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The role of metallothioneins, selenium and transfer to offspring in mercury detoxification in Franciscana dolphins (*Pontoporia blainvillei*)

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ABSTRACT

The concentrations of mercury (Hg), selenium (Se) and metallothioneins (MT) were evaluated in fetuses, calves, juveniles and adults of the endangered coastal Franciscana dolphin (*Pontoporia blainvillei*) from Argentina. Mercury concentrations varied among analyzed tissues (liver, kidney, muscle and brain), with liver showing the higher concentrations in all specimens. An age-dependent accumulation was found in liver, kidney and brain. No significant relationship between Hg and MT concentrations was found for all tissues analyzed. Hepatic Hg molar concentrations were positively correlated with those of Se, indicating a great affinity between these two elements. Furthermore, dark granules of HgSe were observed in Kupffer cells in the liver by electron microscopy, suggesting the role of this macrophage in the detoxification of Hg. A transfer of Hg through placenta was proved. The presence of Hg in brain in all age classes did not show concentrations associated with neurotoxicity.

Mercury is a potentially highly toxic metal that could be released into the environment from both natural and anthropogenic sources (Hong et al., 2012). It is ubiquitous due to atmospheric transport and deposition, and it is considered one of the most concerning metals to marine organisms (Bezerra et al., 2015). Effects reported for Hg in marine mammals, include neurotoxicity (Basu et al., 2009), immune-suppression (Kakuschke et al., 2009) and endocrine disruption (Schaefer et al., 2011; Bechshoft et al., 2015).

Marine mammals, particularly odontocetes, are long-lived top predators that incorporate Hg through the diet, accumulating it in high concentrations in their organs (Nigro et al., 2002). Inorganic mercury (Hg^{2+}) has been identified in tissues, although they are primarily exposed to methylmercury through the fish diet (Das et al., 2003). Marine mammals may be partially protected against the negative effects of Hg through a number of mechanisms, including demethylation (Wintle et al., 2011), excretion (e.g., urine, feces, hair) (Nigro et al., 2002; Correa et al., 2014), interactions with proteins, such as metallothioneins (MT) (Das et al., 2000) and elements such as selenium (Se) (Khan and Wang, 2009). Koeman et al. (1973) reported for the first time a positive correlation between Hg and Se in liver and brain of cetaceans, and it has already been demonstrated that mercury selenide (HgSe) is a final product of the demethylation process, and as a mechanism to cope with mercury toxicity in cetaceans (Nakazawa et al., 2011; Lailson-Brito et al., 2012). Metallothioneins are a conserved family of proteins among mammals that are involved in defending organisms against metals (Harley and O'Hara, 2016). Female dolphins can also detoxify Hg by transferring it to fetus and calf through placenta and milk, respectively (Durden et al., 2007); however, some authors have suggested that the transfer of Hg via placenta is minimal (Endo et al., 2006; de Moura et al., 2012).

Franciscana dolphin (*Pontoporia blainvillei*) is a small endemic dolphin that inhabits the Southwestern Atlantic Ocean. Its geographical distribution ranges from Itaúnas (18°25′S, 30°42′W, Brazil; Siciliano and Santos, 1994) to Golfo Nuevo (42°35′S, 64°48′W, Argentina; Bastida et al., 2007). The International Union for Conservation of Nature (IUCN) has classified the species as Vulnerable A3d throughout its geographical range (Reeves et al., 2012), being the most impacted small cetacean in the Southwestern Atlantic Ocean (Secchi and Wang, 2002). Due to their coastal and estuarine habits, Franciscana dolphins inhabit areas of great human activity, which poses threats to their conservation. Only two studies assessed Hg concentrations in Franciscana dolphins from Argentina (Marcovecchio et al., 1990; Gerpe et al., 2002) but without evaluation of the relationship with Se. This study assessed concentrations of mercury in Franciscana dolphins and evaluated possible mechanisms of mercury detoxification -MT, Se, transference to fetus and calves- in the species.

