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Seasonality and toxins effects on oxidative/nitrosative metabolism in digestive glands of the bivalve *Mytilus edulis platensis*☆

Paula Mariela González, Susana Puntarulo *

Instituto de Bioquímica y Medicina Molecular (IBIMOL)-Universidad de Buenos Aires (UBA), CONICET, Fisicoquímica, Facultad de Farmacia y Bioquímica, Junín 956, C1113AAD, Buenos Aires, Argentina

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ABSTRACT

The hypothesis presented here is that oxidative and/or nitrosative metabolism in the bivalve *Mytilus edulis platensis* is altered by the presence of planktonic toxins. Digestive glands (DG) were isolated from specimens collected in the Argentinean Sea during summer, winter and spring (in the presence of harmful planktonic toxins). The labile iron pool content was not significantly different in DG from animals collected in summer and winter, but was 2.3-fold increased in samples from spring compared to summer collected mollusks. The 2′,7′ dichlorofluorescein diacetate (DCFH-DA) oxidation, ascorbyl radical/ascorbate and lipid radical/α-tocopherol content ratios showed no significant differences between samples collected in winter and summer. However, spring collected samples showed significantly higher DCFH-DA oxidation rate and oxidative ratios in comparison to DG from mollusks collected in summer. Superoxide dismutase activity decreased by 75% in winter, and 93% in spring, compared to samples collected in summer. Glutathione S-transferase activity decreased by 89% in winter, and 30% in spring, compared to samples collected in summer. Catalase activity in winter animals increased by 3.8-fold in comparison to summer values, with no differences between spring and summer collected mollusks. Nitrite plus nitrate content was not significantly different among samples collected in the three seasons, but nitric oxide content was 8.5- and 2.7-fold higher in samples from winter and spring collected mollusks than values obtained in summer, respectively. These results showed the lack of effects of climatic changes on the integrative oxidative indexes; however, under exposure to toxins, both oxidative and nitrosative metabolisms were affected.

1. Introduction

Abiotic and biotic environmental features such as temperature, O_2 consumption, availability of food, endogenous rhythms and algal blooms, may fluctuate according to the annual seasons. These factors might be potential stressors for aquatic organisms via induction of disbalance between the generation and elimination of reactive oxygen species (ROS) and reactive nitrosative species (RNS) that would lead to damage to macromolecules. A baseline quantity of ROS is produced at all times in all aerobic cells, even in aquatic cold blooded species (Lesser, 2006). A fraction of the total cellular iron content called the labile iron pool (LIP) is the source for catalytically active iron, responsible for the generation of ROS, such as hydroxyl radical (•OH) (Harrison and Arosio, 1996). To be able to control hazardous reactive species at low steady state concentrations, the presence of antioxidants and radical scavenging biochemical reactions are required. The antioxidants might be enzymatic (e.g. catalase, CAT; superoxide dismutase, SOD; glutathione S-transferase, GST; etc.) or non-enzymatic, such as hydrophilic (e.g. ascorbic acid, AH^-) and lipophilic (e.g. α-tocopherol, α-T) compounds. Oxidative stress ratios (damage/protection), such as ascorbyl radical ($A^•$)/AH^- content and lipid radical ($LR^•$)/α-T content provide a useful tool for stress diagnosis (Puntarulo et al., 2004; González et al., 2013).

Nitric oxide (NO) is formed mainly from L-arginine by the activity of the enzyme NO synthase (NOS, EC 1.14.13.39) (Knowles, 1997). NOS-like enzymatic activity has already been observed in marine, freshwater and terrestrial mollusks (including prosobranchs, opisthobranchs and pulmonates) (Jacklet, 1997), and in the bivalves *Mya arenaria* and *Laternula elliptica* (González et al., 2008; González and Puntarulo, 2011). ROS and RNS cellular pathways are related through the reaction of superoxide anion (O_2^-) with NO to generate peroxynitrite ($ONOO^-$). $ONOO^-$ is a nitrating agent and a potent oxidant able to modify proteins, lipids and nucleic acids (Lu et al., 2014). NO can also bind iron (Schneppensieper et al., 2001).

Over the 5000 marine phytoplankton species only 2% are considered harmful or toxic (Landsberg, 2002). Algal blooms of these microalgae, known as “harmful algal blooms” or “red tides” are increasing worldwide (Anderson, 2009). It was found a positive correlation between

