A RETROSPECTIVE INVESTIGATION OF THE PREVALENCE AND SIGNIFICANCE OF HEMOSIDEROSIS IN CAPTIVE PINNIPEDS

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Introduction

Iron is a trace element required for the synthesis of haemoglobin and a number of energetic reactions.[1] In most species, iron uptake is primarily regulated by the absorption of iron and protein-addition conversion to ferritin in the mucosal layers of the intestines and the sloughing of mucosal layers when iron levels are replete. However, if this system becomes imbalanced as a result of genetic or environmental factors then iron absorption and accumulation may occur. This excessive iron accumulation results in the development of hemosiderosis, where the excess iron accrues in specific tissues. This may further develop into hemochromatosis, or iron storage disease (ISD), when clinical signs of toxicity accompany the excessive iron.[4,13] Iron accumulation occurs primarily in the liver and spleen, where it is stored as a component of the proteins, hemosiderin and ferritin.[3] Iron accumulation in hepatocytes is normally indicative of excess dietary iron while accumulation in Kupffer cells is more often associated with concurrent infectious or inflammatory processes.[8] Although best documented in human literature, ISD is reported in numerous captive avian and mammalian species including birds of paradise, toucans, and mynahs,[12] and lemurs,[7] bongos, pikas,[9] and fur seals,[6,10,11] respectively. However, its relative absence, whether assumed or suspected, from free-ranging co-specifics suggests that ISD in these species results primarily from environmental factors associated with captivity. The pathological diagnosis of secondary hemosiderosis in tissues obtained during necropsy of eight captive-housed Australian fur seals (*Arctocephalus pusillus doriferus*) provided circumstantial evidence of iron accumulation as a possible cause of mortality in these animals. These results led institutional veterinarians to initiate a retrospective study into the potential incidence of ISD in their populations and determine whether there were sufficient historical data to observe changes in serum associated with the excess accumulation in these eight animals. Therefore it was the aim of this study was to determine the prevalence of ISD in this captive population using archived tissue and blood samples and compare these results to captive management and husbandry during this time. Samples collected from free-ranging pinnipeds were used for comparison.

Materials and Methods

Archived samples were obtained from 16 pinnipeds housed at the Royal Melbourne Zoo, Parkville, Victoria between 1980 and 2007 and from 30 free-ranging pinnipeds captured from the south-eastern coastline of Australia from 1995 to 2007. Epidemiological data on the management of captive pinnipeds were collected via husbandry protocols, animal records, and necropsy reports. Sera were analysed for iron, total iron-binding capacity (TIBC), ferritin, ceruloplasmin, haptoglobin, and transferrin saturation percentage. Serum iron and TIBC were measured

spectrophotometrically using the liquid FerroZine® assay method, which was then used to calculate the transferrin saturation percentage (serum iron/TIBC x 100).[11] Serum ferritin was analysed using an enzyme-linked immunosorbent assay (ELISA).[10] Ceruloplasmin was measured colorimetrically using p-phenylenediamine dihydrochloride (PPD) substrate, and haptoglobin was measured spectrophotometrically using a semiautomatic adapted assay. Statistical analyses of the serological data were performed using simple linear regression models. Iron concentration and hemosiderin accumulation in liver sections were qualitatively assessed using haematoxylin and eosin and prussian blue stain. Liver iron was graded using an adapted quantifiable system based on the estimated quantity of liver (Table 1). Frozen liver samples from three wild pinnipeds found deceased and one captive-reared female subantarctic fur seal were used to quantify total liver iron concentrations using acid digestion and atomic absorption spectroscopy (AAS) analysis. Samples of dietary items were collected and analysed for total iron using the same AAS technique used to quantify liver iron.

