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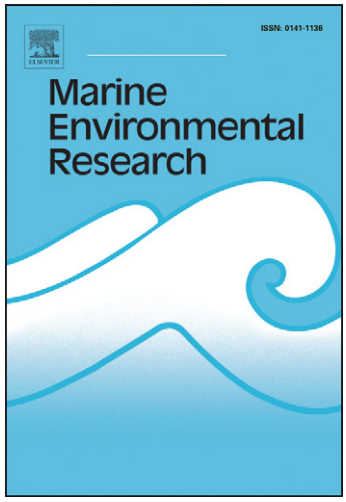

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Heavy metal toxicity of kidney and bone tissues in South Australian adult bottlenose dolphins (*Tursiops aduncus*).

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ABSTRACT

Metallothioneins (MT) concentration, renal damage, and bone malformations were investigated in 38 adult *Tursiops aduncus* carcasses to determine any associations with cadmium, copper, zinc, mercury, lead and selenium. Significantly higher concentrations of cadmium, copper, and zinc in the liver were observed in dolphins showing evidence of more advanced renal damage. No significant differences in metal or selenium concentrations in the liver were observed between groups differing in level of bone malformations. Some dolphins displayed evidence of toxicity and knowledge of metal toxicity pathways were used to elucidate the cause of these abnormalities. Two dolphins had high metal burdens, high MT concentrations, renal damage, and evidence of bone malformations, indicating possible severe and prolonged metal toxicity. One dolphin showed evidence of renal damage, but the lack of any other symptoms suggests that this was unlikely to be caused by metal toxicity. We recommend examining a range of metal toxicity symptoms simultaneously to aid in distinguishing metal toxicity from unrelated aetiologies.

Keywords: Heavy Metals; Toxicity; Mammals; Dolphins; Metallothionein; Kidney; Bone; Cadmium; Mercury; Lead; Zinc

1. INTRODUCTION

Metal concentrations have been recorded in over 60 species of cetaceans (O'Shea, 1999) but few studies have explored the relationship between metals and toxicity. Metal toxicity causes irregular metallothioneins (MT) protein synthesis, renal damage, and

disruption of bone structure in humans and wildlife (Beiglbock et al., 2002; Jarup, 2002; Sato and Kondoh, 2002). Studies of metal toxicity in cetaceans, particularly beach-stranded carcasses, are hindered by difficulties arising from examining tissues that have undergone post-mortem autolysis. Since these are usually the only specimens available, it is important that methods of measuring metal toxicity in decomposed carcasses are developed to allow determination of dolphin health, even in degraded specimens.

Metallothioneins (MT) are metal-binding proteins that preferentially bind to Cd (cadmium) and Zn (zinc), reducing the bioavailability of these elements (Sato and Kondoh, 2002; Das et al., 2006) and providing an early indicator of cellular responses to metal toxicity (Petering et al., 1990). Although MT are often correlated with liver and kidney metal burdens, few attempts have been made to correlate them with markers of heavy metal toxicity in cetaceans. This is an absolute prerequisite to support the use of MT overexpression as a bioindicator of metal toxicity.

Metals are toxic to mammalian renal cells (Wang and Pfeiffer, 2001), causing damage that leads to leakage of phosphates, calcium, glycogen and proteins (proteinuria) from the kidney (Loghman-Adham, 1997). Histological indicators of proteinuria have been used as a preliminary measure of renal pathology in *Tursiops aduncus* in South Australia (Long et al., 1997) but may have been impacted by post-mortem autolysis. In contrast, metal-induced swelling of the Bowman's capsule, and protein leakage within an intact capsule, are not affected by post-mortem decomposition (Beiglbock et al., 2002).

While MT and renal structure swelling may determine toxicity in mildly degraded specimens, bone structure is resistant to post-mortem degradation. Loss of bone mineral structure occurs in cases of Cd, Pb (lead), and Hg (mercury) toxicity in humans (Escribano et al., 1997; Jarup, 2002; Suzuki et al., 2004) while increased and decreased bone mineral density have been observed in response to Zn and Se (selenium) excess and deficiency respectively (Turan et al., 2000; Yun and Zeng-Li, 2002). Loss of bone density is a clinical endpoint marker of chronic metal toxicity and indicates accompanying sub-clinical behavioural and immunological deficits (Staessen et al., 1999) which are difficult to examine directly in free-living cetaceans.

The causes of elevated MT, renal damage, and loss of bone density and complexity are probably interrelated. MT induced by metal toxicants form a large metal-MT complex which leads to damage of renal structures. This results in leakage of calcium, phosphate and proteins from the kidney, hindering bone remodelling and leading to a loss of bone density (bone mineral density) and complexity (bone histomorphometry) (Alfven et al., 2002). Thus, dolphins displaying metal-induced bone malformations should show symptoms of elevated MT synthesis and damage of renal structures, assuming these markers are reflecting metal toxicity and not extraneous influences (e.g., post-mortem cellular degradation, unrelated parasitism, unrelated disease, normal mammalian ageing process). Consistency between MT, renal damage and bone structure should provide a valuable tool for helping to distinguish metal-induced toxicity from other aetiologies.

