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Relating diving behavior and antioxidant *status*: Insights from oxidative stress biomarkers in the blood of two distinct divers, *Mirounga leonina* and *Arctocephalus australis*

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ABSTRACT

Pinnipeds rely upon diving to perform essential activities, including foraging. As pulmonated animals, oxygen privation experienced during submergence represents a considerable challenge both physiologically and biochemically. Routine exposure to hypoxia and the rapid transitions between ischemia/reperfusion of tissues leads to extremely high reactive oxygen species (ROS) production, which must be opposed by antioxidant defenses to avoid oxidative stress. The diving behaviors and capabilities of pinnipeds are very diverse, resulting in distinct metabolic responses among species. To assess whether these characteristics reflect the antioxidant *status* of two marine diving mammals with distinct diving capacities, oxidative stress biomarkers were measured in the blood of *Arctocephalus australis* (n = 11) and *Mirounga leonina* (n = 12). All of the biomarkers analyzed in *M. leonina* were significantly higher than those of *A. australis*, suggesting that higher antioxidant content is needed to counteract the high ROS production associated with the long submergence times (24.3 ± 5.6 min) of this species, which are nearly ten times greater than those of *A. australis* (2.8 ± 0.5 min). Thus, the constitutive antioxidant defenses of both species are of distinct magnitudes due to their inherent diving capacity.

1. Introduction

Modern pinnipeds (Pinnipedia: Otariidae, Phocidae and Odobenidae) represent over a quarter of all marine mammals worldwide (Berta and Sumichs, 1999). Unlike most marine mammals, pinnipeds exhibit "amphibious" behavior, as they are able to survive for long periods of time both in and out of water during resting periods and the reproductive season (Crocker and Costa, 2008). This group of animals displays some of the most remarkable diving capacities among diving mammals (Nowak, 1991; Berta and Sumichs, 1999).

For air breathing mammals such as pinnipeds, extreme diving represents a challenge. The lack of external oxygen sources beneath the water demands a variety of metabolic adaptations (Crocker and Costa, 2008) that allow some species, such as the elephant seals, to stay submerged for long time periods (Andrews et al., 1997). To decrease oxygen consumption and enhance their time underwater, pinnipeds reduce their heart rates during dives using a process known as bradycardia (Kooyman et al., 1981). Its intensity varies among species, in some cases reaching up to 80% of the normal or pre-dive heart rates (Andrews et al., 1997). While diving, pinnipeds also develop widespread vasoconstriction, which redirects blood flow and finite body oxygen stores to essential tissues and organs, such as the brain and heart, leaving the majority of body tissues in a hypoxic condition (Hochachka et al., 1977; Kooyman et al., 1981; Kanatous, 1999). Upon resurfacing, heart rate increases gradually, promptly restoring blood and oxygen flow to these tissues (Thompson and Fedak, 1993; Andrews et al., 1997) and restoring their metabolic functions and oxygen stores before the next dive.

Respiratory chain reactions and ATP production are also rapidly reestablished, leading to elevated reactive oxygen species (ROS) production (Cantú-Medellín et al., 2011). ROS are molecules produced physiologically and continuously at the mitochondrial cristae, mainly as byproducts of oxygen consumption (Boveris and Chance, 1973; Boveris, 1977; Halliwell and Gutteridge, 2007; Nelson and Cox, 2008).

Although these molecules participate in intracellular signaling, an increase in their concentration leads to free radical chain reactions that target and damage proteins (Stadtman and Levine, 2000), lipids (Rubbo et al., 1994), polysaccharides (Kaur and halliwell, 1994) and DNA (Richter et al., 1988).

To quickly dispose of ROS and maintain their intracellular concentration at physiological levels, all aerobic cells possess a panoply of

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antioxidant defenses, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes, which are considered the first enzymatic barrier to neutralize ROS, as well as the co-adjuvant enzymes, glutathione reductase (GR) and glutathione-S-transferase (GST), among other enzymatic and non-enzymatic defenses (Halliwell and Gutteridge, 2007).

Pinnipeds routinely expose their body cells to ischemia (hypoxia)/reperfusion (reoxygenation) (IR) transitions and, consequently, to extremely high ROS. However, they do not suffer from the expected deleterious effects associated with IR events (Wilhelm Filho et al., 2002; Zenteno-Savín et al., 2002; Vázquez-Medina et al., 2006). Accordingly, prior studies revealed that diving mammals exhibit higher constitutive antioxidant defenses compared to non-diving mammals, suggesting that this antioxidant strategy prevents oxidative damage in these animals (Wilhelm Filho et al., 2000, 2002).