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

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bloom events and elevated ocean levels of Fe^{3+}, PO_4^{3-}, and NO_3^- (Hallegraeff, 1993; Bruland et al., 2001; Maldonado et al., 2002; Wells, 2003; Marchetti et al., 2004). Some of the planktonic harmful species found in the Argentinean Sea are *Alexandrium tamarense*, *Gymnodinium catenatum*, *Dinophysis acuminate*, *Pseudo-nitzschia australis*, *Pseudo-nitzschia pseudodelicatissima* (Gayoso, 2001; Reguera, 2002; Vinuesa and Varisco, 2007). Especially, some diatoms of the genus *Pseudo-nitzschia* produce increasing amounts of the domoic acid neurotoxin as a function of iron or copper limitation (Rue and Bruland, 2001; Maldonado et al., 2002; Wells et al., 2005). Marine biotoxins are of great concern to seafood industry, government regulators and health care system, since they enter the food chain through mollusk ingestion to reach humans, generating severe intoxications (Lefebvre et al., 2002) with effects on the central nervous system. Monitoring programs for toxins in shellfish currently conducted in more than 50 countries, such as the United States, Canada, Spain, Argentina and others, have successfully prevented human incidents (Anderson et al., 2001; Santinelli et al., 2002). Even more, several countries have already established regulations for paralytic shellfish poisoning (PSP). Most regulations are set for PSP toxins as a group. In general, limits are set at 400 mouse units (MU)/100 g or 80 μg saxitoxin (STX) eq/100 g (Van Egmond et al., 2004). However, some countries used a different tolerance limit, such as Mexico and Philippines (30 and 40 μg STX eq/100 g, respectively), assessed by the mouse bioassay (Aune, 2001).

The mussel *Mytilus edulis platensis* is a filter feeder species from the South America Atlantic coast. This organism is one of the most important sources in aquaculture of the region having a significant economic importance. The bivalve populations at San Matías Gulf, Río Negro, Argentina, are distributed along the coast from 15 to 50 m depth, forming resident units called banks, over rocky surfaces. *Mytilus edulis* is the most common vector of the diatom *Pseudo-nitzschia* sp. (Bates, 2004; Bates et al., 2008), that can also be present in other shellfish and crustaceans (Quilliam and Wright, 1989; Blanco et al., 2002; Powell et al., 2002). However, no information is currently available on the effects of the harmful planktonic toxins on oxidative and/or nitrosative metabolism in the bivalve *M. edulis platensis*.

The hypothesis of this work is that even though mollusk survival is not affected by harmful planktonic toxins, oxidative and/or nitrosative metabolism in the bivalve *M. edulis platensis* is altered. Total iron and LIP content and oxidative and nitrosative conditions were measured in isolated DG from animals collected in winter, summer and spring (in the presence of harmful planktonic toxins). The A•/AH^- and the LR•/α-T content ratios were determined to assess damage/protection ratio in the hydrophilic and hydrophobic fraction of the tissue, respectively. The 2′,7′ dichlorofluorescein diacetate (DCFH-DA) oxidation rate was determined as a general indication of reactive species generation. Nitrosative metabolism was evaluated by measuring both nitrite (NO_2^-) plus nitrate (NO_3^-) content and NO tissue content.

2. Materials and methods

2.1. Animal collection and maintenance

Adult specimens of *M. edulis platensis* (7.67 ± 0.07 cm shell length and 3.65 ± 0.05 cm shell width) were collected from the Argentine Sea (El Sótano 40° 55′.00 S and 65° 05.00′ O, San Antonio Oeste, San Matías Gulf, Río Negro) during the 2012 summer and winter, and the 2013 spring (in the presence of harmful planktonic toxins). In each studied season, 30 to 40 bivalves were sampled. The animals were kept in aquaria under natural temperature and salinity conditions for one day before dissection. Staying up to three days under laboratory conditions did not changed survival of the animals (data not shown).

2.2. Total iron content

Total iron content was analyzed in DG samples after mineralization according to Du Laing et al. (2003) with modifications. Samples were exposed to controlled temperature increases (100 °C) each 30 min up to 500 °C in a muffle. Iron concentrations in the digests were measured spectrophotometrically at λ = 535 nm after reduction with thioglycolic acid followed by the addition of bathophenanthroline (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid) (Brumby and Massey, 1967). To avoid iron contamination, the materials were all treated with HNO_3 and deeply rinsed with iron free water.

2.3. LIP content

The LIP content was determined by employing a fluorescence modified technique using the iron sensor calcein (Darbari et al., 2003), as described by González et al. (2008). DG were homogenized at 1:9 (*w*/*v*) in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4.

2.4. A• content

A• content measurements were performed at room temperature (18 °C) by Electron Paramagnetic Resonance (EPR) using a Bruker (Karlsruhe, Germany) spectrometer EMX plus 080. Homogenates from DG were prepared in dimethylsulfoxide (DMSO) at 1:20 (*w*/*v*) and were immediately transferred to a Pasteur pipette for A• detection. Instrument settings were as follows: 9.86 GHz microwave frequency, 20 mW microwave power, 50 kHz modulation frequency, 1 G modulation amplitude, 3512 G center field, 40.96 ms time constant, 84 ms conversion time, 1×10^5 receiver gain and 20 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 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).