South Australian adult Indo-Pacific bottlenose dolphins, *Tursiops aduncus,* have been shown previously to have high concentrations of Cd, Hg, and Se in the liver, moderate concentrations of Pb, Cu, and Zn in the liver and moderate concentrations of Cd and Pb in bone compared to dolphins elsewhere (Lavery et al., 2008). This study aims to explore any links between MT, renal damage, bone density and structure, and liver tissue concentrations of Cd, Hg, Pb, Zn, Cu and Se in adult bottlenose dolphins.

2. MATERIALS AND METHODS

2.1 Necropsy procedures and sample selection

Necropsy procedures and determination of Se and metal concentrations by Inductively Coupled Plasma Atomic Emission Spectrometry in 71 *T. aduncus* from South Australia are detailed elsewhere (Lavery et al., 2008). All dolphins were collected by the South Australian Museum (SAMA) and given a museum identification number. Some dolphins had multiple analyses conducted on one tissue, and the mean metal or Se value was used in these cases. Since animal age impacted metal accumulation in these dolphins, only a subset of 38 sexually and / or physically mature adults of tooth category 3 and above (see Kemper and Gibbs, 2001, for details of dolphin development compared to tooth category) were examined in the present study. This sample of 38 adult dolphins consisted of 17 male and 21 female adult dolphins, 16 of which had kidney samples with intact nuclei and glomeruli available for histopathological examination (Geraci and Lounsbury 1993, decomposition code 2 and 3). Kidney (N = 15) and liver (N = 14) samples from a subsample of these animals were analysed for MT. Bone density was measured in four

vertebrae of 30 animals and the histomorphometry of costal rib tips examined from 15 animals.

2.2 Metallothionein quantification

Soft tissues were collected following procedures that minimise cross contamination (Geraci and Lounsbury, 1993). Subsampling of frozen (-20 ^{o}C) tissue entailed collecting approximately 0.5 g of liver and kidney for MT analysis. MT was quantified using the modified cadmium-haemoglobin affinity assay (Eaton and Toal, 1982). Briefly, this methodology requires adding radioactively labelled ^{109}Cd to the tissue supernatant, allowing it to bind to MT present. A 'Cobra Auto-gamma' counter (Packard Company) was used to determine the Cd content of the resultant supernatant, which allows for determination of the equivalent MT concentration, expressed as nmol of Cd bound g^{-1} wet weight.

2.3 Histological examination

Histology was carried out on 16 formalin-fixed kidney tissues: 15 samples were previously frozen (-20 ^{o}C) and one fixed without prior freezing (SAMA: M21243). Haematoxylin and eosin (H&E) preparations were carried out on each of the 16 kidney samples using standard histological methodology (Brancroft and Stevens, 1982). An Olympus BH-2 light microscope was used with 20× magnification. Digital microscope images of 60 glomeruli and Bowman's capsules observed were captured, and software (Motic Images Plus, Version 2.0) was used to measure the area of 20 randomly selected glomerulus and Bowman's capsules from each slide. The area of the space between the

glomeruli and Bowman's capsule, and the presence of proteins within each Bowman's capsule was recorded.

2.4 Quantification of bone density and structure

Bone strength depends on the amount of bone and the organisational structure of the trabeculae (Kleerekoper et al., 1985), so two measures of bone density were obtained; Dual energy X-ray Absorptiometry (DXA) and histomorphometry. DXA measures the total amount of bone (cortical and trabecular bone), but skeletal metal stores can bias results (Puzas et al., 2002). Histomorphometry directly quantifies the 3-dimensional micro-architectural structure and organisation of bone and is not biased by metal burden.

DXA scans were performed on a GE Lunar Prodigy Vision Dual X-ray Absorptiometry, GE Lunar Madison, Wisconsin. Four caudal vertebrae from each of 30 animals were analysed to obtain measurements of bone mineral density, BMD (g cm^{-2}), and bone mineral content, BMC (g). Caudal vertebrae were selected on the basis of being the most posterior four caudal vertebrae with small transverse processes. Small animal software (version 8.10.027) on standard settings was used to examine a maximum area of 25×25 cm. Vertebrae were laid longitudinally to best simulate the longitudinal stress placed on the caudal region of the spine by powerful dorsal and ventral muscles associated with swimming. Bones were laid on a 1 cm thick Lucite tissue equivalent block and scanned with parameters set at 76 kilovolts and 150 μA. Pixel size was 1.05×0.6 mm. BMD and BMC of the vertebrae and posterior process (but not the transverse processes) were determined.

Histomorphometry was performed using a Skyscan 1072 X-ray Microtomograph on the largest costal rib available from each of 15 animals. A piece 2 cm long was sampled from the largest end of the bone and placed in the X-ray Microtomograph with the cut facing down. Pixel size was 18.95 μm while the X-ray parameters were set at 16× magnification, 100 kilovolts and 98 μA. A ring artefact correction was used and a beam hardening correction of 100% was set. Cone reconstruction software was used to reconstruct the 3-dimensional structure of the bone from 1,000 separate 2-dimensional cross section images. Bone density parameters from the 3-dimensional reconstructions were calculated using CTan software (Skyscan). Density parameters calculated by CTan are shown in Table 1.

