low polluted zones (Table 1) [7]. The α-T and β-C antioxidant content values were significantly higher in DG than in gills (Table 1). This observation allowed the authors to conclude that DG might be better protected than gills to the effects of pollution exposure. Cellular antioxidant defense systems in biological systems, when exposed to environmental pollutants, are depleted, but the levels of these antioxidants in living organisms may also rise to redress the imbalance caused by the oxidative damage [24]. Measurements of their depletion as seen in *C. nigra* can be used as biomarkers for adverse health effects by xenobiotics [25, 26].

3.2. Activity of antioxidant enzymes

Several studies in aquatic organisms demonstrated the importance of enzymatic antioxidant defenses in protecting cellular systems from oxidative stress induced by xenobiotics [20]. The primary antioxidant response to variable conditions includes the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) leading to a rapid, ad-hoc response to change. In general, O_2^- is dismutated by SOD into less reactive species, O_2 and H_2O_2, which is the main cellular precursor of the •OH; subsequently H_2O_2 is detoxified by CAT present in peroxisomes, or by glutathione peroxidase (GPx) present in mitochondria and in the cytosol [27]. CAT activity is considered as an important strategy of marine organisms to protect against oxidative stress [28]. H_2O_2 and organic hydroperoxides will also be destroyed in the cytosolic and mitochondrial compartments by GPx in the presence of GSH. GSH, that has been previously oxidized, will be regenerated by glutathione reductase (GR) participating in the turnover of reduced glutathione pools. GST, on the other hand, is involved in the biotransformation of many xenobiotics [29] and some endogenous compounds such as the end-products of lipid peroxidation [30], and more recently, it has been suggested that it may also play a role in metal homeostasis or detoxification [31].

Table 1. Content of non-enzymatic antioxidants in different tissues from limpets.

Species	Environmental condition	Tissue	AH^-	GSH	α-T	β-C	Ref
			(μmol/g FW)		(pmol/mg Prot)		
Nacella concinna		Gill	nd	346 ± 61	nd	nd	[17]
Antarctic	0 h	Foot	0.10 ± 0.06	451 ± 62	nd	nd	[17]
intertidal	24 h air exposure		0.10 ± 0.09	nd	nd	nd	[17]
Nacella concinna		Gill	nd	390 ± 56	nd	nd	[17]
Antarctic	0 h	Foot	0.08 ± 0.01	481 ± 144	nd	nd	[17]
subtidal	24 h air exposure		0.09 ± 0.06	nd	nd	nd	[17]
Nacella magellanica	Winter	DG	30 ± 3	nd	2700 ± 400	5200 ± 600	[15]
Subantarctic	Spring		15 ± 2[a]	nd	1600 ± 800	17000 ± 6000[a]	[15]
intertidal	Summer		17 ± 2[a]	nd	3500 ± 200[a]	7400 ± 900[a]	[15]
	Fall		11 ± 3[a]	nd	4800 ± 800[a]	8000 ± 2000[a]	[15]
	Winter	Gills	17 ± 2[b]	nd	600 ± 100[b]	900 ± 200[b]	[13]
Nacella deaurata	Winter	DG	26 ± 2	nd	5300 ± 800[c]	14000 ± 2000[c]	[14]
Subantarctic	Winter	Gills	14 ± 2[b]	nd	700 ± 200[b]	1400 ± 400[b]	[13]
subtidal							
Cymbula nigra	Low pollution	DG	nd	0.03 ± 0.07	0.75 ± 0.09	4 ± 1	[7]
Southern Spain	High pollution		nd	0.019 ± 0.007	1.4 ± 0.5	2.4 ± 0.5	[7]
	Low pollution	Gills	nd	0.081 ± 0.003	0.10 ± 0.02[d]	0.13 ± 0.03[d]	[7]
	High pollution		nd	0.023 ± 0.003[e]	0.17 ± 0.04[d]	0.08 ± 0.03[d]	[7]

[a]significantly different from values in winter samples ($p < 0.05$), [b]significantly different from DG ($p < 0.05$), [c]significantly different from *N. magellanica* ($p < 0.05$), [d]significantly different from DG ($p < 0.001$), [e]significantly different from low pollution ($p < 0.001$), ANOVA, nd stands for not determined.

