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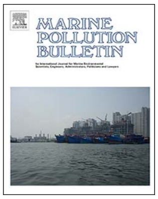

Baseline

Blood cadmium and metallothionein concentrations in females of two sympatric pinnipeds species

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ABSTRACT

Otaria flavescens (SASL) and *Arctocephalus australis* (SAFS) are endemic of South America. The aims were to assess Cd concentrations in red blood cells (RBC) and plasma from free living females of both species; and to establish metallothioneins (MT) levels in blood fractions and the possible relationship between MTs and Cd. Blood of fifteen SASL and eight SAFS females from Isla de Lobos were analyzed (years 2010–2011). All animals showed Cd levels above the detection limit. Cd concentrations on SAFS were higher than those of SASL, however, no significant differences were observed on metal concentrations between cell fractions by species. Metal levels were associated with a natural presence and ecological-trophic habits of the prey items. On SASL the MT concentrations between fractions were similar; whereas, SAFS plasma concentrations were higher than RBC. The results reported constitute the first information on Cd and MT blood levels in these species.

Marine mammals accumulate heavy metals in their tissues and organs as a result of biomagnification process (Bossart, 2011; Polizzi et al., 2013; Schaefer et al., 2015; Romero et al., 2016), being diet the major intake of metals for these top predators (Das et al., 2003; Booth and Zeller, 2005; Gerpe et al., 2009). Cadmium (Cd) is a toxic metal even at low concentrations (Goyer and Clarkson, 2001), and has no known physiological function. In mammals, high Cd concentrations has been associated with numerous harmful effects: physiological and biochemical disorders of several organs, mainly kidney and liver, and decrease of reproductive performance including fertility, abnormal embryonic development, prenatal death, and sexual dysfunction (Obianime and Roberts, 2009; ATSDR, 2012; Sarkar et al., 2013; Marettová et al., 2015). Besides, in marine mammals, this metal has been associated with immunosuppression and viral diseases (morbillivirus) (Kakuschke et al., 2006, 2009). Therefore, some authors related these effects with possible declines in pinniped and cetacean populations (Das et al., 2003; Gavind and Madhuri, 2014). In marine mammals from natural colonies, the information about non-essential elements in the different blood fractions (red blood cells – RBC-, plasma and/or serum) is limited, and most reports refer to whole blood (Griesel et al., 2006, 2008; Kakuschke et al., 2006, 2009; Sarran et al., 2008). This situation could be related to the difficulty to obtain the blood fraction samples in the field.

Metallothioneins (MT) are signal proteins of metal exposure that are widely used in biomonitoring programmes. Their high content of sulphydryl groups (−SH) allows them to bind divalent cations (Hylland et al., 1994), and therefore, play a primary role in the homeostasis of essential metals, such as copper (Cu) and zinc (Zn) (Klaassen, 2001), and the protection against toxic metal, such as Cd (Carpenè et al., 2007; Higashimoto et al., 2009; Klaassen, 2001). These evidences have also been reported in pinnipeds (Ikemoto et al., 2004a; Pillet et al., 2002; Sonne et al., 2009; Teigen et al., 1999).

South American sea lion (*Otaria flavescens*, SASL) and South American fur seal (*Arctocephalus australis,* SAFS) are endemic species of South America. The stocks of both species that breed on coastal islands of Uruguay constitute the most important focal concentration of pinniped females of the geographic distribution. Riet-Sapriza et al. (2013) reported that SASL diet in Uruguay mainly consists of white mouthcroaker (*Micropogonias furnieri*), striped weakfish (*Cynoscion guatucupa*), Brazilian codling (*Urophysis brasiliensis*), largehead hairtail

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(*Trichiurus lepturus*), Argentine croaker (*Umbrina canosai*), and, in less proportion of Argentine conger (*Conger orbignyanus*), and cephalopods (Loligoginidae and *Illex argentinus)*. On the other hand, SAFS preys principally on anchoveta (*Anchoa marinii*), Argentine anchovy (*Engraulis anchoita*), hake (*Merluccius hubbsi*), Argentine red shrimp (*Pleoticus muelleri*), Argentine squid (*Illex argentinus*), striped weakfish, and largehead hairtail (*Trichiurus lepturus*) (Naya et al., 2002; Ponce de León, 2000; Ponce de León and Pin, 2006). Based on dietary differences between SASL and SAFS, the aims of this study were to: 1) to assess Cd concentrations in red blood cells (RBC) and plasma from SASL and SAFS free living females; 2) to establish MT levels in both blood fractions and identify the possible relationship between these proteins and Cd in both pinniped species.