2.5. Oxidation of DCFH-DA by tissue homogenates

DG were homogenized (1:5 *w*/*v*) in a 100 mM Tris–HCl, pH 7.75 with 2 mM EDTA and 5 mM $MgCl_2$ buffer (Gallagher et al., 1992). Measurements were conducted according to Lund Amado et al. (2009) and Viarengo et al. (1999) with modifications. Briefly, the homogenates were centrifuged at 4 °C for 20 min at 10,000 *g* and the supernatants were used for the assay. The reaction was followed in a 30 mM HEPES buffer, pH 7.2 with 200 mM KCl and 1 mM $MgCl_2$. The fluorescent probe DCFH-DA was added to the buffer at a final concentration of 40 μM. Then, after addition of 10 to 15 μl of the supernatant for DG, the reaction mixture was incubated at 35 °C for 10 min. The fluorescent compound DCF, generated by radical-dependent oxidation of the probe, was detected spectrofluorometrically at λ_{exc} = 488 nm and λ_{em} = 525 nm.

2.6. LR• content

The DG homogenates were prepared in a potassium phosphate buffer (pH 7.4) with 130 mM of the spin trap α-(4-pyridyl 1-oxide)-N-t-butyl nitrone (POBN). EPR spectra were measured using the spectrometer described above, operating with the following instrument settings: room temperature (18 °C), 9.81 GHz microwave frequency, 22.6 mW microwave power, 50 kHz modulation frequency, 1.232 G modulation amplitude, 3515 G centered field, 81.92 ms time constant and 1×10^4 receiver gain (Jurkiewicz and Buettner, 1994). Quantification was performed according to Kotake et al. (1996).

2.7. Content of non-enzymatic antioxidants

The AH^- content was measured by reverse phase HPLC with electrochemical detection. DG samples were homogenized in 10% (*w*/*v*)

metaphosphoric acid according to Kutnink et al. (1987). Commercially available AH^- was used as standard. The content of α-T in the homogenates (40 mg/615 μl of a mixture of water, 4% (*w*/*v*) butylated hydroxytoluene and 0.1 M sodium dodecyl sulfate) 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 (Malanga and Puntarulo, 1995). Extraction from the samples was performed with 600 μl methanol and 1 ml hexane. After centrifugation at 2300 *g* for 5 min, the hexane phase was removed and evaporated to dryness under N_2. Extracts were dissolved in methanol:ethanol (1:1 *v*/*v*) and injected for HPLC analysis. HPLC conditions were: isocratic reversed phase; column: Supelcosil LC-8; 15 cm × 4.6 cm × 3 μm; mobile phase: 20 mM lithium perclorate in methanol/water 99/1 (*v*/*v*), flow rate: 1 ml/min. D, l-α-T from synthetic phytol (Sigma) was used as standard.

2.8. Antioxidant enzymatic activities

DG were homogenized in 30 mM potassium phosphate buffer, 120 mM KCl, pH 7.4 (1:9 *w*/*v*) and were 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. 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 λ = 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% (McCord and Fridovich, 1969). CAT activity was determined according to Aebi (1984). The sample 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 λ = 240 nm during 30 s (ε = 40/M cm) at 20 °C. GST activity was determined by measuring the increase in absorbance at λ = 340 nm, incubating reduced glutathione and 1-chloro-2.4-dinithrobenzene as substrates, according to Habig et al. (1974). Protein content was calculated according to Lowry et al. (1951).

2.9. NO content

The NO content was determined by EPR. DG were homogenized 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: 9.88 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 and 1×10^5 receiver gain. Quantification of the spin adduct was performed using TEMPOL.

2.10. NO_2^- and NO_3^- content

The content of $NO_2^- + NO_3^-$ was assessed by the Griess reaction, according to Verdon et al. (1995) with modifications. The isolated DG were homogenized in 14 mM sodium phosphate buffer, pH 7.1 (1:1.6 *w*/*v*). By the addition of the enzyme nitrate reductase and NADPH as cofactor, NO_3^- in the extracts was reduced to NO_2^-. The samples were then incubated for 2 h at room temperature. Later, the Griess reagent was added and incubated for 10 min. The content of $NO_2^- + NO_3^-$ was measured spectrophotometrically at λ = 540 nm. Quantification was performed using two standard curves in the range 0 to 40 μM for NO_2^- and NO_3^-.

2.11. Statistical analyses

Data in the text and tables are expressed as mean ± S.E.M. Statistical tests were carried out using Statview for Windows, ANOVA, SAS Institute Inc., version 5.0.

3. Results

Argentina has a national monitoring program for mussel toxicity in each coastal province involving regional laboratories (Ferrari, 2001). The Regional Unit for Epidemiology and Environmental Health from Atlantic Zone (Senasa, Argentina) reported that non-detectable levels of toxins were found during the studied winter and summer seasons. However, during the 2013 spring time the toxin content was 53.8 μg STS eq/100 g (SENASA local officer, personal communication).