Franciscana dolphins were collected from the coast of Buenos Aires Province, Argentina (Fig. 1). Dolphins were incidentally captured in artisanal fishing nets, being entangled for a period <10 h until sampling. Total length, weight and sex were determined for each specimen. Samples of liver, kidney, muscle and brain were collected,

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Fig. 1. Study area in Argentine continental shelf.

immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Integral analysis during necropsy and Relative Body condition Index de Le Cren close to 1 (one) revealed no significant indication of an unhealthy condition of any dolphin. Age of the same dolphins was determined in a parallel study by Denuncio et al. (2013) using dentine and cementum dental layers to determine Growth Layer Groups (GLGs). According to Polizzi et al. (2013), the estimated fine scale age was determined and dolphins were classified into four categories of maturity (Table 1).

Metallothioneins were previously determined by Polizzi et al. (2014) in the same specimens analyzed according to the spectrometric method described by Viarengo et al. (1997). For selenium determination, lyophilized tissues samples were subjected to microwave-assisted digestion in Teflon™ vessels with 2 ml HNO_3 (65%), 1 ml H_2O_2 (30%) and 5 ml of 18.2 $M\Omega \cdot cm^{-1}$ deionized water. After cooling, samples were diluted to 50 ml with 18.2 $M\Omega \cdot cm^{-1}$ deionized water in a volumetric flask. Se concentrations were determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, PerkinElmer, Sciex, DCR 2). An internal standard (^{103}Rh, CertiPUR®, Merck) was added to each sample and calibration standard solutions. Total Hg (THg) concentrations were measured by atomic absorption spectroscopy (DMA-80, Direct Mercury Analyzer, Milestone).

Quality control and quality assurance included field blanks, method blanks, and certified reference materials (CRMs: NIST 1566b, NIST 2976, DOLT-3, BCR 60 and BCR 61) (Table 2). The reported concentrations of elements were expressed in $\mu g \cdot g^{-1}$ dry weight as mean ± standard deviation.

For ultrastructural observations, formalin-fixed samples of liver were cut into 1 cm^3 cubes. Afterwards, samples were cut into smaller sections (1 mm^3) using glutaraldehyde 2% in phosphate buffer (PBS) to wash. Post-fixation was performed with osmium tetroxide (OsO_4) 1% and then fragments were dehydrated in ascending grades of ethanol (50, 70, 80, 95 and 100%) for 10 min each, with a last bath with acetone. Finally, the fragments were embedded in Epon resin. Ultra-thin sections (60 nm) were obtained in an ultramicrotome (Supernova) and transferred to copper grids. These sections were observed in a JEM 1200EX II (Jeol).

Data were tested for a normal distribution using Kolmogorov-Smirnov's test and homoscedasticity of data was checked by Levene's test. After that, statistical differences were assessed by *t*-test and ANOVA where appropriate. Spearman's correlation coefficient was determined between mercury and selenium. All analyses were conducted with software STATISTICA® 6.0 (Statsoft, Inc.).

Table 1
Estimated fine scale age, total weight and length range of *Pontoporia blainvillei*. n = number of dolphins.

	Estuarine dolphins			
	n	Estimated age (year)	Weight (kg)	Total length (cm)
Fetuses	2	–	1.9–2.4	51–60
Calves	9	0.1–0.4	4.1–15.9	74–108
Juveniles	22	1.0–3.4	11.5–23.0	91–129
Adults	9	3.5–10.5	16.4–31.1	114–140

Table 2
Precision and accuracy for Hg and Se on certified reference materials. Data are expressed as mean ± SD $\mu g \cdot g^{-1}$ dry weight.

Element	Hg		Se	
	Certified value	Measured value	Certified value	Measured value
NIST 1566b	–	–	2.06 ± 0.15	2.09 ± 0.07
NIST 2976	–	–	1.08 ± 0.15	2.04 ± 0.04
DOLT-3	3.37	3.62 ± 0.02	–	–
BCR 60	0.34	0.36 ± 0.03	–	–
BCR 61	0.23	0.25 ± 0.04	–	–

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Table 3
Mercury and selenium concentration (mean ± SD, $\mu g \cdot g^{-1}$, dry weight) in liver, kidney, muscle and brain in age classes of *Pontoporia blainvillei*. Equal letter demonstrates a significant difference between age classes ($p < 0.05$).