CAT and SOD activities measured in DG and gills in *N. concinna* populations exposed for 24 h either to air or hypoxia showed no effect or a decrease as compared to control (0 h) values, as shown in Table 2. Even though CAT activity was similar between tidal populations under control condition in both tissues, SOD activity showed significant differences [17]. Malanga *et al*. [13-15] showed that in *N. magellanica* and *N. deaurata* CAT and SOD have tissue specific activities which were significantly different between DG and gills. SOD activity in DG was not altered around the year, but CAT activity was significantly lower in winter time as compared to the rest of the seasons (Table 2). SOD and CAT activities from southern Chile limpets reported by Pöhlmann *et al*. [6] are in line with the study performed in gill tissues over *N. magellanica* and *N. deaurata* from Ushuaia [13]. Thus, antioxidant enzyme activities in South American limpets are a tissue specific phenomenon which, in gills, reflects the oxygenation levels that the animals encounter in their respective habitats. The antioxidant activities in DG of the Antarctic congener *N. concinna* [17], however, revealed considerably higher levels of both antioxidants. SOD activities twice as high in Antarctic intertidal *N. concinna* than in South American intertidal *N. magellanica* may represent an adaptation to the harsh environment in the Antarctic high intertidal zones, where extremely low temperatures, thermal fluctuation and fresh water run-off may call for special metabolic adaptations [32]. In colder environments, investing in antioxidant defense seems to be important for survival in the intertidal and in the subtidal zones to minimize the risk of oxidative damage during emersion.

In *C. nigra* CAT activity was significantly different among sites in gills and DG (Table 2). SOD activity did not differ between both tissues. Even though GST antioxidant activity was significantly higher in DG than in gills in both low and high polluted areas, this activity did not show changes between zones [7].

The activity of SOD, CAT, GST and GPx were studied in DG from intertidal *N. concinna* after 24, 48 and 168 h of exposure to seawater containing 0%, 0.05% or 0.1% diesel [33]. GPx showed the clearest effect; its activity significantly increased in the 0.1% diesel group as compared to values obtained in the control group. GST activity in the haemolymph of the limpet *Patella caerulea* when tested as a biomarker of exposure to heavy metals in sampling sites along the Israeli Mediterranean coast [34], showed high activities in limpets from Qiryat Yam and Shemen beach confirming that Haifa-Shemen site suffers from chronic pollution.

4. Oxidative damage to lipids

ROS production can lead to oxidative damage to DNA, proteins and lipid membranes and impair the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions. Thus, subcellular structures may be disturbed and eventually these effects could limit the survival of the organisms. Peroxidation of lipids results in different structural and functional membrane alterations. ROS production and the generated oxidative damage have been a matter of recent interest, as a mechanism of toxicity in aquatic organisms [35], since relatively little information is available on the mechanistic aspects of redox cycling in these organisms.

In molluscs, the DG is a major site of uptake of natural (dietary) and organic xenobiotic pro-oxidant chemicals; also O_2 is mainly taken up through the gills, making these tissues an important target for oxidative injury. Since a variety of stable products are formed during lipid peroxidation, several assays with different specificity and sensitivity have been developed over the years to assess these products in order to evaluate lipid peroxidation, as biomarkers of biochemical perturbations resulting from oxidative stress. The most recently used assay is the determination of malondialdehyde (MDA) formation with the thiobarbituric acid reactive substances test (TBARS). In limpets, Malanga *et al*. [13, 14, 36] reported the DG and gill oxidative status of three limpets from Subantarctic and Antarctic environments exposed to different abiotic conditions in their natural habitats. Table 3 summarizes TBARS content showing that DG in subtidal populations had significantly higher lipid damage than intertidal populations. Gills showed no significant differences between populations, however, the TBARS content

Table 2. Antioxidant enzymatic activities in different tissues from limpets.