Total iron content in DG of the bivalve *M. edulis platensis* collected in the Argentinean Sea did not show any significant difference in samples isolated in winter compared to data obtained in samples from animals collected in summer (Fig. 1). In DG of animals collected during spring in the presence of toxins, the total iron content was not significantly different from the measured values in samples collected during summer; however, it was lower than the iron content determined in winter collected samples (Fig. 1). The LIP content was not significantly different in the DG from bivalves isolated during the winter and was 2.3-fold increased in samples from the animals collected during spring compared to data from DG of mollusks collected in summer, respectively (Fig. 1).

A typical EPR spectrum for A• was measured in DG samples from mollusks isolated during summer, winter and spring. The EPR spectrum showed the two characteristic lines at g = 2.005 and a_H = 1.8G, in accordance with computer simulated signals obtained using the parameters previously indicated (Fig. 2A). No spin adduct was observed when DMSO was examined by itself (Fig. 2A e). A• content, assessed by quantification of the EPR signals, was 42% and 29% lower in DG from animals collected during winter and spring compared to values obtained in samples from bivalves collected in summer, respectively (Fig. 2B). The AH^- content was determined in samples from animals during the studied seasons, and the antioxidant content was 55% lower in DG from *M. edulis platensis* in winter and spring than in summer bivalves (Fig. 2B).

The A•/AH^- content ratio, understood as an index of oxidative stress in the hydrophilic medium (González et al., 2013), was not significantly different between the obtained data in winter and summer (Fig. 3). However, the A•/AH^- content ratio was significantly different in samples collected during spring compared to data determined in samples taken during summer. In agreement with this observation, the oxidation rate of the dye DCFH-DA, taken as a general indicator of the

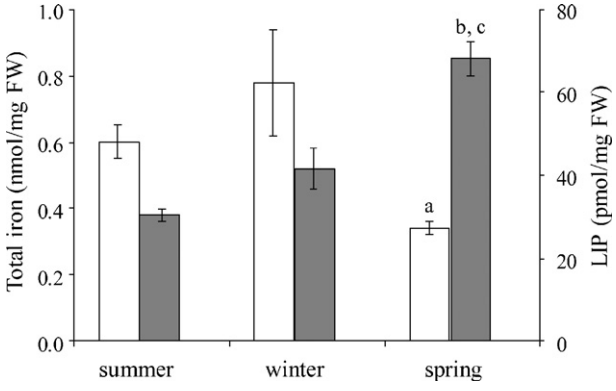

Fig. 1. Iron content in DG of *M. edulis platensis*. Total iron content (□) LIP content (■). Measurements from five (5) samples taken by duplicate in each season. [a]Significantly different to winter ($p < 0.01$), ANOVA. [b]Significantly different to winter ($p < 0.001$). [c]Significantly different to summer ($p < 0.0001$), ANOVA.

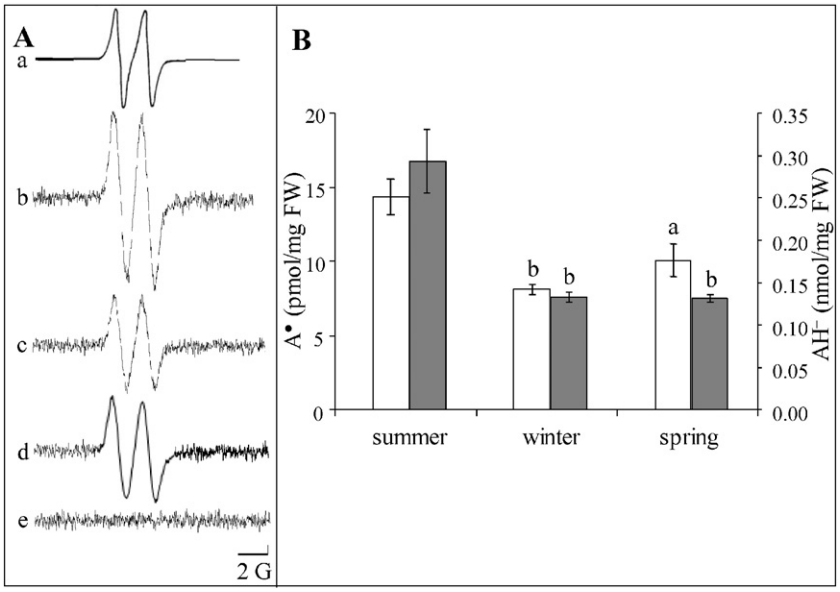

Fig. 2. A: Detection of A• measured by EPR, spectra from: a) computer-simulated employing the following spectral parameters g = 2.005 and a_H = 1.8 G, b) DG homogenates from summer, c) DG homogenates from winter, d) DG homogenates from spring and e) DMSO without tissue. B: A• content (□) and AH^- content (■) in GD of *M. edulis platensis*. Measurements from five (5) samples taken by duplicate in each season. [a]Significantly different to summer ($p < 0.01$), ANOVA. [b]Significantly different to summer ($p < 0.001$).

oxidative stress status (McDowell et al., 2013), was not significantly different in DG form samples collected in summer and winter (Fig. 3). In DG from bivalves collected during spring in the presence of detectable levels of toxins, the oxidation rate of DCFH-DA was significantly different in comparison to both, summer and winter samples.

