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Evidence of chronic cadmium exposure identified in the critically endangered Christmas Island flying-fox (*Pteropus natalis*)

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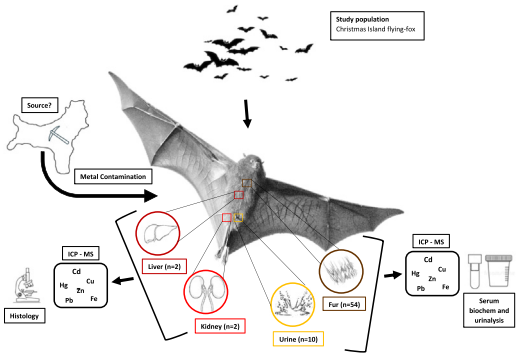
HIGHLIGHTS

- Evidence of chronic cadmium (Cd) exposure was found in Christmas Island flying-foxes.
- Bone lesions and renal dysfunction were identified and are suggestive of Cd toxicity.
- Renal iron excretion appears to be how flying-foxes adapt to an iron-rich diet.

GRAPHICAL ABSTRACT



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ABSTRACT

The Christmas Island flying-fox (*Pteropus natalis*) is the last native mammal on Christmas Island and its population is in decline. Phosphate mining occurs across much of the eastern side of Christmas Island. The phosphate deposits are naturally rich in cadmium, and potentially other metals, which may be threatening the Christmas Island flying-fox population. To test this, concentrations of metals (cadmium, copper, iron, mercury, lead, and zinc) were measured in fur and urine collected from Christmas Island flying-foxes and interpreted concurrently with urinalysis and serum biochemistry data. In addition, metal concentrations in liver and kidney samples from two Christmas Island flying-foxes and associated histological findings from one of these individuals are reported. Fur cadmium concentrations were significantly higher in the Christmas Island flying-fox compared to concentrations found in flying-foxes in mainland Australia. Additionally, 30% of Christmas Island flying-foxes had urine cadmium concentrations exceeding maximum concentrations previously reported in flying-foxes in mainland Australia. Glucosuria and proteinuria were identified in two Christmas Island flying-foxes, suggestive of renal dysfunction. In one aged flying-fox, kidney cadmium concentrations were four-fold higher than toxic thresholds reported for domestic mammals. Microscopic evaluation of this individual identified bone lesions consistent with those described in laboratory animals with chronic cadmium poisoning. These results suggest that Christmas Island flying-foxes are being exposed to cadmium and identification of these sources is recommended as a focus of future research. Unexpectedly, urine iron concentrations in Christmas Island flying-foxes were higher compared to previous studies of Australian mainland flying-foxes, which suggests that urinary excretion of iron may be an important aspect of iron homeostasis in this species whose diet is iron rich.

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1. Introduction

The critically endangered Christmas Island flying-fox (*Pteropus natalis*) is the only extant native mammal on Christmas Island. The current population is estimated to be less than 3800 individuals (Todd, 2020), a decline from an estimated population of 6000 individuals in the 1980s (Tidemann, 1985b). While factors contributing to the decline of the Christmas Island flying-fox are unknown, intoxication with the heavy metal cadmium (Cd) has been identified as a potential threatening process (Walshe et al., 2012). Phosphate deposits on Christmas Island are naturally rich in Cd (see David et al., 1978; Loganathan et al., 2003; Ruthrof et al., 2018), and potentially other metals. Therefore, ongoing phosphate mining operations on the eastern part of Christmas Island are a potential source of Cd exposure for Christmas Island flying-foxes through ingestion of cadmium containing dust via contaminated food (fruit, leaves, and flowers) or during grooming.

Chronic and acute exposure to Cd can have serious consequences in humans and other mammals. In humans, chronic Cd exposure results in renal tubular dysfunction (Järup et al., 1998; Johri et al., 2010; Satarug, 2018). Additionally, Cd can disrupt calcium metabolism resulting in varying degrees of osteoporosis and osteomalacia in humans and laboratory animals, which in advanced stages, can result in pathological bone fractures (Chakraborty et al., 2013; Johri et al., 2010; Trzcinka-Ochocka et al., 2010). Acute and chronic Cd exposure can also result in significant neurological impairment, interference with immune and reproductive function and carcinogenesis (see Järup and Akesson, 2009; Peralta-Videa et al., 2009). For example, in captive rat-tailed bats (*Rhinopoma kinneari*), exposure to Cd resulted in testicular necrosis, atrophy of the seminiferous tubules, and decreased spermatogenesis (Dixit and Lohiya, 1974).