2.5 Statistical analyses

Our primary aim was to determine if metal concentrations were associated with markers of toxicity. To examine the influence of metal concentrations on kidney structures a hierarchical cluster analysis (data not shown) was employed which sorted 14 individuals into two groups based on their concentrations of MT and their levels of renal toxicity. Between groups linkage was used to designate groups based on all available information and squared Euclidian distances were used to derive proximity measurements. Values were transformed using Z scores due to variables being measured on different scales. Once groups were designated, an independent samples t-test was then used to determine any significant differences in log transformed metal concentrations, dolphin length, or blubber thickness between the two groups. This process was then repeated, using cluster

analysis to sort bone structure variables of 15 individuals into two groups, and a t-test to determine differences in log transformed metal concentrations, dolphin length, and blubber thickness between the groups. All analyses were conducted using SPSS version 14.0.

2.6 Qualitative analyses

Our second aim was to examine the datasets for outlier animals with extreme levels of toxicity which could be examined to shed light on the aetiology of the observed toxicity. Three outlier animals were clearly identifiable from scatter plots (data not shown) and examined. We assume here that metal toxicity aetiology will be highlighted by consistency along the metal toxicity pathway (high metal concentrations, high MT concentrations, advanced renal damage and advanced bone malformations). We assume one of these indicators alone suggests an aetiology that is unrelated to metal toxicity.

3. RESULTS

High levels of variation among individuals were observed in all toxicity markers (Table 2) and qualitative differences among animals were observed for histological indicators (Figure 1) and costal rib histomorphometry (Figure 2).

3.1 Renal damage

Cluster analysis classified individuals into two groups based on differences in their measured renal parameters (renal structure swelling, protein leakage, and MT

concentrations). Renal Group 1 had low scores for indicators of renal damage, whereas Renal Group 2 was characterised by high levels of renal damage, with advanced renal structure (glomerulus, Bowman's capsule, and the space between glomerulus and Bowman's capsule) swelling, higher levels of protein leakage, and higher MT (liver and kidney) concentrations (Table 3). Renal Group 2 had significantly higher levels of Cd ($p = 0.045$), Cu ($p = 0.047$) and Zn ($p = 0.047$) than Renal Group 1. No significant difference in dolphin length or blubber thickness existed between the groups ($p > 0.05$).

3.2 Bone malformations

Classifications based on bone parameters were less clear than those based on renal parameters. Bone Group 1 was defined by lower spinal bone mineral density whereas Bone Group 2 had lower levels of rib bone trabecular organisation (Table 4). Metal concentrations, animal length, and blubber thickness were not significantly different between the groups ($p > 0.05$).

3.3 Consistency among toxicity markers

Three dolphins showed extreme values in one or more of the measured markers (MT, liver histology, bone density or bone organisation). A 2 m female dolphin (SAMA: M22410) from lower Spencer Gulf showed consistency among the measured parameters. This dolphin had the lowest recorded spinal bone mineral density, highest tissue concentrations of Cd and Cu (Table 2), moderately high levels of Zn in the liver (209.35 mg kg^{-1}), the highest concentration of liver MT, the largest area between Bowman's

capsule and glomeruli, the highest percentage of protein leakage within the Bowman's capsule (Table 2), the second highest Bowman's capsule area (19477 μm^2) and third highest glomerulus area (11703 μm^2) recorded. Examination of the skeleton revealed serious malformations in the lumbar-caudal region. The last lumbar vertebra had no transverse processes on the right side. The first caudal vertebra had no transverse processes at all, while the second and third caudal vertebrae were missing transverse processes on the left side. Neural spines were thickened on the second, third and fourth caudal vertebrae.

A 2.1 m male dolphin (SAMA: M20877) from upper Spencer Gulf had the lowest recorded bone complexity values (BV/TV, Tb.Pf, SMI) (Table 2) and also showed consistency among measured parameters. This male dolphin had the second highest recorded scores for concentrations of liver MT (876 nmol of Cd bound g^{-1} wet weight), glomerulus area (11922 μm^2), and area of space between Bowman's capsule and glomerulus (6675 μm^2). It had the third largest Bowman's capsule area (18597 μm^2), and second highest recorded liver Zn burden (270 mg kg^{-1}).

A 2.3 m male (SAMA: M21243) from Gulf St Vincent had the highest recorded glomerulus and Bowman's capsule areas (Table 2) but this was not consistent with other measured parameters. Values for tissue metal burdens and other markers of toxicity were very close to the mean and there were no other signs of toxicity in this dolphin.

4. DISCUSSION

4.1 Renal damage

Concentrations of Cu and Cd in the liver were significantly higher in the cluster of animals defined by high levels of renal damage. Cadmium is a known nephrotoxicant (Wang and Pfeiffer, 2001). Exposure to Cd is known to alter Cu metabolism (Peraza et al., 1998) and the close association between Cd, Cu and renal damage in the present study raises the possibility that alteration of Cu metabolism may be a mechanism for the observed damage. The relationship between MT and renal damage provides support for the use of MT overexpression as a suitable biomarker of Cd-induced renal damage in cetaceans.