Pinnipeds, encompassing thirty-eight species, exhibit a variety of diving behaviors. As examples of the extremes reached within this group, *Arctocephalus australis* (Pinnipedia: Otariidae), the South American fur seal, reaches depths of approximately 26 to 175 m and can remain underwater for an average of approximately 3 min (2.80 ± 0.50 min) (Trillmich et al., 1986). In contrast, *Mirounga leonina* (Pinnipedia: Phocidae), the southern elephant seal, dives for an average of approximately 25 min (24.30 ± 5.60 min), reaching depths of ca. 400 to 800 m (Slip et al., 1994).

The main goal of this study was to evaluate the antioxidant *status* of *A. australis* and *M. leonina* to determine whether it is related to the mean diving duration of each species.

2. Material and methods

Blood samples were collected in the field through the cubital vein of juvenile *A. australis* (n = 11) and recently weaned (i.e., within 2 to 3 days of weaning) (n = 4), post-weaned (i.e., 15 and 30 days after weaning) (n = 3) and adult (n = 5) *M. leonina* in heparinized syringes. All *A. australis* were males, and samples were collected from April to November of 2011 at CRAM-FURG (Rio Grande, southern Brazil). Samples of *M. leonina* were obtained at Punta Delgada (Peninsula Valdes, Chubut, Argentina; 42°30′S; 64°30′W) from adult females and pups of both sexes. None of the animals sampled was molting and only *M. leonina* pups were fasting.

Samples were centrifuged (5000 *g* for 3 min) in the field to separate the plasma and erythrocytes, which were used to determine thiobarbituric acid-reactive substances (TBARS) and enzyme content, respectively, and immediately frozen. The aliquots of plasma and erythrocytes were sent to the Laboratório de Ecofisiologia Respiratória/UFSC in Florianópolis/SC, Brazil, where they were stored in liquid nitrogen until analyses. For enzymatic assays, red cells were washed twice with cold saline solution; hemolysates were obtained by freezing/thawing using Na/K 20 mM buffer (pH 7.4), and supernatants were collected by centrifugation (5000 *g* for 10 min).

2.1. Enzymatic antioxidant assays

Enzymatic antioxidant assays were performed in duplicate according to previously described protocols: SOD (EC 1.15.1.1) activity was evaluated by measuring cytochrome *c* reduction (Flohé and Ötting, 1984); CAT (EC 1.11.1.6) activity was evaluated by measuring the decrease in a freshly prepared solution (10 mM) of hydrogen peroxide (240 nm) (Aebi, 1984); GPx (EC 1.11.1.9) activity was measured according to the method described by Flohé and Günzler (1984), using *tert*-butyl hydroperoxide as substrate; GR (EC 1.8.1.7) activity was assessed according to the method described by Calberg and Mannervik (1985), by measuring NADPH oxidation rate; and GST (EC 2.5.1.18) was determined according to the method described by Habig et al. (1974), using CDNB as substrate.

2.2. Lipid oxidation

Lipid oxidation was evaluated in the plasma by determining TBARS, according to the protocol described by Ohkawa et al. (1979), measuring the formation of stable compounds as a result of the reaction between lipid peroxides and thiobarbituric acid (TBA 0.73%). Briefly, trichloroacetic acid (TCA 12%) was added to plasma samples, followed by centrifugation (5000 *g* for 5 min). Supernatants were added to thiobarbituric acid, maintained in boiling water for 60 min, cooled and measured spectrophotometrically at 535 nm in triplicate.

2.3. Statistical analyses

The Shapiro–Wilk test was used for distribution fitting analysis. For normality accommodations, log or inverse transformations were performed whenever necessary.

To evaluate the influence of distinct species-specific diving profiles (i.e., reported mean submergence times), the mean values obtained for each marker (for both *M. leonina* and *A. australis*) were compared using independent *t*-tests (i.e., $p < 0.05$) (Zar, 1974) with Welch's correction, not assuming similarity among variances. For a clearer observation of the similarity between individuals, cluster analysis was performed based on the standardized values of all of the biomarkers analyzed.