Results and Discussion

Viable serum samples were available for sixteen individual seals, nine of which were kept as part of the permanent collection, and seven that were wild-caught rehabilitation seals, for a total of 36 samples. The greatest majority of these animals supplied only one or two serum samples, but of the nine permanent collection animals, seven provided periodic samples. Samples were catalogued based on collection type; non-medical (16) and medical (20). Non medical included any samples taken during training or prophylactic treatment, whereas medical included any additional treatments, normally associated with some use of anaesthesia. Due to these small sample sizes, comparisons were made based on individual animals and among age categories.

Although serum ferritin has previously been reported to be the most reliable indicator of iron stores in humans and other species,[1,12] no significant elevations in serum ferritin levels were apparent in individual animals diagnosed with pathological hemosiderosis, compared with animals that did not exhibit any clinical signs (Table 2). Furthermore, high concentrations of ferritin were observed in animals with no clinical signs of hemosiderosis, nor were they associated with elevations in other markers of acute-phase reaction, such as ceruloplasmin and/or haptoglobin. Unlike previous research stating that ferritin is a useful measure of total body iron,[6,8,11] these data suggest that ferritin may not be the most effective measure of estimating ISD in fur seals. Insufficient sample size would limit this supposition, but given that this research is the first to provide serum in association with pathology, these preliminary results justify additional research and consideration.

Hemosiderosis was mentioned in the histopathology reports of eight seals within the institution, but hemochromatosis was not diagnosed in any cases. Histopathological slides for these and several other animals were examined as a qualitative means of analysing the deposition of hemosiderin in the liver and other tissues. The qualitative assessment of one of the high grade seals was supported by the extremely high liver iron concentration measured quantitatively (Fe=12,770.09 ng/ml) from the same liver. Although there were insufficient cases to attempt any statistical analysis of these observations, the correlation between the staining and the AAS method supports the additional gathering of data. Whereas all six wild-caught rehabilitation seal livers revealed low iron grades, all of the captive-housed seals had moderate to high liver iron

contents. The three captive seals with the highest degree of iron accumulation had all been held at the institution in excess of ten years and were in excess of 20 years of age. The major issue with this comparison was all of the wild-rehabilitation seals were young (<10 years) whereas all of the captive animals were greater than 15 years of age, thus the risk factors associated with age could not be discounted.[1]

Individual dietary items were analysed for total iron content and are presented on a dry matter basis. These results reveal Californian squid contained between half and one-third the total iron composition of the other main dietary ingredients (Table 3), consistent with the findings of other researchers.[2] Since free-ranging co-specifics consume much larger quantities of cephalopod taxa than what is offered in captivity[5] these much higher quantities of fish, could be a factor in the development of ISD in this captive population. Although there are no comparative studies showing differences in the incidence of ISD between seals consuming diets containing high and/or low cephalopod intake, these data would appear to suggest the increasing of the ratio of squid to fish in the diet may provide a method of reducing the consumption of excess iron.

The major constraint to this research was the general lack of background information and insufficient biological data, which limited the ability to make significant conclusions. Improved record-keeping practices, the additional collection of routine samples, and additional samples from free-ranging animals within this institution would greatly benefit the strength of this research. Furthermore, this type of study should be expanded to multiple institutions to more accurately represent the historical incidence of ISD within captive pinniped population. The aim of this study was to determine whether historical data could be used to identify the presence of ISD in a captive population, however although this study demonstrated hemosiderosis in several animals, the association of serum analytes with the incidence of hemosiderosis and the total iron stores was not confirmed statistically.

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Table 1. Grading system developed to assess visually the iron deposition in specific tissues using haematoxylin and eosin (H&E) and Prussian blue (PB) staining, under a microscope at 10x, 40x and 100x magnification.

Grade	Description
1	No iron noticeable on H&E staining. No or very little iron seen on PB staining. Normal tissue structure maintained.
2	Small amount of iron seen on H&E and PB staining. No associated tissue damage.
3	Moderate (obvious) amount of iron seen on H&E and PB staining, with little or no associated tissue damage.
4	Large amount of iron seen, with little or no associated tissue damage.
5	Large amount of iron seen (>50%) on both H&E and PB staining, with associated* tissue damage.