With the exception of phosphate mining and human settlement, Christmas Island remains a pristine environment with 70% of its area designated as national park. Given the relative absence of industrialisation and urbanisation on the island, it is possible that concentrations of other heavy metals including copper (Cu), iron (Fe), mercury (Hg), lead (Pb), and zinc (Zn) would be expected in lower concentrations in the Christmas Island flying-fox than would be seen in flying-foxes feeding in more urbanized areas. This study provides a unique opportunity to establish baseline reference values in a flying-fox species considered relatively unaffected by anthropogenic impacts with application of these values to flying-fox species worldwide.

The aim of this study was to determine if the Christmas Island flying-fox is exposed to Cd, Cu, Fe, Hg, Pb or Zn using a recently validated minimally invasive screening method in fur and urine (Pulscher et al., 2020) and develop reference values in this species. In addition, hepatic and renal metal concentrations were determined in a subadult Christmas Island flying-fox that died spontaneously and an older (estimated age 11–13 years-old) female Christmas Island flying-fox that was euthanized as the result of a femoral fracture. To determine potential health impacts on the population, bone and kidney were examined histologically for evidence of lesions caused by Cd poisoning in the older female Christmas Island flying-fox. Further, urinalysis and serum biochemistry analysis were undertaken in a subset of Christmas Island flying-foxes to determine if Cd exposure was interfering with renal function.

2. Material and methods

2.1.1. Study location

Christmas Island is an Australian external territory located in the Indian Ocean, at 10°25′S and 105°43′E, approximately 380 km south of Java, Indonesia, and 1500 km off the coast of western Australia. The island is small with a land area of 135 km^2 and is composed of tertiary limestone overlaying basalt volcanic rock that rises 361 m above sea level. Approximately 25% of the island has been cleared, primarily for phosphate mining. Of this cleared area, approximately 13% is currently under lease for phosphate mining, 2% is reclaimed mining land currently undergoing rehabilitation by national parks, and the remaining 10% is regrown to varying degrees (Fig. 1). Christmas Island flying-foxes utilize the entire island (Todd, 2020), including vegetation surrounding phosphate mines and rehabilitated reclaimed mining land.

2.2. Sample collection

Christmas Island flying-foxes were captured at foraging and roost sites on Christmas Island (Fig. 1) between July 2016–March 2019 and anaesthetised with 2% isoflurane in 1 L/min oxygen (Isoflurane 100%, Zoetis, Australia) via mask as previously described (Hall et al., 2014; Todd et al., 2018). Sex, body mass (g), and forearm length (mm) were determined. Age was determined based on morphometric measurements, extent of tooth wear and reproductive status/sexual characteristics as previously described (Todd et al., 2018). Approximately 50–100 mg of fur was collected with scissors from the nucha (nape) of the neck (n=54), placed in individual labelled envelopes and stored at room temperature until analysis. Scissors were cleaned between individuals with 70% ethanol. Whole blood was collected from the uropatagial vein and placed into plain tubes and stored at −4 °C overnight. Whole blood samples were centrifuged (Qik Spin, Edwards Group Pty Ltd., Narellan, NSW, Australia) for 10 min at 6000 rpm and serum was frozen at −20 °C. Urine was collected opportunistically from flying-foxes (n=10) who urinated during capture, or by bladder expression during anaesthesia. Urine was frozen at −20 °C. Of the 10 urine samples collected, two individuals had both urine and fur collected for analysis, all other samples were independent.

Liver and kidney samples (approximately 50 g each) were collected from one Christmas Island flying-fox that was found dead in July 2016 and from a second Christmas Island flying-fox who was found to have a broken femur upon capture and was subsequently euthanized in September 2017. All frozen samples were transported to the Taronga Conservation Society where they were stored at −80 °C until analysis. For both animals, sex, body mass (g), forearm length (mm), and age group were recorded, and a routine necropsy performed. For the Christmas Island flying-fox euthanized in 2017, representative tissues were formalin-fixed, paraffin-embedded, sectioned at 4 μm and the sections stained with haematoxylin and eosin and examined by light microscopy. The femur was demineralized in a calcium EDTA solution before sectioning. A set of canine teeth was also extracted from the fixed skull of this individual for cementum aging (Matson's Laboratory, Manhattan, MT, USA).