The study was conducted in Isla de Lobos (35°01′ 50″ S, 54°53′ 00″ W, Uruguay) in May 2010 and June 2011. Fifteen SASL and eight SAFS females were captured by the staff of DINARA (National Direction of Aquatic Research, Uruguay), and held in a big corral under veterinarian observation during 24 h. The animals were anesthetized with isoflurane using a mask (oxygen 5–10 L min^{-1} and 5% isoflurane), and afterwards with an endotracheal tube (constant oxygen flow of 2 L min^{-1} oxygen and 0.75–1.5% isoflurane). Animals breathed regularly by themselves. Heart and breathing rates, body temperature, electrocardiographic recording and oral mucosa condition were monitored each 5 min with both manual techniques and a multi-parametric monitor (Guoteng, Science and Technology). All females were classified as ASA 1 (American Society Anesthesiologists, www.asahq.org/clinical/physicalstatus.html) by a Veterinary Doctor. Total weight and length were registered (Table 1). The SASL sexual maturity - adult and subadult - was estimated according by Grandi et al. (2010). As no significant differences were found between adults and subadults for both Cd and MT concentrations, data were analyzed as a single group. All SAFS individuals were sexually mature.

Blood samples were collected from an interdigital vein of the flipper using an intravenous catheter (BD Angiocath 18G × 1.16″, SASL and BD Angiocath 20G × 1.16″, SAFS) and syringe with sodium heparin. Blood was centrifuged at 3000 rpm during 10 min for fraction separation. Plasma and RCB were immediately frozen in liquid nitrogen and stored at − 80 °C until analysis. Cadmium concentrations were determined by Atomic Absorption Spectrometry (Shimadzu AA6800); samples were digested with perchloric and nitric acid (Merck, analytical grade, 1:3) according to the method of FAO/SIDA (1983). The accuracy of Cd analyses was checked using the certified reference material (CRM) LUTS-1 (lobster hepatopancreas, National Research Council of Canada); the recovery rate was 91 ± 6%. Blanks were also analyzed for potential contamination and they were treated under the same conditions of samples and CRM. The detection limit (LOD) was 0.015 µg·g^{-1} wet weight (w.w.), and Cd concentrations were expressed in ng·g^{-1} (w.w.). For the statistical analyses, when the values were below of the LOD, they were replaced with a value equal to half the LOD (LOD/2) (Wood et al., 2011).

Table 1
Morphometrics parameters and sexual maturity stages of the South American sea lion (SASL) and South American fur seal (SAFS) females.

Sea Lion	Sexual maturity	Weight (kg)	Length (cm)
SASL 1	Adult	77.0	153.0
SASL 2	Adult	71.4	151.0
SASL 3	Adult	145.8	193.0
SASL 4	Adult	79.0	159.0
SASL5	Adult	109.6	184.0
SASL6	Adult	64.1	153.0
SASL7	Adult	121.0	189.0
SASL8	Adult	69.2	162.0
SASL8	Adult	67.4	151.0
SASL9	Adult	64.8	152.0
SASL 10	Subadult	62.0	145.5
SASL 11	Subadult	56.3	143.0
SASL 12	Subadult	61.0	142.0
SASL 13	Subadult	56.4	142.0
SASL 14	Subadult	55.3	137.0
SAFS 1	Adult	46.0	137.0
SAFS 2	Adult	46.5	136.0
SAFS 3	Adult	48.5	126.0
SAFS 4	Adult	40.0	124.0
SAFS 5	Adult	37.0	125.0
SAFS 6	Adult	40.5	128.0
SAFS 7	Adult	48.8	133.0
SAFS 8	Adult	40.1	128.0

The MT assay was performed according to the spectrometric method described by Viarengo et al. (1997). The absorbance was read at 412 nm, and MT concentrations were quantified using reduced glutathione (GSH) as a reference standard. The amount of MT was calculated based on cysteine content of rabbit MT (18 cysteines/mol), assuming a similar SH group content in pinniped MT. All samples were analyzed by duplicate, blanks were performed and MT concentrations were reported as nmol MT·µl^{-1} of wet tissue.

Mean values and standard deviation (SD) were calculated. Homoscedasticity of data was checked by Levene test ($p < 0.05$) and normality was checked with Shapiro-Wilk's test ($p < 0.01$). Statistical differences between Cd concentrations of adult and subadult SASL females were checked by Student's *t*-test, as no differences were found ($p > 0.05$), specimens were analyzed as an only data set. The differences about Cd concentrations between species were performed by non-parametric U-Mann Whitney test. Spearman's correlations were performed between Cd and MT concentrations, total length and weight. All analyses were conducted with Statistica 6.0 (Statsoft, Inc.).

The mean concentrations, SD and range of Cd and MT in RBC and plasma of both studied species are showed in Table 2. All specimens showed metal levels above the detection limit in at least one of the blood fractions. Blood metal concentrations reflect circulating levels and define the actual body burden, indicating a recent assimilation of the metal (Kakuschke et al., 2006; ATSDR, 2012).