The cluster defined by high levels of renal damage also had significantly higher levels of hepatic Zn. Zinc is an essential metal but it can be toxic in high concentrations (Walsh et al., 1994). Large anthropogenic quantities of Zn (> 30,000 kg) are released into the marine environment annually in Spencer Gulf, South Australia (NEPC, 2003), and in the long term could result in unusually high Zn accumulation in dolphin tissues and toxic impacts. The significance of renal structure swelling has not been studied in cetaceans specifically; however, laboratory mammal and human studies have found an increase in annual mortality risk of 40 – 80% in human patients with metal-induced renal pathology (Iwata et al., 1992; Nakagawa et al., 1993; Nishijo et al., 1999).

4.2 Bone malformations

The bone demineralising effects of environmental contaminants are well documented. East Greenland polar bear and Baltic grey seal populations show evidence of bone mineral disruption associated with high body burdens of organochlorine contaminants (Lind et al., 2003; Sonne et al., 2004). While laboratory studies suggest that metals also influence bone structure and mineralisation (Suzuki et al., 2004), associations between metals and bone in wildlife studies are less readily observed (Sonne-Hansen et al., 2002; although see Beiglbock et al., 2002)

Rather than sorting individuals based on high or low levels of bone damage, cluster analysis sorted dolphins into groups based on different types of bone organisation. Bone Group 1 was characterised by low levels of spinal bone density and Bone Group 2 were characterised by low levels of rib bone trabecular organisation. Perhaps due to this method of clustering, and despite the known impacts of metals on bone, there were no significant differences in metal concentrations between groups, suggesting metals may be exerting no influence on bone structure in this study (although see sections 4.3 below).

Alternatively, it is known that Pb preferentially accumulates in the skeleton, leading to overestimates of bone mineral density and complexity (Puzas et al., 2002). Thus, animals with high bone mineral density scores here may be biased by bone metal content. Interpretation of these results is hindered by the fact that measurements were taken in different bones. Histomorphometry was measured in peripheral rib bones while bone mineral density was measured in the axial spinal skeleton. The axial skeleton is more sensitive to the effects of toxicants than the peripheral skeleton (Laan et al., 1993).

Future increases in microtomograph scanning size may allow for quantification of spinal histomorphometry and would aid in clarifying the relationship between metals, bone density, and bone organisation.

4.3 Consistency among toxicity markers

Three dolphins had evidence of toxicity and these animals were examined more closely to reveal possible aetiologies. A 2.3 m male (SAMA: M21243) from Gulf St Vincent had high levels of renal structure swelling but no other markers of toxicity suggesting this animal was probably not suffering from metal toxicity, but from an undetermined renal condition.

A female dolphin from Spencer Gulf (SAMA: M20877) had high Cd and Cu concentrations in the liver, MT overexpression, renal damage and extreme bone malformations. While difficult to prove, the consistency amongst toxicity markers may suggest this dolphin was suffering from severe metal toxicity. This animal had a tooth category of five, indicating that it should be both physically and sexually mature (Kemper and Gibbs, 1997). However, examination of ovaries and skeleton revealed that this animal was physically and sexually immature. It is possible, therefore, that the high levels of metal toxicants have delayed sexual and physical maturity in this animal. However, due to the non-experimental nature of these observations, it is impossible to rule out the presence of an extraneous influence that either caused the observed pathology, or rendered this dolphin more susceptible to the effects of metal toxicity.

A 2.1 m male dolphin (SAMA: M20877) from upper Spencer Gulf with high Zn tissue concentrations showed decreased bone complexity, high concentrations of liver MT, and renal structure swelling. This dolphin had a tooth category of 5 and was sexually and physically mature (Kemper and Gibbs, 1997). The consistency between toxicity markers suggests this dolphin may have been suffering from metal toxicity. Zinc toxicity in humans occurs above a threshold level of 465 mg kg^{-1} dry weight (Anon, 1998), which is well below the dry weight Zn concentration of 999 mg kg^{-1} recorded in this animal.

The relationship between metals, toxicity of the kidney, and bone malformations, has been demonstrated in experimental and natural studies of humans and wildlife (Staessen et al., 1999; Alfven et al., 2002; Jarup and Alfven, 2004). A study of 100 ringed seals (*Phoca hispida*) in Greenland, however, found no relationship between cadmium, degree of bone mineralisation, and nephropathy (Sonne-Hansen et al., 2002) and the authors concluded that high dietary intakes of vitamin D and calcium (among other minerals) in these ringed seals may protect seals from cadmium toxicity. However, the sample of ringed seals consisted mainly of animals younger than 10 years. Cadmium-induced renal damage typically occurs only after 10 years exposure (Friberg et al., 1986) and the effect of Cd on bone is more pronounced in older bone (Alfven et al., 2002). While accurate ages of the bottlenose dolphins examined here are unknown, all are tooth category 3 and above, and would be older than 10 years (Kemper and Gibbs, 2001).

Experimental studies of metal toxicity in dolphins are undesirable, necessitating the use of natural experiments to infer relationships between health and toxicants. Natural

experiments must contend with a lack of experimental control and the presence of confounding factors (e.g., reproductive status, nutritive condition etc). Results obtained from natural studies must be viewed cautiously but can provide important information that can guide future research despite the absence of controlled experimental data. The influence of age on metal accumulation (Lavery et al., 2008) and bone mineral density (Butti et al., 2007) have been minimised in the present study by including only adult animals but not eliminated as knowledge of the accurate ages of each dolphin included in this study is not known.