Species	Stress	Tissue	CAT	SOD	Ref
			(U/mg prot)		
Nacella concinna	0 h	DG	48 ± 14	11 ± 3	[17]
Antarctic	24 h air exposure		21 ± 9[a]	9.5 ± 0.3	[17]
intertidal	24 h hypoxia		53 ± 10	5.5 ± 0.5[b]	[17]
		Gills	2.6 ± 0.8	5.1 ± 0.5	[17]
	24 h air exposure		3 ± 1	2.5 ± 0.1[c]	[17]
	24 h hypoxia		3.4 ± 0.9	3.4 ± 0.6[c]	[17]
Nacella concinna		DG	48 ± 10	18 ± 6[e]	[17]
Antarctic	0 h air exposure		49 ± 10[f]	8 ± 2[c]	[17]
subtidal	24 h hypoxia		38 ± 8	9 ± 1[c]	[17]
	0 h	Gills	1.7 ± 0.8	2.8 ± 0.5[e]	[17]
	24 h air exposure		1.2 ± 0.8[d]	2.3 ± 0.2	[17]
	24 h hypoxia		2.0 ± 0.2[d]	2.5 ± 0.4[d]	[17]
Nacella magellanica	Winter	DG	15 ± 1	2.1 ± 0.3	[15]
Subantarctic	Spring		22 ± 4[g]	1.5 ± 0.1	[15]
intertidal	Summer		35 ± 5[g]	1.6 ± 0.3	[15]
	Fall		29 ± 3[g]	2 ± 1	[15]
	Winter	Gills	3 ± 1[h]	4 ± 1[h]	[13]
Nacella deaurata	Winter	DG	1.7 ± 0.2[i]	1.4 ± 0.2[i]	[14]
Subantarctic	Winter	Gills	4 ± 1[h]	3 ± 1[h]	[13]
subtidal					
Cymbula nigra	Low pollution	DG	6 ± 1	0.014 ± 0.013	[7]
Southern Spain	High pollution		13 ± 1[j]	0.015 ± 0.007	[7]
	Low pollution	Gills	5 ± 2	0.023 ± 0.019	[7]
	High pollution		20 ± 2[j]	0.013 ± 0.011	[7]

[a]significantly different from values at 0 h ($p < 0.05$), [b]significantly different from values at 0 h ($p < 0.001$), [c]significantly different from values at 0 h ($p < 0.0001$), [d]significantly different from intertidal *N. concinna* ($p < 0.05$), [e]significantly different from intertidal *N. concinna* ($p < 0.001$), [f]significantly different from intertidal *N. concinna* ($p < 0.0001$), [g]significantly different from values in winter samples ($p < 0.05$), [h]significantly different from DG ($p < 0.05$), [i]significantly different from *N. magellanica* ($p < 0.05$), [j]significantly different from low pollution ($p < 0.001$), ANOVA.

Table 3. Lipid damage in different tissues from limpets.

Species	Environmental conditions	Tissue	TBARS (pmol/mg FW)	LR• (pmol/mg FW)	Ref
Nacella concinna		DG	491 ± 102	nd	[36]
Antarctic		Gills	33 ± 9[b]	nd	[13]
intertidal					
Nacella concinna		DG	1242 ± 367[a]	nd	[36]
Antarctic		Gills	42 ± 7[b]	nd	[13]
subtidal					
Nacella magellanica	Winter (2002)	DG	15 ± 2[c,d]	122 ± 29[c]	[14, 15]
Subantarctic	Spring (2002)		nd	358 ± 30[e]	[15]
intertidal	Summer (2003)		nd	204 ± 29[e]	[15]
	Fall (2003)		nd	314 ± 45[e]	[15]
	Winter (2002)	Gills	nd	80 ± 36[c]	[13]
Nacella deaurata	Winter (2002)	DG	23 ± 4[d]	192 ± 32	[14]
Subantarctic	Winter (2002)	Gills	nd	412 ± 98[b]	[15]
subtidal					

[a]significantly different from intertidal *N. concinna* ($p < 0.05$), [b]significantly different from DG ($p < 0.05$), [c]significantly different from *N. deaurata* ($p < 0.05$), [d]significantly different from the same Antarctic habitat population ($p < 0.001$), [e]significantly different from values in winter samples ($p < 0.05$), ANOVA, nd stands for not determined.

was significantly lower in gills as compared to DG in both groups.