3. Results

All distributions were considered normal, with the following *p*-values: *A. australis*: CAT − p = 0.62; GPx − p = 0.91; SOD − p = 0.19; GST − p = 0.91; GR − p = 0.97; and TBARS − p = 0.46. *M. leonina*: CAT − p = 0.97; GPx − p = 0.75; SOD − p = 0.21; GST − p = 0.48; GR − p = 0.99; and TBARS − p = 0.67. Table 1 summarizes the *t*-test results for all the analyses, showing the mean and standard deviation values for both species, *t*-value, degrees of freedom (df), *p* and *p* value for variances. Despite the three groups sampled for *M. leonina*, only GR activity in recently weaned and GPx activity in post-weaned pups showed any significant difference from adult values, showing lower values for pups in both cases ($p < 0.05$). Therefore, biomarker values for the specie were aggregated and compared predominantly under the scope of interspecies distinction.

Regarding that comparison, all of the antioxidant markers analyzed displayed significantly greater mean concentrations and/or activity values in *M. leonina* compared to *A. australis* (Figs. 1–6). SOD concentration varied dramatically among species (Fig. 1); the mean value for *M. leonina* (1240.26 ± 92.76 USOD·mL^{-1}) was nearly four times the value obtained for *A. australis* (301.56 ± 66.46 USOD·mL^{-1}) [t = 8.23; df = 19,23; $p < 0.01$]. Mean CAT activity values were over five times greater in *M. leonina* (68.14 ± 5.88 mmol·min^{-1}·mL^{-1}) compared to *A. australis* (13.02 ± 3.38 mmol·min^{-1}·mL^{-1}) [t = 8.13; df = 17,38; $p < 0.01$] (Fig. 2). The mean GPx value obtained for *M. leonina* (1.81 ± 0.16 μmol·min^{-1}·mL^{-1}) was more than three times the value observed for *A. australis* (Fig. 3) (0.55 ± 0.03 μmol·min^{-1}·mL^{-1}) [t = 7.89; df = 11,81; $p < 0.01$]. GR and GST activities showed a significant, yet smaller difference between species. The mean GR value for *M. leonina* (0.39 ± 0.08 μmol·min^{-1}·mL^{-1}) was higher than that of *A. australis* (Fig. 4) (0.20 ± 0.01 μmol·min^{-1}·mL^{-1}) [t = 2.30; df = 11,55; $p < 0.05$]. The standard deviation of *M. leonina* GR activity was high due to ontogenetic differences, which will be discussed later in this article. The mean GST value for *M. leonina* (21.78 ± 2.07 μmol·min^{-1}·mL^{-1}) was also greater than that of *A. australis* (Fig. 5) (12.11 ± 3.14 μmol·min^{-1}·mL^{-1}) [t = 2.59; df = 17,59; $p < 0.05$].

In *A. australis*, the mean GST activity also presented a high standard deviation, as the value obtained for one specimen (2601) was over four times the value obtained for the other *A. australis* sample. Finally, the TBARS concentration of *M. leonina* (60.77 ± 5.43 nmol·mL^{-1}) was

Table 1
t-Test results summary of biomarkers of oxidative stress measured in the blood of *Arctocephalus australis* and *Mirounga leonina*.

	Mean (*A. australis*)	Std. error (*A. australis*)	Mean (*M. leonina*)	Std. error (*M. leonina*)	*t*-Value	df	p	p-Value variances
SOD (USOD·mL^{-1})	301.56	66.46	1240.26	92.76	−8.23	19.53	0	0.2455
CAT (mmol·min^{-1}·mL^{-1})	13.02	3.38	68.14	5.88	−8.13	17.38	0	0.0700
GPX (μmol·min^{-1}·mL^{-1})	0.55	0.03	1.81	0.16	−7.89	11.80	0	0
GR (μmol·min^{-1}·mL^{-1})	0.20	0.01	0.39	0.08	−2.30	11.55	0.04	0
GST (μmol·min^{-1}·mL^{-1})	12.12	3.14	21.78	2.07	−2.59	17.59	0.02	0.2393
TBARS (nmol·mL^{-1})	17.17	2.20	60.77	5.43	−7.45	13.21	0	0.0087

four times greater than the mean value of *A. australis* (Fig. 6) (17.17 ± 2.20 nmol·mL^{-1}) [t = 7.45; df = 13,21; p < 0.01].