There was a large hiatus between the highest Cd levels (M22410, 98.58 mg kg^{-1}) which seem to be associated with toxicity, and the second highest (M21231, 22 mg kg^{-1}) which showed no association with toxicity. Thus, toxicity thresholds for *T. aduncus* could not be determined. Future discoveries of such intermediate Cd tissue burdens in South Australian *T. aduncus* may allow for the establishment of a toxicity threshold which will aid in interpreting the relevance of metal concentrations already determined throughout the world (see O'Shea, 1999, for a review).

5. CONCLUSION

Higher metal concentrations were found in a group of animals suffering from renal damage. No differences in metal concentrations were found between groups sorted on the basis of bone density and organisation. Knowledge of the toxicological pathway of metals can aid in distinguishing symptoms of metal toxicity from other, unrelated aetiologies. While only adult dolphins were examined here, the influence of fine scale

changes in dolphin age and undetected pathologies represent a methodological limitation in carcasses studies such as this. Extraneous influences are difficult to determine or overcome but we suggest that, by examining a range of metal toxicity symptoms concurrently, future studies can better allow for determination of whether observed health deficits are related to metal toxicity, or an unrelated aetiology.

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REFERENCES

Alfven, T., Jarup, L., Elinder, C.G., 2002. Cadmium and lead in blood in relation to low bone mineral density and tubular proteinuria. Environmental Health Perspectives, 110, 699–702.

Anon., 1998. National irrigation water quality program. *Information report No. 3*, US Department of Interior.

Beiglbock, C., Steineck, T., Tataruch, F., Ruf, T., 2002. Environmental cadmium induces histopathological changes in kidneys of roe deer. Environmental Toxicology and Chemistry, 21, 1811 – 1816.

Brancroft, J., Stevens, A., 1982. Theory and practice of histological techniques. 2nd Edition. New York: Churchill-Livingstone.

Butti, C., Corain, L., Cozzi, B., Podesta, M., Pirone, A., Affronte, M., Zotti, A., 2007. Age estimation in the Mediterranean bottlenose dolphin Tursiops truncatus (Montagu 1821) by bone density of thoracic limb. Journal of Anatomy, 211, 639 – 646.

Das, K., De Groff, A., Jauniaux, T., Bouquegneau J.-M., 2006. Zu, Cu, Cd and Hg binding to metallothioneins in harbour porpoises *Phocoena phocoena* from the southern North Sea. BMC Ecology, 6, 2.

Eaton, D.L., Toal, B.F., 1982. Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. Toxicology and Applied Pharmacology, 66, 134 – 142.

Escribano, A., Revilla, M., Hernandez, E.R., Seco, C., Gonzalez-Riola, J., Villa, L.F., Rico, H., 1997. Effect of lead on bone development and bone mass: A morphometric, densitometric, and histomorphometric study in growing rats. Calcified Tissue International, 60, 200 – 203.

Friberg, L., Elinder, C.-G., Kjellstrom, T., Nordberg, G.F., 1986. Cadmium and health: A toxicological and epidemiological appraisal, vol. II. Florida: CRC Press Inc.

Geraci, J.R., Lounsbury, V.J., 1993. Marine Mammals Ashore: A field guide for strandings. Texas: A&M Sea Grant Publications.

Iwata, H., Saito, H., Moriyama, M., Nakano, A., 1992. Follow up study of renal tubular dysfunction and mortality among residents of an area polluted with cadmium. British Journal of Industrial Medicine, 49, 736 – 737.

Jarup, L., 2002. Cadmium overload and toxicity. Nephrology Dialysis Transplantation, 17, 35 – 39.

Jarup, L., Alfven, T., 2004. Low level cadmium exposure, renal and bone effects – the OSCAR study. Biometals, 17, 505 – 509.

Kemper C.M., and Gibbs, S.E, 1997. A study of the life history parameters of dolphins and seals entangled in tuna farms near Port Lincoln, and comparisons with information from other South Australian dolphin carcasses. *Report to Environment Australia,* pp. 98.

Kemper, C., Gibbs, S., 2001. Dolphin interactions with tuna feedlots at Port Lincoln, South Australia and recommendations for minimising entanglements. Journal of Cetacean Research and Mangement, 3, 283 – 292.

Kleerekoper, M., Villanueva, A.R., Stanciu, J., Sudhaker Rao, D., Parfitt, A.M., 1985. The role of three-dimensional trabecular microstructure in the pathogenesis of vertebral compression fractures. Calcified Tissue International, 37, 594 – 597.

Laan, R.F.J.M., Buijs, W.C.A.M., van Erning, L.J.T.O., Lemmens, J.A.M., Corstens, F.H.M., Ruijs, S.H.J., van de Putte, L.B.A., van Riel, P.L.C.M., 1993. Differential effects of glucocorticoids on cortical appendicular and cortical vertebral bone mineral content. Calcified Tissue International, 52, 5 – 9.

Lavery, T.J., Butterfield, N., Kemper, C.M., Reid, R.J., Sanderson, K., 2008. Metals and selenium in the liver and bone of three dolphin species from South Australia, 1988 – 2004. Science of the Total Environment, 390, 77 – 85.

Lind, P.M., Bergman, A., Olsson, M., Orberg, J., 2003. Bone mineral density in male Baltic grey seal. Ambio, 32, 385 – 388.