There were no significant difference ($p > 0.05$) in Cd concentrations between cell fractions by species (Table 2). However, Cd concentrations were significantly higher in both blood fractions of SAFS than those of SASL (RBC, $Z = -2.64654$, $p = 0.008132$; plasma, $t = -3.36182$, $p = 0.003275$). As previously mentioned, diet plays an important role as the main metal source to marine mammals, and prey preferences can influence the heavy metal contents of predators (Stavros et al., 2008; Kakuschke et al., 2009). SAFS presents, as main prey items, species with ability for Cd accumulation, such as cephalopods (Miramand and Bently, 1992; Caurant and Amiard-Triquet, 1995; Bustamante et al., 1998; Gerpe et al., 2000) and anchovy (Gerpe, 2006).

Among prey cephalopods, there are two taxonomic groups with opposite capacities to accumulate Cd, Loliginidae squids, whose levels are extremely low, and Ommastrephidae squids, whose accumulate Cd at very high levels as normal characteristic (Bustamante et al., 1998). In this last group, *Illex argentinus* is found, whose digestive gland reaches concentrations of 485.01 µg·g^{-1} (Gerpe et al., 2000). With respect to Argentine anchovy, visceral tissues reached Cd levels as high as 3.32 µg·g^{-1} (wet weight) (Gerpe et al., 2006), as consequence of being a zooplanktophagous species (Angelescu, 1982; Sabatini, 2004). Based on this information, we suggest that, the presence of Cd in the waters to inhabit both pinnipeds species is not associated with anthropogenic sources. Several studies have reported that sub Antarctic oceanographic water mass, origin of the continental shelf waters of the Southwestern Atlantic Ocean is naturally rich in Cd (Honda et al., 1987; Westerlund and Ohman, 1991). Cd levels reported in particulate matter (0.038–0.163 µg·g^{-1}, w.w.) and zooplankton (from 0.07 to 4.33 µg·g^{-1}, w.w.) of northern Argentine shelf waters (Gerpe, 2006) evidence its presence. Both, this natural presence of Cd and the trophic and ecological habits of *I. argentinus* and *E. anchoita* to concentrate it, are responsible for trophic availability of Cd for the two studied species.

In marine mammals, MT has been associated with the homeostasis

Table 2
Cadmium (ng·g^{-1}, w.w.) and metallothionein concentrations (nmol·µl^{-1}, wet tissue) in red blood cell (RBC) and plasma of *Otaria flavescens* (SASL) and *Arctocephalus australis* (SAFS). SD: standard deviation, n: number of seals.

	SASL		SAFS	
	Mean ± SD	Range	Mean ± SD	Range
Cadmium				
RBC	41 ± 24	< 15–102	105 ± 65	22–241
Plasma	26 ± 31	< 15–105	88 ± 55	< 15–178
Metallothioneins				
RBC	0.035 ± 0.009	0.023–0.052	0.027 ± 0.006	0.016–0.038
Plasma	0.029 ± 0.003	0.022–0.036	0.038 ± 0.007	0.031–0.049

of essential metals and protection against toxic metal like Cd, in both pinnipeds (Ikemoto et al., 2004a; Pillet et al., 2002; Sonne et al., 2009; Teigen et al., 1999) and cetaceans (Das et al., 2002, 2004, 2006; Falconer et al., 1983; Ikemoto et al., 2004b). Metallothionein mean concentrations of SASL in RBC were similar to plasma (Table 2, $p > 0.05$); whereas, SAFS MT concentrations between both fractions showed significant differences ($t = -3.41144$, $p = 0.004216$), being plasma concentrations higher than RBC. It has been reported in rats exposed to Cd that MT plasma levels rise in the presence of the metal and appearance of this proteins in the body fluids is an early effect of cadmium exposure (Tohyama and Shaikh, 1981). Although this metal induces MT even at extremely low concentrations (Das et al., 2000; Klaassen, 2001), there was no relationship between Cd and MT concentrations in both blood fractions from SASL and SAFS. This could be due to: 1) Cd is not linked exclusively to MT, because it also has a close relationship with erythrocyte membranes and albumin (Suzuki et al., 1986; Ashour, 2014); 2) Cd blood levels are smaller than those usually reported in liver and kidney, target organs of this metal. Therefore, it is feasible that the observed SASL and SAFS Cd levels are not high enough to induce MT synthesis, despite being a powerful inducer; 3) Finally, increasing the number of specimens analyzed may be a factor that improves the interpretation of results. There are few studies about Cd and MT blood levels on marine mammals, therefore, the results reported here constitute a new contribution on blood values of these proteins in marine mammals, and in turn, constitute the first information on Cd and MT blood levels in *O. flavescens* and *A. australis*.

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