TBARS has a limited validity, since a variety of different substances like carbohydrates, nucleic acids and aminoacids can react with thiobarbituric acid (TBA), representing a sensitive but non-specific method [37]. Electron paramagnetic resonance (EPR) spectroscopy has shown the capacity of detecting, in the presence of exogenous traps, LR• formed during peroxidation, by yielding unique and stable products. Even though EPR detection of LR• could be considered a finger-print of radical presence, spin trapping studies cannot really distinguish among ROO•, RO• and alkyl (R•) adducts. In limpets, Malanga *et al.* [13-15] reported the DG and gill lipid damage of two limpets exposed to different tidal level and seasonal variations. Tissues from subtidal *N. deaurata* had significantly higher LR• content than the intertidal *N. magellanica*. Bulk LR• content in *N. magellanica* DG was significantly lower in winter than in spring (193%), summer (67%) and fall (157%), respectively (Table 3). Rivera-Ingraham [38] analyzed the LR• content in the DG and gills from the limpet *C. nigra* from low and high-polluted areas. No significant differences were observed between zones; however, gills showed significantly higher LR• content than the DG ($p < 0.001$, ANOVA).

Weihe *et al.* [17] measured MDA concentrations using HPLC in *N. concinna* populations after hypoxia and air exposure, and an accumulation of MDA in intertidal limpet foot muscle during 12 h of exposure to air was observed. This reaction might indicate that O_2 uptake occurs directly over the surface of the foot during air exposure. Indeed, these authors have shown previously that intertidal limpet foot muscle is maintained aerobic during air exposure, whereas subtidal limpets switch to anaerobic metabolism [32], documenting insufficient adaptation to life under intertidal exposure conditions in this population.

Ansaldo *et al*. [33] measured lipid hydroperoxides (FOX assay) in DG of the limpet *N. concinna* intertidal population. Lipid damage did not show significant differences between animals exposed to 0, 0.05 and 0.1% diesel at 24 and 48 h. However, after 168 h animals in contact with 0.05% diesel showed significantly higher lipoperoxide levels as compared to control animals, but surprisingly no significant differences were found between control and 0.1% diesel exposed groups.

5. Update on nitric oxide (NO) participation in limpet oxidative metabolism

NO is a radical intermediate of many reactions. NO has been shown to act as intracellular or transcellular signal, and as a cytotoxic host defense compound [39]. NO is generated in mammal cells and tissues from L-arginine by the activity of the enzyme nitric oxide synthase (NOS, EC 1.14.13.39) [40]. NOS-like enzyme activity has been detected in marine, freshwater and terrestrial molluscs [41-44]. Johansson and Carlberg [45], and Moroz and Gillette [46] summarized data on NOS-like activity detected by NADPH-diaphorase histochemistry in the central nervous system of mollusc species. Moreover, hemocytes are capable of producing NO in response to zymozan, lipopolysaccharide or pathogenic bacteria [47], also the gene coding for NO-generating enzymes was suggested in invertebrates [48-51].

Nitrite (NO_2^-) and NO content have been evaluated in DG in limpets from the Beagle Channel. The NO_2^- content was 1.6 ± 0.2 and 1.8 ± 0.3 nmol/mg FW for *N. magellanica* and *N. deaurata*, respectively [14]. NO content was analyzed by EPR with homogenates supplemented with the spin trap MGD-Fe. A distinctive EPR signal for the adduct Fe–MGD–NO (g = 2.03 and aN = 12.5 G) was detected in the homogenates taken from tissues of both animals. The spectra obtained showed a broad signal and included a signal that could be part of the EPR spectrum of the [$(MGD)_2$/Cu] complex, resulting from the chelation of Cu present in the mollusc. Quantification of the EPR signal showed a significant difference in the content of the Fe–MGD–NO adduct in DG of *N. magellanica* and *N. deaurata* (491 ± 137 and 839 ± 63 pmol/g FW, respectively).