Loghman-Adham, M., 1997. Renal effects of environmental and occupational lead exposure. Environmental Health Perspectives, 105, 928 – 938.

Long, M., Reid, R.J., Kemper, C.M., 1997. Cadmium accumulation and toxicity in the bottlenose dolphin, the common dolphin, and some dolphin prey species in South Australia. Australian Mammalogy, 20, 25 – 33.

Nakagawa, H., Nishijo, M., Morikawa, Y., Tabata, M., Senma, M., Kitagawa, Y., Kawano, S., Ishizaki, M., Sugita, N., Nishi, M., Kido, T., Nogawa, K., 1993. Urinary B_2-microglobulin and mortality in a cadmium polluted area. Archives of Environmental Health, 48, 428 – 435.

NEPC, National Environment Protection Council., 2003. The national pollutant inventory. A public database on pollutant emissions. Environment Australia.

Nishijo, M., Nakagawa, H., Morikawa, M., Tabata, M., Miura, T., Yoshita, K., Higasgiguchi, K., Seto, T., Kido, T., Nogawa, K., Mizukoshi, K., Nishi, M., 1999.

Relationship between urinary cadmium and mortality among inhabitants living in a cadmium polluted area in Japan. Toxciology Letters, 108, 321 – 327.

O'Shea, T.J., 1999. Environmental contaminants and marine mammals. (pp. 485 – 564) In: Reynolds, III J.E., Rommel, S.A., editors. Biology of Marine Mammals. Melbourne: Melbourne University Press.

Peraza, M.A., Ayala-Fierro, F., Barber, D.S., Casarez, E., Rael, L.T., 1998. Effects of micronutrients on metal toxicity. Environmental Health Perspectives, 106, 203 – 216.

Petering, D.H., Goodrich, M., Hodgman, W., Krezoski, S., Weber, D., Shaw III, C.F., Spieler, R., Zettergren, L., 1990. Metal-binding proteins and peptides for the detection of heavy metals in aquatic organisms, In: McCarthy, J.F., Shugart, L.R., Biomarkers of Environmental Contamination, Florida: Lewis Publishers.

Puzas, E., Campbell, J., O'Keefe, R.J., Schwarz, E.M., Rosier, R.N., 2002. Lead in the skeleton interferes with bone mineral density measurements. Journal of Bone and Mineral Research, 17, S314.

Sato, M., Kondoh, M., 2002. Recent studies on metallothionein: Protection against toxicity of heavy metals and oxygen free radicals. Tohoku Journal of Experimental Medicine, 196, 9 – 22.

Sonne, C., Dietz, R., Born, E.W., Riget, F.F., Kirkegaard, M., Hyldstrup, L., Letcher, R.J., Muir, D.C.G., 2004. Is bone mineral composition disrupted by organochlorines in East Greenland polar bears (*Ursus maritimus*)? Environmental Health Perspectives, 112, 1711 – 1716.

Sonne-Hansen, C., Dietz, R., Leifsson, P.S., Hyldstrup, L., Riget, F.F., 2002. Cadmium toxicity to ringed seals (*Phoca hispida*) – an epidemiological study of possible cadmium induced nephropathy and osteodystrophy in ringed seals (*Phoca hispida*) from Qaanaaq in Northwest Greenland. Science of the Total Environment, 295, 167 – 181.

Staessen, J.A., Roels, H.A., Emelianov, D., Kuznetsova, T., Thijs, L., Vangronsveld, J., Fagard, R., 1999. Environmental exposure to cadmium, forearm bone density and risk of fractures: prospective population study. Lancet, 353, 1140 – 1144.

Suzuki, N., Yamamoto, M., Watanabe, K., Kambegawa, A., Hattori, A., 2004. Both mercury and cadmium directly influence calcium homeostasis resulting from suppression of scale bone cells: The scale is a good model for the evaluation of heavy metals in bone metabolism. Journal of Bone and Mineral Research, 22, 439 – 446.

Turan, B., Bayari, S., Balcik, C., Severcan, F., Akkas, N., 2000. A biomechanical and spectroscopic study of bone from rats with selenium deficiency and toxicity. BioMetals, 13, 113 – 121.

Walsh, C.T., Sandstead, H.H., Prasad, A.S., Newberne, P.M., Fraker, P.J., 1994. Zinc: Health effects and research priorities for the 1990s. Environmental Health Perspectives, 102, Suppl 2, 5 – 46.

Wang, A., Pfeiffer, C.J., 2001. Cytopathology induced by mercuric chloride and methylmercury in cultured renal cells of Atlantic spotted dolphin (*Stenella plagiodon*). Journal of Submicroscopic Cytology & Pathology, 33, 7 – 16.

Yun, L., Zeng-Li, Y., 2002. Effect of zinc on bone metabolism in fetal mouse limb culture. Biomedical and Environmental Science, 15, 323 – 329.

Figure Captions.

Table 1 Description of trabecular microarchitectural parameters acquired by histomorphometric analyses.

Table 2 Copper, cadmium, lead, mercury, selenium and zinc concentrations in the liver, cadmium and lead concentrations in the bone, MT (metallothioneins) concentrations in liver and kidney, and markers of health (renal damage and bone structure) measured in adult Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) carcasses collected from South Australia, 1989 – 2004.