Age class	Tissue	Hg	Se	Se/Hg
Fetus	Liver	2.54–5.04	2.54–3.86	2.16–2.52
	Kidney	1.10–2.54	1.09–5	3.86–5.89
	Muscle	1.32–2.93	1.41–2.16	1.22–2.09
	Brain	1.16	1.24	2.71
Calf	Liver	1.29 ± 0.79^{ab}	1.88 ± 0.49^{fg}	4.68–2.18
	Kidney	0.76 ± 0.72	3.57 ± 1.23i	21.18–13.86
	Muscle	1.03 ± 1.09^{d}	1.15 ± 0.37	4.64 ± 2.69
	Brain	0.16 ± 0.05^{e}	0.87 ± 0.13	14.81–6.28
Juvenile	Liver	3.62 ± 3.46^{ac}	6.14 ± 2.65^{fh}	5.49 ± 1.94
	Kidney	1.35 ± 1.11	11.65 ± 6.07i	27.49 ± 13.68
	Muscle	1.49 ± 0.72	4.75 ± 5.26	7.87 ± 7.26
	Brain	0.39 ± 0.17^{e}	1.38 ± 0.50	9.81 ± 3.86
Adult	Liver	26.88 ± 22.25^{bc}	31.76 ± 24.48^{gh}	5.62 ± 4.49
	Kidney	3.78 ± 4.35	7.48 ± 1.07	14.12 ± 21.00
	Muscle	3.10 ± 1.96^{d}	1.48 ± 0.43	4.88 ± 5.86
	Brain	0.38–3.85	0.82–2.21	9.89 ± 6.14

Mercury and Se concentrations, as well as their molar ratio in liver, kidney, muscle and brain are presented in Table 3. They are evaluated by age classes, without distinction of sex, due to the absence of significant differences between them ($p > 0.05$).

Mercury concentration in liver, kidney and muscle from calves, juveniles and adults of Franciscana dolphins were according to those found by Gerpe et al. (2002) in specimens of the same area; however hepatic concentrations were lower than those reported by Marcovecchio et al. (1990), although these results were derived from only two juvenile dolphins. Liver concentrations of Hg were similar (Lailson-Brito et al., 2002) or higher (Kunito et al., 2004; de Carvalho et al., 2008; Moreira et al., 2009; Seixas et al., 2009; Di Beneditto et al., 2011) to those found in Franciscana dolphins from Brazil. In all these reports the liver was the principal organ for Hg accumulation compared to other tissues, as well as in the specimens analyzed in this study.

An age-dependent accumulation of Hg was found in liver ($r = 0.69$; $p < 0.001$; Fig 2A), kidney ($r = 0.55$; $p < 0.01$; Fig. 2B) and brain ($r = 0.59$; $p < 0.01$; Fig. 2C). Several studies found a positive correlation between Hg concentrations with age (Gerpe et al., 2002; García-Alvarez et al., 2015).

No significant relationship between Hg and MT concentrations was found for all tissues analyzed ($p > 0.05$). In terrestrial mammals, such as rats and mice, binding of Hg to MT has been demonstrated and induction of these proteins after Hg exposure (Morcillo and Santamaria, 1993; García-Sevillano et al., 2015; Orct et al., 2015). However, the role of MT in the detoxification of Hg is controversial; studies in marine mammals have indicated that a small percentage of Hg is bound to MT in liver and kidney (Decataldo et al., 2004; Ikemoto et al., 2004a, 2004b). All specimens analyzed here had Hg concentrations below the tolerance limit for cetaceans (100–400 $mg \cdot kg^{-1}$ w.w. liver, Wagemann and Muir, 1984), and therefore, the Hg concentrations were likely not high enough to induce the synthesis of MT.

On the other hand, in the liver of Franciscana dolphins, Hg molar concentration was positively correlated with those from Se ($r^2 = 0.86$; $r = 0.90$; $p < 0.001$; Fig. 2D), indicating a great affinity between these two elements. Furthermore, the Se/Hg molar ratio was higher than 1 for all tissues in all age classes indicating a potentially adequate Se supply for normal function and protection against Hg toxicosis (Correa et al., 2014).