Data of the NO_2^- content in the DG over the year in *N. magellanica* was significantly decreased during winter, between a 30 and 100% as compared to the content measured in samples taken from animals in other seasons [15]. However, EPR analysis indicated that NO content was significantly higher during summer (768 ± 90 pmol/mg FW) than the rest of the seasons. The authors conclude that seasonal changes not only affect ROS generation but also reactive nitrogen species (RNS) formation. O_2^- plays a key role in the interaction between ROS and RNS, since through the reaction with NO, peroxynitrite ($ONOO^-$) is generated at a rate close to diffusion, and $ONOO^-$ acts as both a nitrating agent and powerful oxidant capable of modifying proteins (formation of nitrotyrosine), lipids (lipid oxidation, lipid nitration) and nucleic acids (DNA oxidation and DNA nitration) [52].

6. CONCLUSIONS

The importance of free radical reactions and ROS in the physiological processes of limpets and in the mechanisms of toxicity by exposure to environmental stressors, such as an alteration in the antioxidant system and/or oxidative damage in limpets tissues has been of interest in the last few years enhancing this knowledge as compared to bivalves which had been more intensively studied. A variety of environmental stressors, natural or anthropogenic, together with a periodical lack of seawater in the intertidal habitat increases the probability of temporal stress coincidence and thus interaction of pro-oxidant factors. This combination should lead to important effects in the metabolism of limpets.

Oxidative stress ratios (damage/protection) reflect successfully the actual state of a fraction of the oxidative metabolism and provide an early and simple diagnosis of stress in these aquatic organisms. These indexes could be considered as a powerful method for detection of the initial stages of oxidative stress in the cellular lipophilic and hydrophilic media due to the impact of environmental factors on aquatic communities. The dependability of these parameters, mostly by combining it with other biochemical strategies, drastically improves the value of these procedures [53].

Adaptation to high shore environment involves extended periods of metabolic reduction which may reduce the overall rate of metabolically produced O_2 radicals compared to subtidal limpet species. Organisms experiencing periodically recurring stress situations often develop a state of hardening, which improves their chance of survival during severe stress, but is attained at the cost of significantly increased energetic investments, such as the antioxidant system, into cellular protection and maintenance [54-56]. Antioxidant defenses are ubiquitous in aerobic species and vary in different tissue-types. Thus, environments and metabolic requirements of tissues of different species should result in different profiles in the oxidative cellular status. Comparisons of enzymatic antioxidants showed higher CAT and SOD activities in Antarctic as compared to Subantarctic limpet species, but it is unclear whether the enzyme activity is highly operative at low temperatures. In concordance with [35] the data shown in the present review leads us to speculate that some molecular adaptations may have taken place to compensate for antioxidant enzyme activity at low temperatures. Even more, tissue specific antioxidant systems were observed in the studied species. The higher antioxidant enzymatic activities in Antarctic limpets as compared to Subantarctic animals was accompanied with an increased lipid damage. In this regard, the peculiarities of membrane lipids in marine organisms, such as high contents of unsaturated fatty acids [57], suggest a special pattern for lipid peroxidation [36]. Moreover, the complex cellular scenario including ROS and RNS should be taken into account. In this regard, seasonal-dependent simultaneous increase in ROS and RNS may drastically enhance potential cellular damage. It is also important to point out that NO could exert a dual effect avoiding lipid oxidation by chelating the catalytically active Fe and limiting the chain reaction. On the other hand, to make this scenario even more complex, the generation of $ONOO^-$ could act to suppress possible signaling effects of O_2^- and NO, as it was suggested in several biological systems. Further, deeper studies are urgently required to fully understand the global chain of responses that takes place in these invertebrates that achieve survival in these hostile conditions.

ACKNOWLEDGMENTS

This study was supported by grants from the University of Buenos Aires, National Agency of Science and Scientific Promotion (ANPCYT) and National Council for Science and Technology (CONICET). S. P. and G. M. are career investigators from CONICET, P. M. G. is a postdoctoral fellow from CONICET.

CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest of any category for the present publication.

REFERENCES

1. Hofmann, G. E. 1999, Am. Zool., 39, 889-900.
2. Tomanek, L. and Somero, G. 1999, J. Exp. Biol., 202, 2925-2936.
3. Denny, M. W., Miller, L. P. and Harley C. D. G. 2006, J. Exp. Biol., 209, 2420-2431.
4. Helmuth, B., Harley, C. D. G., Halpin, P. M., O'Donnell, M. J., Hofmann, G. E. and Blanchette, C. A. 2006a, Science, 298, 1015-1017.
5. Helmuth, B., Broitman, B. R., Blanchette, C. A., Gilman, S., Halpin, P. M., Harley, C. D. G., O'Donnell, M. J., Hofmann, G. E., Menge, B. and Strickland, D. 2006b, Ecol. Monogr., 76, 461-479.
6. Pöhlmann, K., Koenigstein, S., Alter, K., Abele, D. and Held, C. 2011, Cell Stress and Chaperones, 16, 621-632.

7. Rivera-Ingraham, G. A., Malanga, G., Puntarulo, S., Pérez, A. F., Ruiz-Tabares, A., Maestre, M., González-Aranda, R., Espinosa, F. and García-Gómez, J. C. 2013, Water Air Soil Pollut., 224, 1458-8.
8. Jones, D. P. 2006, Antioxid. Redox Signal, 8, 1865-79.
9. Galleano, M., Aimo, L. and Puntarulo, S. 2002, Toxicol. Lett., 133, 193-201.
10. Malanga, G., Perez, A., Calvo, J. and Puntarulo, S. 2009, Mar Biol., 156, 763-770.
11. Kozak, R. G., Malanga, G., Caro, A. and Puntarulo, S. 1997, J. Plant Physiol. (Life Sci. Adv.), 1, 233-239.
12. Estevez, M. S., Malanga, G. and Puntarulo, S. 2001, Plant Sci., 161, 9-17.
13. Malanga, G., Estevez, M. S., Calvo, J. and Puntarulo, S. 2004, Aquatic Toxicol., 69, 299-309.
14. Malanga, G., Estevez, M. S., Calvo, J., Abele, D. and Puntarulo, S. 2005, Sci. Mar., 69, 297-304.
15. Malanga, G., Estevez, M. S., Calvo, J., Abele, D. and Puntarulo, S. 2007, Comp. Biochem. Physiol. A, 146, 551-558.
16. Han, D., Hanawa, N., Saberi, B. and Kaplowitz, N. 2006, Am. J. Physiol. Gastrointest. Liver Physiol., 291, 1-7.
17. Weihe, E., Kriews, M. and Abele, D. 2010, Mar. Environ. Res., 69, 127-13.5.
18. Fenech, M. and Ferguson, L. R. 2001, Mutat. Res., 475, 1-6.
19. Young, I. S. and Woodside, J. V. 2001, J. Clin. Pathol., 54, 176-186.
20. Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. 2006, Ecotox. Environ. Saf., 64, 178-189.
21. Fuchs, J. 1998, Free Rad. Biol. Med., 25, 848-873.
22. Campbell, S. A. 1969, Mar. Biol., 4, 227-232.
23. Marsh, A. G., Gremare, A., Dawson, R. and Tenore, K. R. 1990, Mar. Ecol. Prog. Ser., 67, 301-304.
24. Winston, G. W. and Di Giulio, R. T. 1991, Aquat. Toxic., 19, 137-167.
25. Lemaire, P. and Livingstone, D. R. 1993, Trends Comp. Physiol., 1, 1119-1150.
26. Livingstone, D. R. 1991, Adv. Comp. Environ. Physiol., 7, 45-185.
27. Diguiseppi, J., Fridovich, I. and McCord, J. M. 1984, Critical Reviews in Toxicology, 12, 315-342.
28. Regoli, F., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I., Focardi, S. and Winston, G. W. 2002, Mar. Environ. Res., 54, 419-423.
29. Eaton, D. L. and Bammler, T. K. 1999, Toxicol. Sci., 49, 156-164.
30. Leaver, M. J. and George, S. G. 1998, Mar. Environ. Res., 46, 71-74.
31. Yoshinaga, M., Ueki, T. and Michibata, H. 2007, Biochimica et Biophysica Acta (BBA) - General Subjects, 1770, 1413-1418.
32. Weihe, E. and Abele, D. 2008, Aquatic Biol., 4, 155-166.
33. Ansaldo, M., Najle, R. and Luquet, C. M. 2005, Mar. Environ. Res., 59, 381-390.
34. Yawetz, A., Manelis, R., Goldbart, O., Kuplik, Z. and Zilberman, B. 2006, United Nations Environment Programme Mediterranean Action Plan, Technical Reports Series, 166, 83-100.
35. Abele, D. and Puntarulo, S. 2004, Comp. Biochem. Physiol. A, 138, 405-415.
36. Malanga, G., González, P. M., Estévez, M. S., Abele, D. and Puntarulo, S. 2008, The Antarctic ecosystem of Potter Cove, King-George Island (Isla 25 de Mayo), C. Wiencke, G. A. Ferreyra, D. Abele and S. Marenssi (Eds.), West Sussex, Reino Unido, 208-215.
37. Halliwell, B. and Gutteridge, J. 1989, Free Radicals Biology and Medicine, B. Halliwell and J. Gutteridge (Ed.), University Press, London.
38. Rivera-Ingraham, G. 2010, Tesis doctoral, Universidad de Sevilla, España, 480p.
39. Moncada, S., Palmer, R. M. J. and Higgs, E. A. 1991, Pharmacol. Rev., 43, 109-142.
40. Knowles, R. G. 1997, Biochem. Soc. Trans., 25, 895-901.
41. Jacklet, J. W. 1997, Invertebr. Neurosci., 3, 1-14.
42. Stefano, G. B. and Ottaviani, E. 2002, Brain Res., 924, 82-89.
43. Gonzalez, P. M., Abele, D. and Puntarulo, S. 2008, Aquat. Toxicol., 89, 122-128.
44. Gonzalez, P. M. and Puntarulo, S. 2011, Comp. Biochem. Physiol. C, 153, 243-250.
45. Johansson, K. U. I. and Carlberg, M. 1995, Adv. Neuroimmunol., 5, 43l-442.