Table 3 Copper, cadmium, lead, mercury, selenium and zinc concentrations in the liver, cadmium and lead concentrations in the bone, and renal parameters measured in two groups of adult bottlenose dolphins (*Tursiops aduncus*) differentiated by cluster analysis on the basis of renal damage.

Table 4 Copper, cadmium, lead, mercury, selenium and zinc concentrations in the liver, cadmium and lead concentrations in the bone, and bone parameters measured in two groups of adult bottlenose dolphins (*Tursiops aduncus*) in two groups differentiated on the basis of bone structure.

Figure 1 Examples of renal parameters measured in adult bottlenose dolphin (*Tursiops aduncus*) carcasses collected from South Australia. A: no space between glomeruli and surrounding Bowman's capsule. B: enlarged space between the glomeruli and surrounding Bowman's capsule. C: enlarged space between glomeruli and Bowman's capsule with no proteins enclosed. D: glomeruli with protein leakage inside the Bowman's capsule.

Figure 2 Cross sectional image of two costal ribs of adult bottlenose dolphins (*Tursiops aduncus*) generated by μCT (×16) showing trabecular microarchitecture. A: SAMA M21282 had low liver cadmium (0.49 mg kg^{-1}) and plate shaped trabeculae. B: SAMA M22410 had high liver cadmium (98.58 mg kg^{-1}) and rod shaped trabeculae characteristic of osteoporosis.

Table 1

Name	Abbreviation	Description	References.
Bone volume / tissue volume	BV/TV	Trabecular bone volume relative to marrow volume (%).	(Parfitt et al., 1983)
Bone surface / bone volume	BS/BV	Bone surface to bone volume ratio. Describes complexity (mm^{-1})	(Parfitt et al., 1983)
Bone surface / total bone	BS/TB	Total surface of the bone over total amount of bone (mm^{-1}).	(Parfitt et al., 1983)
Trabecular thickness	Tb.Th	Mean dimensions of individual trabeculae (mm).	(Parfitt et al., 1983)
Trabecular separation	Tb.Sp	Thickness of the marrow cavities between trabeculae (mm).	(Parfitt et al., 1983)
Trabecular pattern factor	Tb.Pf	Measures trabecular bone connectivity (mm^{-1}).	(Hahn et al., 1992)
Trabecular number	Tb.N	The number of traversals across trabeculae (mm^{-1}).	(Parfitt et al., 1983)
Structural model index	SMI	Relative frequency of rods and plates (no units).	(Hildebrand and Ruegsegger, 1997)

Table 2

Measurement	N	Minimum	Maximum	Mean	SD
Liver Cu (mg kg^{-1} wet weight)	21	8.35	36.21*	19.56	7.76
Liver Cd (mg kg^{-1} wet weight)	30	0.26	98.58*	8.64	18.17
Liver Pb (mg kg^{-1} wet weight)	30	0.04	14.15	0.68	2.55
Liver Hg (mg kg^{-1} wet weight)	30	66	2651	932	677
Liver Se (mg kg^{-1} wet weight)	30	33	1188	355	263
Liver Zn (mg kg^{-1} wet weight)	30	34	347	97	73
Bone Pb (mg kg^{-1} wet weight)	35	0.29	16.00	2.84	3.10
Bone Cd (mg kg^{-1} wet weight)	15	0.005	0.33	0.05	0.08
Glomerulus (μm^2)	16	3067	14880 §	6856	3349
Bowman's capsule (μm^2)	16	4756	20873 §	10862	5020
Space between glomerulus and Bowman's capsule (μm^2)	16	1690	7774*	4006	1829
Bowman's capsules with proteins (%)	16	10	95*	43	28
Liver MT (nmol of Cd bound g^{-1} wet weight)	14	2	1347*	219	394
Kidney MT (nmol of Cd bound g^{-1} wet weight)	15	7	193	67	61
Bone Mineral Density (g cm^{-2})	30	1.21*	1.80	1.53	0.16
Bone Mineral Content (g)	30	15.58	42.17	31.45	5.76
Bone volume / tissue volume (%)	15	23.29 ‡	38.85	30.97	4.00
Bone surface / bone volume (mm^{-1})	15	0.33	0.44	0.37	0.04
Bone surface / tissue volume (mm^{-1})	15	0.09	0.13	0.11	0.02
Trabecular pattern factor (mm^{-1})	15	-0.09	0.02 ‡	-0.05	0.03
Structural model index	15	-0.38	0.96 ‡	0.17	0.37
Trabecular thickness (mm)	15	7.81	9.57	8.95	0.52
Trabecular number (mm^{-1})	15	0.03	0.04	0.03	0.01
Trabecular separation (mm)	15	21.59	30.74	25.97	3.30

Metal and Se concentrations in liver and bone are a subsample of those measured during a previous study (Lavery et al., 2008). * = minimum or maximum values recorded from M22410; ‡ = minimum or maximum values recorded from M20877; § = maximum values recorded from M21243.