Associated tissue damage includes signs of fibrosis or cirrhosis, necrosis or nodular degeneration, which may either precede the iron accumulation, or result from iron damage to the liver. The order of these events cannot be determined on histological analysis

Table 2a. Serum ferritin concentrations for nine male and female captive pinnapeds collected during either training or medical procedures throughout the lifespan of the individual. Where appropriate, cause of death and liver iron concentrations are also provided. Average ferritin concentrations collected from thirty healthy juvenile (<5 years old) free-ranging Australian fur seals from the southern coastline of Victoria, Australia.

Animal	Sex	Location	Sampling			Assessment	
			Age (yrs)	Reason	Assessment	Ferritin (ng/ml)	Liver (grade)
1	F	Captive	2.0	Training	Healthy	28	
			5.3	Medical	Wound	23	
			6.5	Medical	Euthanized – (Bacterial, 2nd ISD)	1750	3
2	F	Captive	4.0	Training	Healthy	25	
			6.0	Medical	Heartworm test	36	
			8.5	Medical	Died - (Tracheitis, 2nd ISD)		3
3	F	Captive	14.1	Training	Healthy	111	
			17.0	Training	Healthy	28	
			18.2	Medical	Mouth exam	48	
			19.9	Medical	Eye exam	48	
			27.5	Medical	Euthanized - (Gradual decline, some ISD)		4
4	M	Captive	18.6	Medical	Died – (Not ISD, multifocal enteritis)	101	5
5	F	Captive	7.0	Medical	Testing	229	
			12.2	Medical	Dermatitis	N/A	
			21.5	Medical	Died – (Not ISD, Adenocarcinoma)		4/5
6	F	Captive	1.5	Training	Healthy	304	
			6.5	Training	Healthy	45	
			17.3	Medical	Wound	114	
7	F	Captive	8.0	Medical	Wound	37	
			10.0	Medical	Died – (Not ISD, sepsis)		5
8	F	Captive	2.4	Training	Healthy	18	
			4.7	Training	Healthy	26	
			5.5	Training	Healthy	24	
			6.5	Medical	Testing	50	
			7.3	Training	Healthy	34	
			10.8	Medical	Wound	48	
9	M	Captive	1.1	Medical	Testing	40	
			5.3	Medical	Wound	47	
			8.9	Medical	Died – (Not ISD, peritonitis)		N/A

Table 2b. Serum ferritin concentrations for seven male and female rehab-release pinnapeds collected during either medical procedures. Where appropriate, cause of death and liver iron concentrations are also provided. Average ferritin concentrations collected from thirty healthy juvenile (<5 years old) free-ranging Australian fur seals from the southern coastline of Victoria, Australia.

Animal	Sex	Location	Sampling			Assessment	
			Age (yrs)	Reason	Assessment	Ferritin (ng/ml)	Liver (grade)
10	M	Release	0.66	Medical	Mouth infection	13	
11	M	Release	1.59	Medical	Parasites	11	
12	F	Release	1.82	Medical	Wound	11	
13	M	Release	4.29	Medical	Wound	27	
14	F	Release	0.49	Medical	Euthanized - (Not ISD, pneumonia)	36	N/A
15	F	Release	7.22	Medical	Testing	24	
16	M	Release		Medical	Wound	48	
All		Wild	<5.0	Research	Body Condition	33.35 ± 26.4	

Table 3. Average total iron (Fe) concentration of dietary ingredients offered to captive-housed pinnapeds at the Royal Melbourne Zoo in 2007 (dry matter basis).

Sample	Average Fe (mg/kg)
Californian Squid	34.92
Goatfish	123.16
Silago Whiting	122.45
Tommy Ruff	72.53
WA Pilchard	194.72
Yellow Tail	110.13