Based on the standardized measures for all biomarkers analyzed, the cluster obtained (Fig. 7) in a horizontal tree plot grouped individuals of *M. leonina* and *A. australis* by similarities in their antioxidant *status*.

4. Discussion

All mean enzymatic antioxidant activity levels and TBARS contents were significantly higher in the blood of *M. leonina* compared to *A. australis*.

SOD, CAT and GPx (Figs. 1–3) presented the most contrasting values (p < 0.001); thus, they were the main oxidative stress enzymatic biomarkers for use in interspecies comparisons.

As the first enzymatic antioxidant barrier of aerobic organisms, SOD is responsible for superoxide anion ($O_2^{\bullet -}$) dismutation, which is continuously and physiologically generated from oxygen utilization within the mitochondria cristae. As a by-product of this reaction, hydrogen peroxide (H_2O_2) is formed, and CAT and GPx function to convert ROS into oxygen and water (Halliwell and Gutteridge, 2007).

Whenever aerobic organisms must cope with high ROS production, SOD activity is increased, and due to the close interaction among these three antioxidant enzymes, CAT and GPx activities also increase to deal with high endogenous H_2O_2 production and avoid the generation of further deleterious hydroxyl radicals (HO•), the strongest known oxidant, through Fenton–Haber–Weiss reactions (Halliwell and Gutteridge, 2007). As expected, we observed this relationship among these antioxidant enzymes in the present study.

GR (p < 0.05) and GST (p < 0.05) activities were significantly higher in *M. leonina*. GR showed a high standard deviation probably due to ontogenetic differences in its expression. In this regard, GR activity was the only marker that showed significant difference between age classes, reaching lower values in wean and post-wean pups (unpublished results). Accordingly, Vázquez-Medina et al. (2011) showed that reduced glutathione (GSH) biosynthesis increases in elephant seal pups after weaning.

Glutathione metabolism is continuously adjusted, balancing GSH synthesis, recycling from GSSG and utilization (Kidd, 1997; Griffith, 1999). Because GR recycles GSSG to GSH, we hypothesized that in weaning and recently weaned pups, an increase in GSH biosynthesis from cellular precursors would diminish the need for intensive GSSG recycling; thus, for high GR activity in fasting pups, yet still maintaining the necessary levels of cellular GSH.

Although ontogenetic developments play an important role in GR activity, *M. leonina* showed higher values for this enzyme, despite the fact that recently weaned individuals were included in this study. Therefore, we believe that the interspecies differences in this enzyme would be even more contrasting if only freely diving individuals were analyzed.

In *M. leonina* adults, M2a displayed GR activity approximately five times higher than that of the other specimens. This increase in the catalytic activity of GR may be related to greater GSH consumption in order to deal with higher levels of environmental pollutants (Machala et al., 1997; Elia et al., 2006; Kanerva et al., 2012) or to ontogenetic differences (Vázquez-Medina et al., 2011).

GST activity showed greater similarity between *A. australis* and *M. leonina*, possibly reflecting the primary role of this enzyme in xenobiotic detoxification (Jakoby, 2006). GST participates in phase II xenobiotic biotransformation reactions, catalyzing the conjugation of GSH with the oxidized products from phase I, enabling compound excretion (Vrzal et al., 2004).

Thus, we believe that the distinctions in GST activity found in the present study could be more closely related to environmental contamination and/or other conditions than to physiological and/or behavioral patterns. For instance, between the two individuals (2601 of *A. australis* and M2a of *M. leonina*) with high dispersion in the tree plot, specimen 2601 of *A. australis* presented abnormally high GST

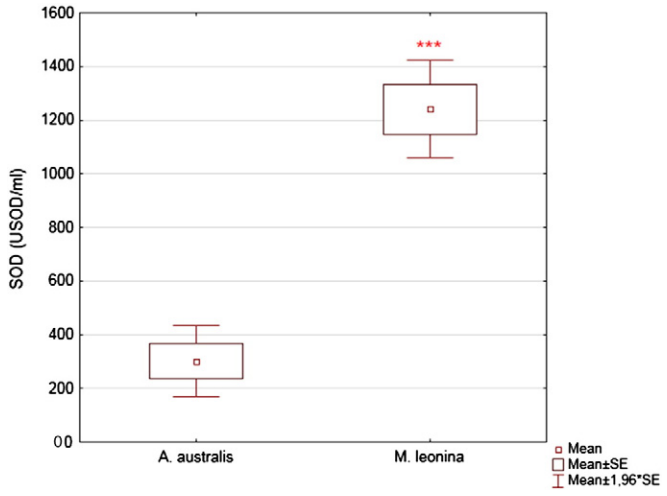

Fig. 1. Box plot of SOD concentration (USOD mL^{-1}) for *A. australis* and *M. leonina*, ***p < 0.001.