The LR• from *M. edulis platensis* DG in combination with the spin trap POBN form adducts gave a typical EPR spectrum with hyperfine coupling constants of a_N = 15.8G and a_H = 2.6 G, in concordance with computer simulated signals (Fig. 4A). POBN alone was examined and no adducts were observed (Fig. 4A e). LR• content, assessed by quantification of the EPR signals, was 2-fold higher in the DG samples from animals collected in winter and spring compared to values obtained in samples collected during summer (Fig. 4B). Even more, the content of the lipid soluble antioxidant α-T was 55% higher in samples taken in winter compared to values in DG from animals collected in summer, but it was not significantly different between spring and summer values (Fig. 4B). The LR•/α-T content ratio, considered as an indicator of the balance between free radical damage and antioxidant protection in the hydrophobic medium (Malanga et al., 2009), was not significantly different between samples obtained in winter and summer (Fig. 4B). However, in DG from bivalves collected during spring, the LR•/α-T content ratio was significantly different from the data obtained in samples taken during summer (Fig. 4B).

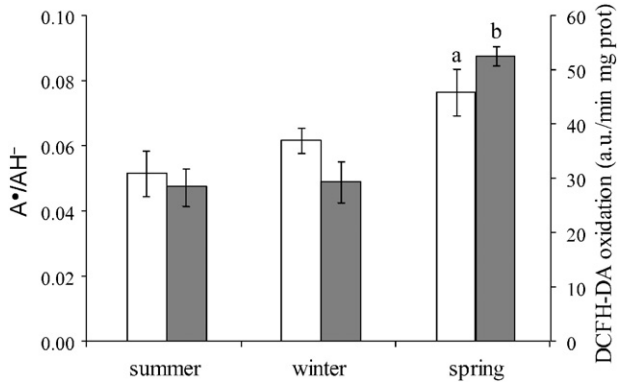

Fig. 3. A•/AH^- ratio (□) and DCFH-DA oxidation rate (■) in DG of *M. edulis platensis*. Measurements from five (5) samples taken by duplicate in each season. [a]Significantly different to summer ($p < 0.05$), ANOVA. [b]Significantly different to summer and winter ($p < 0.001$).

The contribution of the antioxidant cellular capacity seems as a key factor to keep the steady-state concentration of reactive species in living cells. SOD activity was decreased by 75% and 93% in DG from animals collected in winter and spring compared to values obtained in DG from bivalves collected in summer, respectively (Table 1). GST activity was decreased by 89% in DG from animals collected in winter and by 30% in samples from animals collected during spring compared to DG isolated from samples collected in summer, respectively (Table 1). On the other hand, CAT activity was increased by 3.8-fold in samples from winter and showed non-significantly differences in spring collected mollusks compared to values in DG from animals sampled in summer, respectively (Table 1).

The $NO_2^- + NO_3^-$ content gives an useful estimation of the NO metabolism (Granger et al., 1995). The $NO_2^- + NO_3^-$ content was not significantly different among samples collected in winter, spring and summer (Fig. 5B). The EPR signal of the NO adduct with MGD-Fe in DG is characterized by an isotropic triplet signal at g = 2.03 and a_N = 12.5 G, enabling a fingerprint-like detection of the NO (Fig. 5A). The EPR signal was not detectable in the absence of samples (Fig. 5A e). The content of NO in the samples, assessed by quantification of EPR signals, was 8.5- and 2.7-fold higher in samples from winter and spring collected mollusks than values obtained in summer, respectively (Fig. 5B).

4. Discussion

Temperature and seasonality of food availability are among the many factors that affect the metabolic rates, locomotory activity and growth rates in marine ectotherms from temperate waters (Ahmad and Chaplin, 1979; Kirchin et al., 1992). As food resources for most animals are more abundant during spring and summer in seasonality variable environments, many benthic grazers and detritus feeders cease feeding for weeks to months in winter when metabolic rates may decrease to a minimum level (Brockington and Peck, 2001). In temperate animals the effect of seasonal temperature changes on metabolic rates is generally pronounced because of the high seasonal temperature variability. The monthly average temperature in the benthic water in the sampled area (El Sótano) during 2007 and 2008 showed values of 11.5, 13.5 and 18.5 °C, during winter, spring and summer