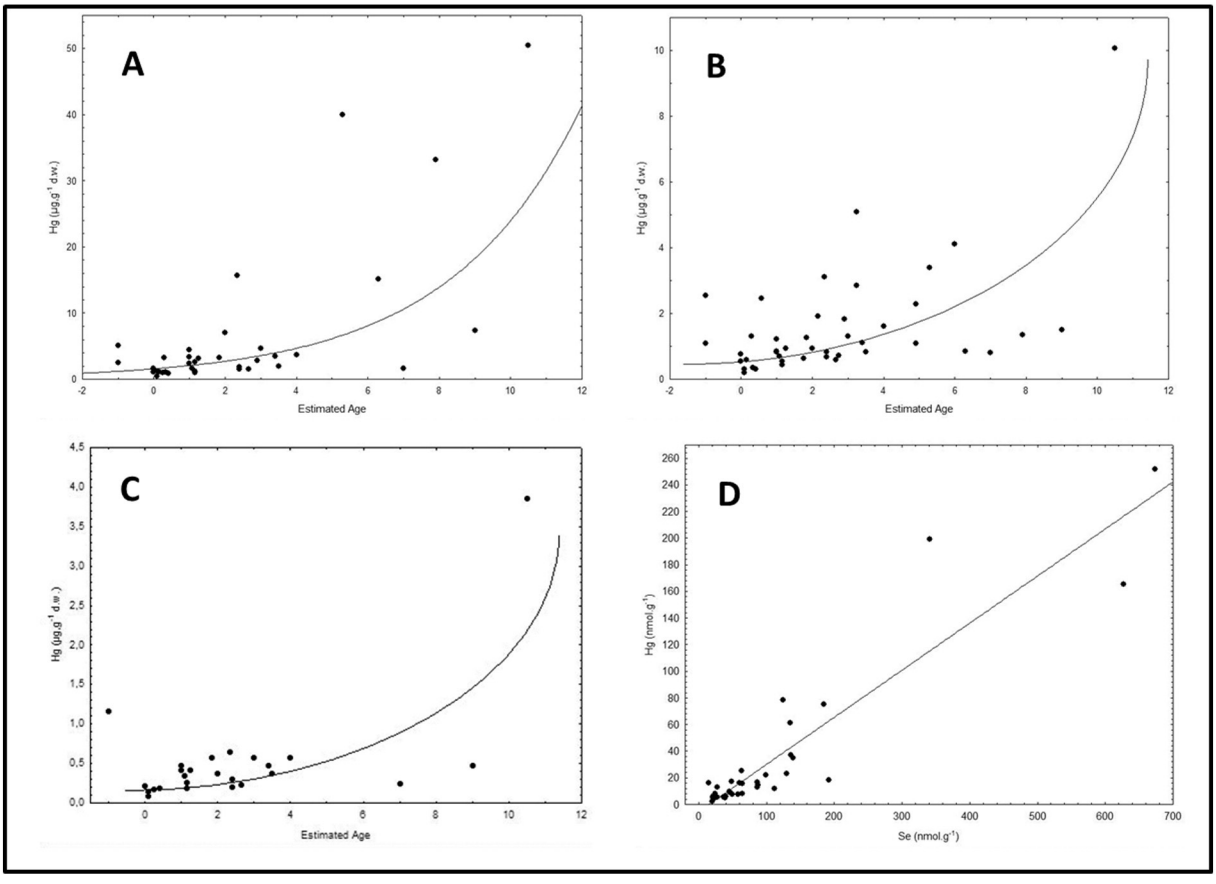

Fig. 2. Distribution of Hg ($\mu g \cdot g^{-1}$ d.w.) in *Pontoporia blainvillei* vs estimated age (A: liver; B: kidney; C: brain) and molar relation between Hg and Se ($nmol \cdot g^{-1}$) (D).