46. Moroz, L. L. and Gillette, R. 1995, Acta Biol. Hung., 46, 169-182.
47. Tafalla, C., Gómez-León, J., Novoa, B. and Figueras, A. 2003, Dev. Comp. Immunol., 27, 197-205.
48. Regulski, M. and Tully, T. 1995, Proc. Natl. Acad. Sci. USA, 92(20), 9072-9076.
49. Luckhart, S., Vodovotz, Y., Cui, L. and Rosenberg, R. 1998, Proc. Natl. Acad. Sci. USA, 95(10), 5700-5705.
50. Nighorn, A., Gibson, N. J., Rivers, D. M., Hildebrand, J. G. and Morton, D. B. 1998, J. Neurosci., 18, 7244-7255.
51. Imamura, M., Yang, J. and Yamakawa, M. 2002, Insect Mol. Biol., 11, 257-265.
52. Gisone, P., Dubner, D., Pérez, M. R., Michelin, S. and Puntarulo, S. 2004, In vivo, 18, 281-292.
53. Malanga, G., Aguiar, M. B. and Puntarulo, S. 2012, Oxidative Stress in Aquatic Ecosystems, D. Abele, T. Zenteno-Savín, and J. P. Vázquez-Medina (Eds.), Willey-Blackwell, West Sussex, Reino Unido, 458-464.
54. Somero, G. N. 2002, Integr. Comp. Biol., 42,780-789.
55. Hofmann, G. E. 2005, Integr. Comp. Biol., 45, 247-255.
56. Dong, Y., Miller, L. P., Sanders, J. G. and Somero, G. N. 2008, Biol. Bull, 215, 173-181.
57. Joseph, J. D. 1982, Prog. Lipid Res., 21, 109-153.