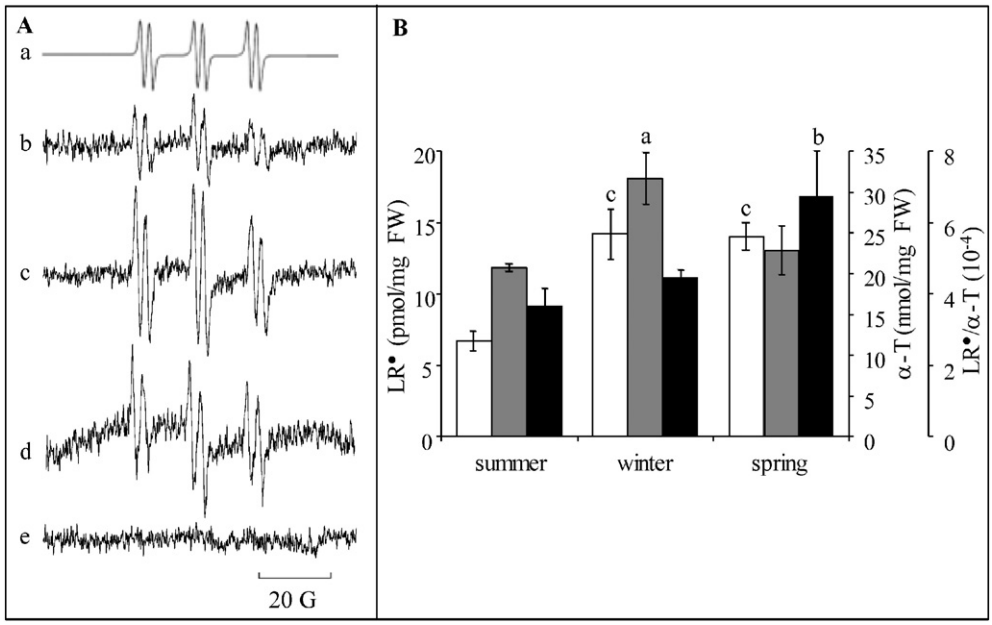

Fig. 4. A: Detection of LR• measured by EPR, spectra from: a) computer-simulated employing the following spectral parameters $a_N = 15.8$ G and $a_H = 2.6$ G, b) DG homogenates from summer, c) DG homogenates from winter, d) DG homogenates from spring and e) POBN without tissue. B: LR• content (□), α-T content (■) and LR•/α-T content ratio (■) in GD of *M. edulis platensis*. Measurements from five (5) samples taken by duplicate in each season. [a]Significantly different to summer and spring ($p < 0.05$), ANOVA. [b]Significantly different to summer ($p < 0.05$). [c]Significantly different to summer ($p < 0.0001$).

seasons, respectively (Zaidman, 2013). However, the effects of air exposure, feeding, and somatic and gonadal growth, are also important on the oxidative metabolism in invertebrates (Pérez et al., 2011; Romero et al., 2011; Schvezov et al., 2013). These seasonal variations in metabolic rate are assumed to entail alterations in ROS formation (Abele and Puntarulo, 2004). Seasonal changes in free radical metabolism have frequently been reported in marine ectotherms, including various bivalve species (Viarengo et al., 1991; Solé et al., 1995; Wilhelm Filho et al., 2001). Specifically, a decrease in the activity of antioxidant defenses in the DG of *M. edulis* has been observed in winter, accompanied by an increase in the lipid peroxidation marker, thiobarbituric acid reactive substances (TBARS) (Viarengo et al., 1991), and also the lipid content of these organisms changed significantly due to seasonal and metabolic factors (Okumuş and Stirling, 1998). Wilhelm Filho et al. (2001) reported in brown mussels (*Perna perna*) from Southern Brazil, an increase in oxygen consumption, endogenous antioxidants and TBARS in summer compared to winter measurements.

Lesser and Kruse (2004) showed no seasonal-dependent changes in either SOD activity or protein levels in the subtidal horse mussels, *Modiolus modiolus* (Isle of Shoals, New Hampshire), although respiration rates were high during summer. However, seasonal variability of antioxidant parameters evaluated in the DG of the ribbed mussel *Aulacomya atra atra* (Giarratano et al., 2013) showed that the antioxidant activities of both GST and CAT were induced in summer compared to winter, meanwhile SOD activity and A• content were parameters seasonally-independent. Giarratano et al. (2011) reported in the Beagle Channel mussels *Mytilus edulis chilensis* a significant effect of season-dependent variation on biological responses, in metal bioaccumulation, and antioxidant enzymes activity. SOD activity was higher in winter than in summer organisms meanwhile CAT activity was not altered in *M. edulis chilensis* (Giarratano et al., 2011). On the other hand, in the polychaete *Arenicola marina* Keller et al. (2004) reported a 5-fold increase in SOD activity in summer vs. winter animals. The data reported here for *M. edulis platensis* showed that SOD activity was increased by 4- and 15-fold in summer compared to winter and spring samples, respectively. Thus, this wide array of effects on the antioxidant enzymatic activities upon season changes indicate that these components are only a limited fraction of a more complex scenario in the cellular network responses when facing climatic changes.