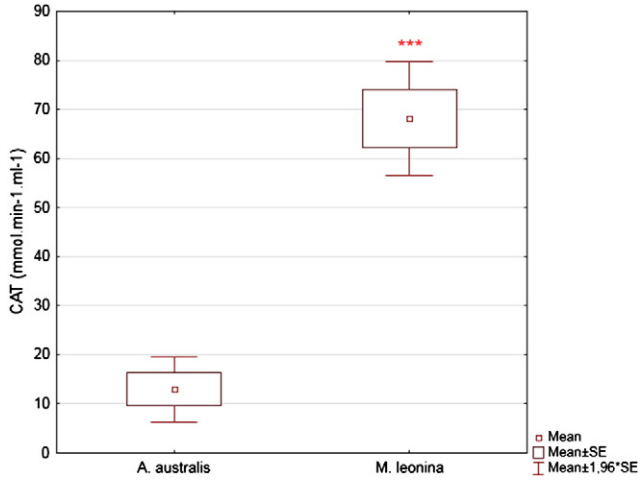

Fig. 2. Box plot of CAT activity (mmol·min^{-1}·mL^{-1}) for *A. australis* and *M. leonina*, ***p < 0.001.

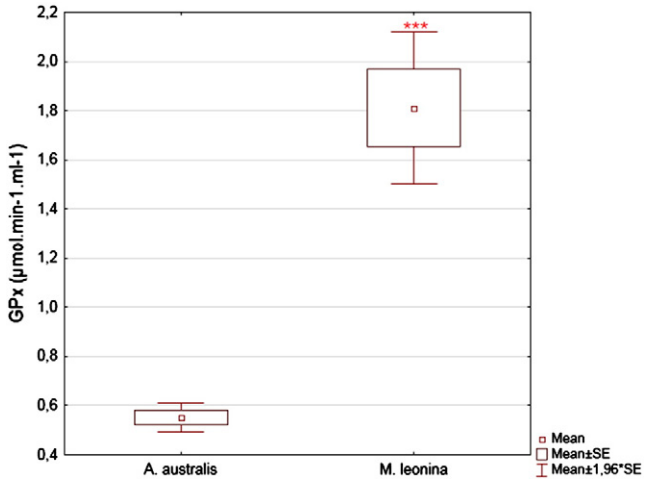

Fig. 3. Box plot of GPx activity ($\mu mol \cdot min^{-1} \cdot mL^{-1}$) for *A. australis* and *M. leonina*, $^{***}p < 0.001$.

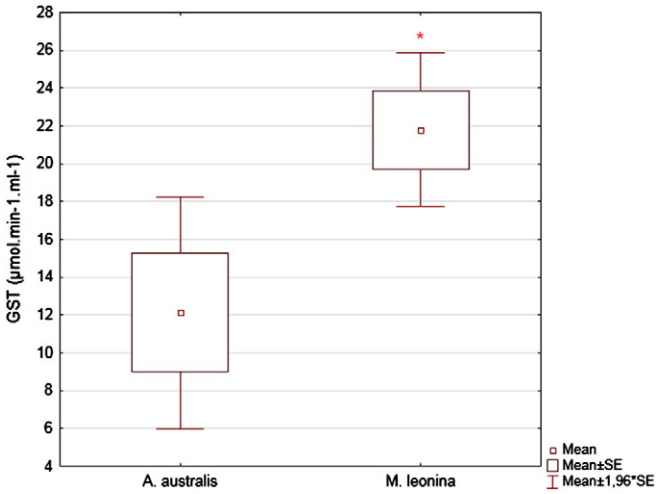

Fig. 5. Box plot of GST activity ($\mu mol \cdot min^{-1} \cdot mL^{-1}$) for *A. australis* and *M. leonina*, $^{*}p < 0.05$.

values, up to four times greater than the mean value obtained for the other samples of *A. australis*. Accordingly, previous studies indicated a significant increase in GST activity, especially for the CDNB induced isoenzyme, in the organs of various groups of animals exposed to high pollution rates (Lenártová et al., 1996; Machala et al., 1997; Canesi et al., 1999; Elia et al., 2006; Farombi et al., 2007).