Table 3

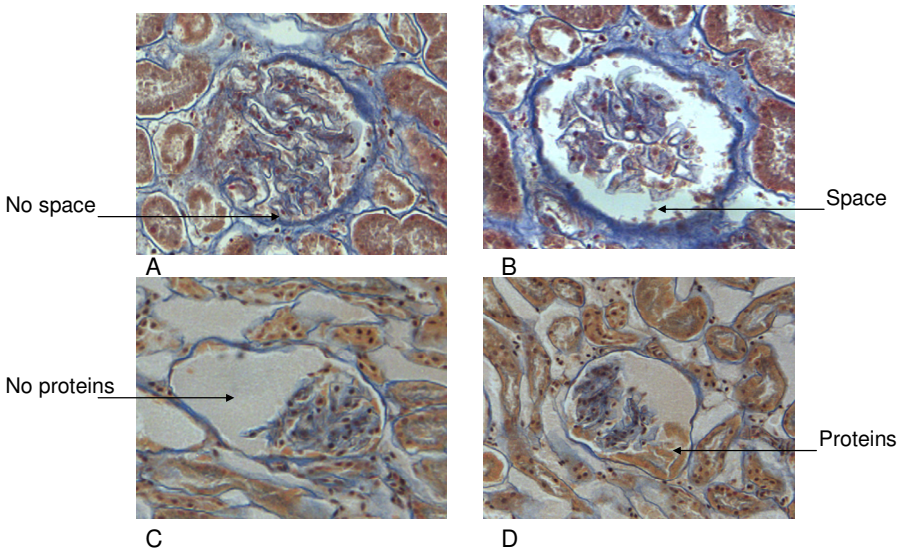
Measurement	Renal Group 1 Low renal damage			Renal Group 2 High renal damage		
	N	Mean	SD	N	Mean	SD
Liver Cu *	9	16.02	5.55	2	29.72	9.18
Liver Cd *	11	4.55	6.27	3	37.00	53.33
Liver Pb	11	0.17	0.13	3	0.12	0.09
Liver Hg	11	800	637	3	1359	687
Liver Se	11	291	255	3	446	175
Liver Zn *	11	73	37	3	178	111
Bone Pb	11	2.85	2.83	3	1.47	0.46
Bone Cd	4	0.10	0.16	0		
Glomerulus	11	5546	1461	3	12835	1774
Bowman's capsule	11	9065	2657	3	19649	1148
Space between glomerulus and Bowman's capsule	11	3519	1279	3	6814	898
Protein leakage	11	43	28	3	52	38
Liver MT	11	76	66	3	745	678
Kidney MT	11	60	58	3	101	81

* = metal concentrations are significantly different between groups ($p < 0.05$)

Table 4

Measurement	**Group 1**			**Group 2**		
	Low bone BMD, BMC			**Low bone organisation**		
	N	**Mean**	**SD**	**N**	**Mean**	**SD**
Liver Cu	9	19.41	8.39	4	16.31	5.33
Liver Cd	9	17.87	31.39	5	3.35	2.90
Liver Pb	9	0.23	0.25	5	0.29	0.13
Liver Hg	9	731.8	531.6	5	1082	706
Liver Se	9	258	175	5	481	263
Liver Zn	9	79	58	5	97	97
Bone Pb	10	2.76	2.91	5	2.91	2.05
Bone Cd	0			0		
BMD	10	1.45	0.14	5	1.59	0.10
BMC	10	28.70	4.02	5	34.54	2.37
BV/TV	10	32.59	3.45	5	27.72	3.07
BS/BV	10	0.37	0.03	5	0.36	0.04
BS/TV	10	0.12	0.01	5	0.10	0.004
Tb.Pf	10	-0.07	0.01	5	-0.01	0.02
SMI	10	0.09	0.34	5	0.33	0.41
Tb.Th	10	8.85	0.56	5	9.16	0.38
Tb.N	10	0.04	0.003	5	0.03	0.002
Tb.Sp	10	24.29	2.61	5	29.33	1.27

Figure 1



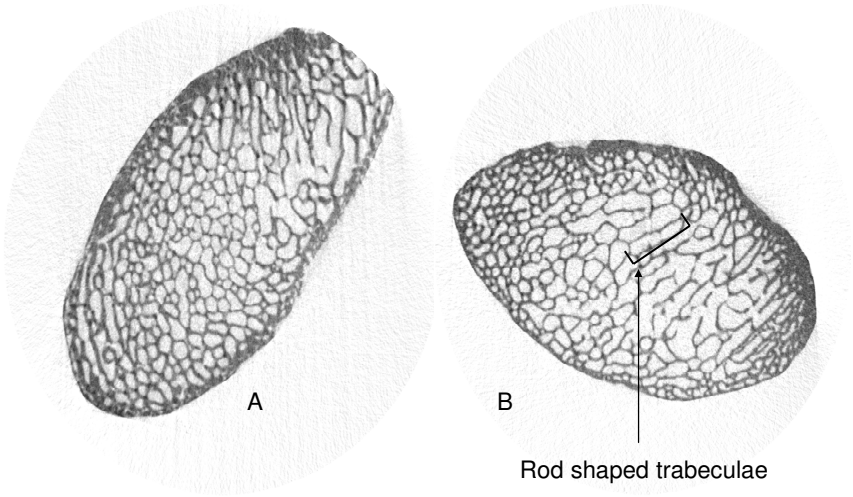


Figure 2