Penchaszadeh (1971) suggested that the enzymatic activity changes corresponded to the modification in the metabolic rates of the whole animals (Schöttler, 1989) and the mitochondria during the reproductive season (August to November). However, Malanga et al. (2009) documented that there were no significant differences on neither the metabolic rate nor the oxidative stress parameters in the limpet *Nacella magellanica* assessed in animals collected in either spring, summer or fall, in agreement with the uniformity of the water temperature throughout this period. Nevertheless, in animals collected in winter, those parameters were affected; suggesting that when the difference in temperature reach a threshold, this factor is a critical issue in the development of oxidative conditions.

In the study reported here, the animals were collected from El Sótano, where there are seasonal fluctuations of temperature, as it was mentioned before. Between summer and winter the average difference in temperature is of 7 °C, and even though many parameters were significantly different between these two seasons, such as LIP, A•, LR•, NO content and antioxidant activities, a critical data is that neither the $A^•/AH^-$ content nor the LR•/α-T content ratios were affected. This uniformity suggested that the oxidative balance was successfully maintained under physiological conditions. Moreover, the oxidation rate of the DCFH-DA, that reflects the production of many reactive species such as O_2^-, H_2O_2, •OH, and $ONOO^-$, among others (Tarpey et al., 2004; McDowell et al., 2013), was not changed between summer and winter in *M. edulis platensis*. Thus, it can be concluded that even though

Table 1
Antioxidant enzymatic activities in DG of *M. edulis platensis*.

	Summer	Winter	Spring
SOD (U SOD/mg prot)	56 ± 6	14 ± 3[c]	3.7 ± 0.9[c]
GST(μM/min mg prot)	122 ± 15	14 ± 1[c]	85 ± 4[a,d]
CAT (pmol/mg prot)	0.42 ± 0.04	1.6 ± 0.3[b]	0.58 ± 0.02

Measurements from five (5) samples taken by triplicate in each season.
[a] Significantly different to summer ($p < 0.05$), ANOVA.
[b] Significantly different to summer and spring ($p < 0.001$), ANOVA.
[c] Significantly different to summer ($p < 0.0001$), ANOVA.
[d] Significantly different to winter ($p < 0.001$), ANOVA.

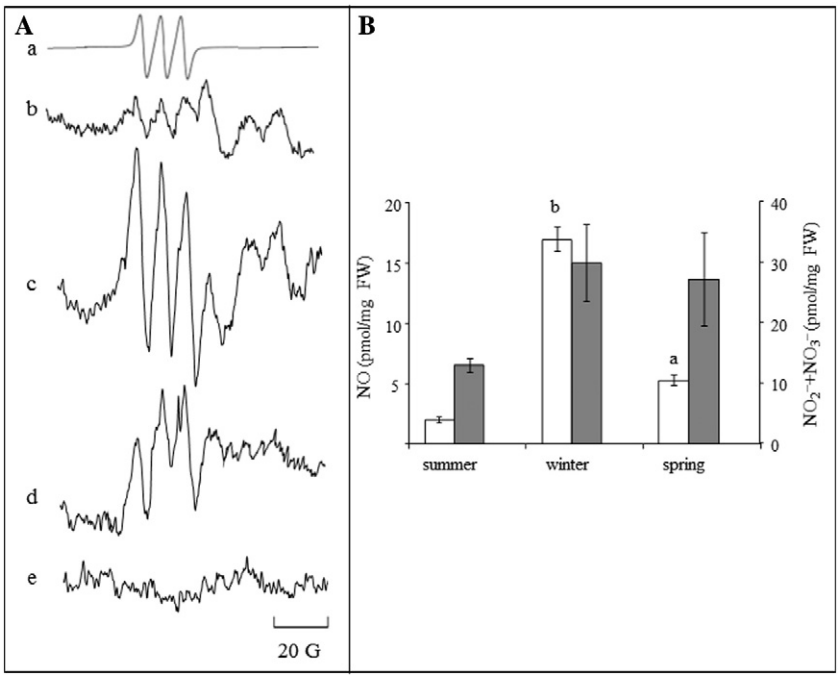

Fig. 5. Nitrosative metabolism in DG of *M. edulis platensis*. A: Detection of NO measured by EPR, spectra from: a) computer-simulated employing the following spectral parameters g = 2.03 and a_N = 12.5 G, b) DG homogenates from summer, c) DG homogenates from winter, d) DG homogenates from spring and e) MGD-Fe without tissue. B: NO content (□) and $NO_2^- + NO_3^-$ content (■). Measurements from five (5) samples taken by duplicate in each season. [a]Significantly different to summer ($p < 0.01$). [b]Significantly different to summer and spring ($p < 0.0001$), ANOVA.

natural environmental conditions have an important influence on oxidative and nitrosative metabolisms, adaptive mechanisms (such as changes in antioxidants content and activities) are physiologically triggered to avoid damaging effects potentially responsible for injury and/or cell death.