We believe that this unusually high GST value might be an enzymatic response to environmental contamination that was specific to this individual, possibly in its colony of origin.

Despite the fact that all enzymatic antioxidants presented higher activities, TBARS values also increased in *M. leonina*. Accordingly, comparing to other diving mammals, *M. leonina* exhibited the highest lipoperoxidation level, which contrasts with its higher blood antioxidant capacity (Wilhelm Filho et al., 2002), reinforcing the concept that this species probably produces greater physiological ROS due to its enhanced diving capacity, even considering its high antioxidant *status*. Apparently, *M. leonina* possesses also a high tolerance to relatively high levels of lipoperoxidation as occurs in other vertebrates such as sharks and rays (Wilhelm Filho et al., 2000).

M. leonina dives for significantly longer times than *A. australis* (Riedman, 1990; Bennett et al., 2001; Hindell and Perrin, 2008; Trillmich et al., 1986). Greater submersion periods demand more intense physiological responses (Hindle et al., 2010) to optimize its finite oxygen stores in the absence of an outer source.

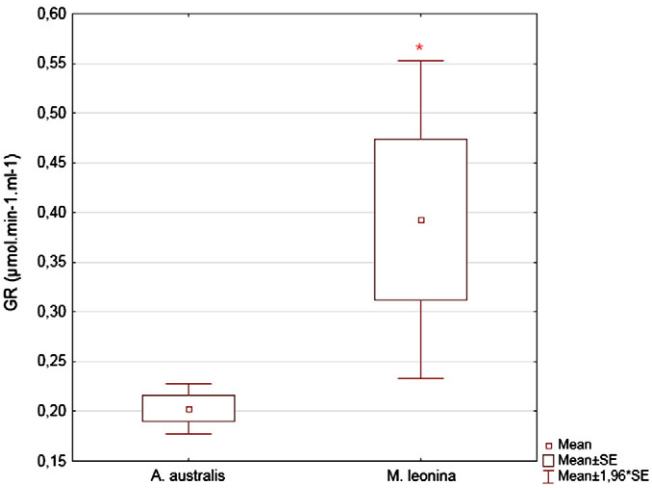

Fig. 4. Box plot of GR activity ($\mu mol \cdot min^{-1} \cdot mL^{-1}$) for *A. australis* and *M. leonina*, $^{*}p < 0,05$.

In general, phocids are excellent divers. Within this group, dives usually reach, and frequently exceed, the Aerobic Dive Limit (ADL) established for the species (Castellini et al., 1992; Thompson and Fedak, 2001) and are commonly followed by very short intervals in the surface (particularly for *M. leonina*) (Le Boeuf et al., 1988). Oxygen consumption in those dives is diminished, but because dives are frequently active (Kooyman and Campbell, 1972; Kooyman, 1985), body oxygen stores are usually depleted in each dive (Le Boeuf et al., 1988).

In contrast, short divers, such as *A. australis*, rarely reach the ADL possibly due to a direct benefit of "abandoning" unsuccessful forage strikes (Thompson and Fedak, 2001).

The early termination of these dives, before the biological limitations are reached, have been shown to be accompanied by the maintenance of heart rates and organ function at their resting state (Kooyman and Campbell, 1972; Kooyman, 1985), reinforcing the fact that the shallow and below ADL dives *A. australis* perform only require mild metabolic responses.

A. australis and *M. leonina* antioxidant profiles were clearly distinguished in the tree plot cluster (Fig. 7), as shown by the two main groups formed by the analysis, indicating that both species developed antioxidant strategies with distinct magnitudes. It is well known that different diving amplitudes are reflected in distinct physiological responses. Individuals that remain underwater for extremely long periods