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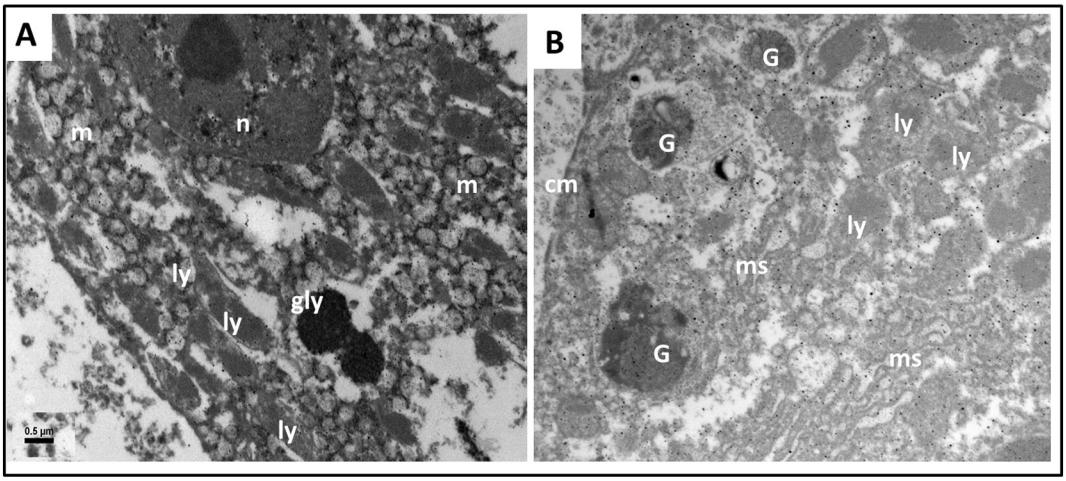

Fig. 3. Ultrastructure of Franciscana dolphin liver. A: electron microscopy of a hepatocyte showing numerous mitochondria (m) and glycogen granules (gly). B: Kupffer cell and its organelles, showing electro-dense granules (G) within lysosomes (ly). n: nucleus, ms: membrane system, cm: cellular membrane.

The structure of a dolphin hepatocyte (Fig. 3A) evidenced great amounts of mitochondria and lysosomes, besides the presence of glycogen in the form of dark granules. Furthermore, a Kupffer cell (macrophages) could be recognized by its ultrastructure and presented electro-dense granules within the lysosomes which would correspond to intracellular deposits of Hg—Se (Fig 3B). Although some studies found mercury deposits in hepatocytes of bottlenose dolphins (*Tursiops truncatus*, Rawson et al., 1993) and pilot whales (*Globicephala melas*, Stoltenberg et al., 2003), the primary cell involved in storage of HgSe is suggested to be the Kupffer cell (Nigro, 1994; Nigro and Leonzio, 1996; Lailson-Brito et al., 2012). Our results support this role of macrophages in the detoxification process of Hg in liver.

The presence of Hg in tissues of the fetus of Franciscana dolphins analyzed here proved a transfer of the metal through placenta. Gerpe et al. (2002) suggested that the maternal contribution of mercury via placenta was not significant in this species, due to no metal concentrations found in fetal tissues (below detection limit: 0.05 $\mu g \cdot g^{-1}$ w.w.). In cetaceans, Hg, especially the organic form, could cross the placental barrier and accumulate in the fetus tissues (Durden et al., 2007). On the other hand, it could not be certain that Hg concentrations found in lactating calves are due to a prior placental transfer or a new contribution through milk. The hepatic Hg burdens in fetuses (n = 2; 156–216 µg) and lactating calves (n = 2; 170–307 µg) were similar, and specimens presented a narrow age difference. Concentrations of mercury in dolphin milk need to be determined.

Sensitive fetuses and calves exposed to Hg could lead to neurodegenerative diseases or dysfunction of the central nervous system followed by premature death (Gaeta and Hider, 2005). Basu et al. (2006) showed that subclinical neurological changes were observed from 0.78 to 1.3 $mg \cdot kg^{-1}$ (w.w.) Hg in mink (*Mustela vison*) brain. However the values of Hg in Franciscan dolphin brains were lower than those mentioned above, The organic form could efficiently cross the placental barrier (Noël et al., 2016) and demethylating mechanisms develop with increasing age (Wintle et al., 2011), leaving fetal and very young dolphins more vulnerable to organic Hg toxic effects when compared to adult mammals (Correa et al., 2014).

In conclusion, a process of bioaccumulation of Hg and transfer through placenta were confirmed in Franciscana dolphins from Argentina. Furthermore, the formation of HgSe granules in the liver is likely to be the main mechanism for mercury detoxification due to the strong positive relationship between Hg and Se. In addition, the lack of relation between Hg and metallothioneins would support the importance of the above mentioned mechanism. The presence of Hg in the brain of all age classes suggests the need to investigate the potential effects.

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