Nutrient pollution in coastal areas contributes to the advance and maintenance of harmful planktonic toxins; however, physical, biological, and other chemical factors may promote harmful responses (Gobler et al., 2002; Sellner et al., 2003; Vadstein et al., 2004; Glibert et al., 2005, Heisler et al., 2008). Intoxication in wild animals and algal toxins outbreaks in coastal waters in many regions were documented (Anderson et al., 2001; Santinelli et al., 2002; Takahashi et al., 2007; Pulido, 2008). Thereby, toxic algae not only cause a global risk to human health and safety but also to wildlife including fishes, sea lions, whales, sea otters and sea birds (Hallegraeff, 1993). It was reported that the toxicity of harmful algae increased oxidative stress in fishes and bivalves (Mazmancı and Çavaş, 2010; Hégaret et al., 2011). In this regard, the potential for ROS production in mollusks may be particularly significant in the lipid rich DG (Joseph, 1982) that is the major site of uptake of natural and organic pro-oxidant compounds from diet, and thus, is the main target for oxidative disruption in mollusks.

Although long daytime light duration and higher food availability in early spring may also trigger elevated activity and spawning in *M. edulis platensis*, the contribution of harmful planktonic toxins to oxidative and nitrosative stress development in the mollusk is not clearly established. The difference in environmental temperature was of 5 °C when comparing oxidative parameters obtained from spring and summer collected animals. Thus, no effect should be seen due to the exposure temperature since the magnitude of this difference is lower than the recorded values when comparing data from winter and summer collected animals (7 °C) where alterations in cellular redox balance were not observed. However, the results presented here showed that, compared to summer samples, the $A^{\bullet}/AH^{-}$ and the $LR^{\bullet}/\alpha$-T content ratios and the DCFH-DA oxidation rate were significantly affected. Taken as a whole, it seems that both hydrophilic and lipophilic cellular media are oxidative altered by the exposure to harmful planktonic toxins.

An interesting point is that even though total iron content in DG from animals collected in spring was not significantly different from values in animals collected summer, the LIP content was 2.3-fold higher in spring collected mollusks than in summer animals. This significant change in the distribution pattern of cellular iron is quite interesting since the availability of catalytic active iron is increased. This could be one of the factors contributing to the value of the NO steady state concentration observed during spring in the presence of toxins. Compared to summer collected samples, NO content in DG from mollusks isolated during spring is 2.7-fold increased; however, this enhancement is significantly lower than the value reached during winter. Thus, seasonality seems to be one factor responsible for altering NO content in *M. edulis platensis* meanwhile, oxidative status was kept constant during winter and summer, even though temperature (among other factors) was significantly changed. It can be proposed that at least a fraction of the NO could be forming Fe-nitro-complexes (such as mononitrosyl and dinitrosyl-complexes) to prevent catalytic active iron release in the cellular media and thus, partially avoid damaging effects produced by ROS and RNS.

Fig. 6 shows a qualitative scheme summarizing, during winter and spring compared to summer, the effect on the general indexes of cellular

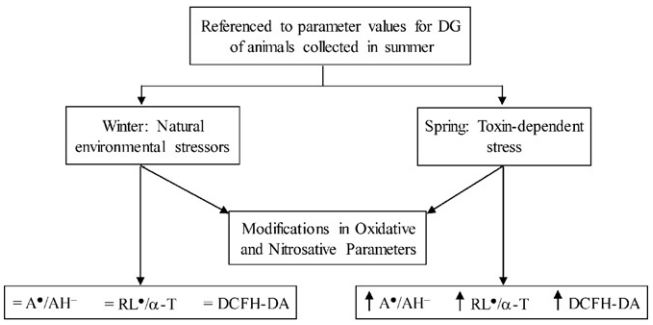

Fig. 6. Brief scheme summarizing in a comparative way to summer-dependent values the effect on the general indexes of cellular redox balance for animals sampled during winter season and in the spring season in the presence of toxins for *M. edulis platensis*.

redox balance which showed no changes during winter, but are significantly increased by the exposure to toxins during spring. Thus, only natural cyclic events were endogenously controlled and cellular damage was successfully avoided.

5. Conclusions

Taken as a whole the data presented here strongly suggested that, under physiological conditions, the overall network of oxidants and antioxidants seems to be adequately controlled to keep steady state concentrations of damaging reactive species far from dangerous levels. However, during the spring season, in the presence of harmful planktonic toxins, the bivalves were facing oxidative stress, as indicated by the increases measured on both hydrophilic and lipophilic cellular media indexes of redox balance and DCFH-DA oxidation rate. The assessment of integrative indexes seems as a critical issue to fully describe the cellular condition, since the partial evaluation of either of the oxidative or the antioxidant features does not reflect the real situation faced by the biological system, and as a consequence wrong conclusions could be reached.

The knowledge of the triggering mechanism of oxidative stress by planktonic toxins will be the following step to get a better understanding of the changes associated to the physiology and biochemistry of the mollusks. These data would add key knowledge in terms of ecological and economical studies allowing a fast and realistic adjustment of the period of restriction of capture and commercialization without risk for the population.

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