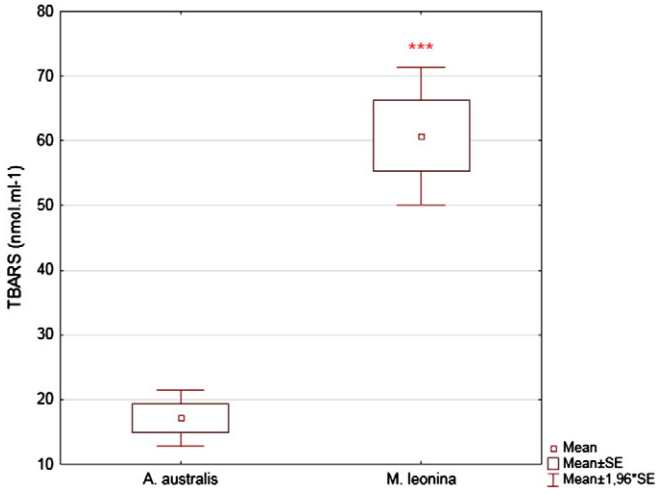

Fig. 6. Box plot of TBARS concentration ($nmol \cdot mL^{-1}$) for *A. australis* and *M. leonina*, $^{***}p < 0.001$.

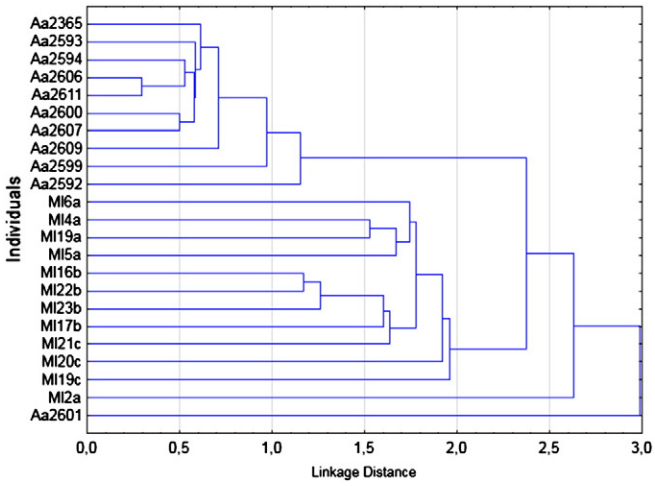

Fig. 7. Horizontal tree plot, displaying individual's grouping based on the similarities found for the standardized measures of the following biomarkers: SOD, CAT, GPx, GR, GST and TBARS. *M. leonina* individuals identified by MI initials, *A. australis* identified by Aa initials.

of time tend to exhibit a more intense bradycardia than shallow/short divers, and they usually deplete their body oxygen stores. These stores must be recovered quickly upon resurfacing, demanding an intense reestablishment of respiratory chain reactions.

In that sense, *M. leonina* must be routinely exposed to ROS production levels much higher than those of *A. australis*, producing greater potential IR injury for this species (Halliwell and Gutteridge, 2007). This condition is most likely associated with their mean submergence time, as *M. leonina* exhibits almost ten times greater capacity (24.30 ± 5.60 min) (Slip et al., 1994) than *A. australis* (2.8 ± 0.5 min) (Trillmich et al., 1986).

Considering that an elevated ROS production may lead to lipid peroxidation, membrane degeneration, mutagenesis and enzymatic inactivation (Moreno et al., 1980), the higher constitutive antioxidant profile in *M. leonina* might act as a way of preventing systemic oxidative stress.

Among vertebrates, torpor and hibernation are processes that resemble the hypoxic condition experienced by diving mammals (Budzádzic et al., 1990; Hermes-Lima and Storey, 1993). In these situations, an increase in the antioxidant capacity of an organism is commonly detected prior to their return to normoxia. Moreover constitutively increased antioxidant capacity has been reported in organisms that experience routinely drastic changes in oxygen consumption (Wilhelm Filho et al., 2000), suggesting an anticipatory defense against the high ROS production rates that occur once oxygen flow is reestablished (Di Meo and Venditti, 2000).

Therefore, our findings suggest that the reestablishment of oxygen flow to highly hypoxic tissues and the potential IR injury that is continuously experienced by *M. leonina* after dives demands a higher constitutive antioxidant *status*.

5. Conclusion

The two species of marine diving mammals analyzed in this study presented markedly distinct constitutive antioxidant profiles. *A. australis*, which dives commonly within its ADL limits and for short time periods, displayed lower antioxidant enzymatic activities, likely due to the shorter/shallower dives. In contrast, *M. leonina*, which routinely dives for long periods well over its ADL limit, demanding intense metabolic/physiological responses, including higher ROS production upon resurfacing, displayed higher antioxidant capacity. Therefore, the constitutive antioxidant defenses of *M. leonina* and *A. australis* indicate that the blood antioxidant *status* is correlated with the diving capacities of each species.

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