Christmas Island flying-foxes were captured under permits issued by the Christmas Island National Park (Permit Nos. CINP_2015-16_1 & CINP_2018_2) and the Australian Government Environment Protection and Biodiversity Conservation Regulations 2000 license to access biological resources in a Commonwealth area for non-commercial purposes (Permit No AU-COM2018-414). Animal capture protocols and sample collection was approved by the Animal Care and Ethics Committee of Western Sydney University (Project Protocol Nos A11140 & A12791).

2.3. Sample processing and inductively coupled plasma mass spectroscopy (ICP-MS) analysis

In accordance with the Australian Department of Agriculture and Water Resources quarantine requirements, fur was fixed in 70% ethanol and urine and tissue samples were gamma irradiated at 50 KGY (Steritech Pty Ltd., Wetherill Park, NSW, Australia) prior to analysis. Two 10 mg fur samples for each individual (serving as technical replicates), urine, and tissue samples were sent to a hospital laboratory accredited to perform trace element and heavy metal analysis on blood and body fluids (National Association of Testing Authorities – no. 2146) and processed as described previously (Pulscher et al., 2020) and stored at −4 °C until analysed by ICP-MS. Urine and digested fur and tissue samples

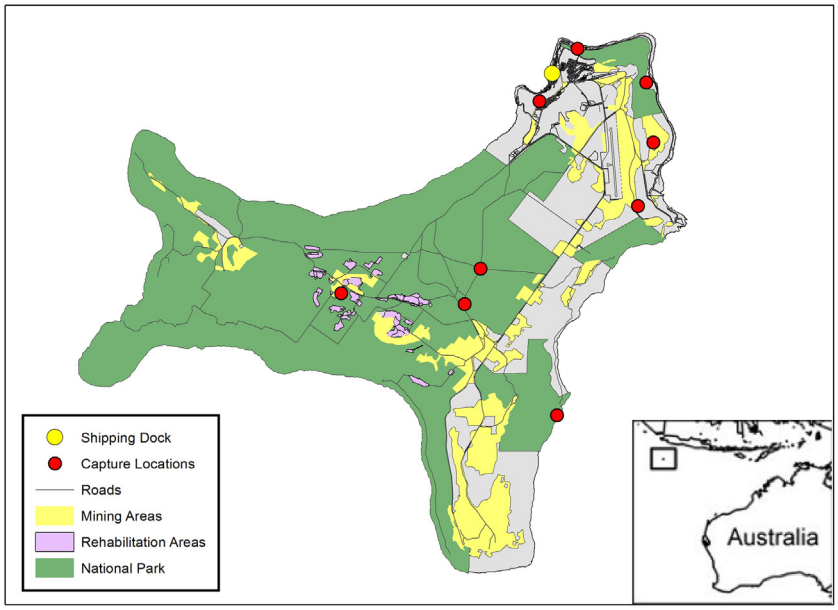

Fig. 1. Map of Christmas Island indicating mining areas and Christmas Island flying-fox (*Pteropus natalis*) capture locations.

were vortexed and 100 μL of each sample was diluted with 3900 μL internal diluent consisting of 2 g EDTA (Ethylenediaminetetraacetic acid, diammonium salt hydrate 97%, Aldrich Chemical Company Inc., Milwaukee, USA), 2 g tritonX-100 (t-Octylphenoxypolyethoxyethanol Sigma-Aldrich Co., St. Louis, MO, USA), 50 mL ammonia (di-Ammonium hydrogen orthophosphate AnalR® DBH Limited Poole, England), and 4 mL internal standard (Rhodium 1000 ppm, Atomic Spectroscopy Standard, PerkinElmer, USA) and analysed for Cd, Cu, Fe, Pb, Hg and Zn on an ICP-MS (Agilent 7500Ce ICP MS, Santa Clara, California, USA) with the limit of detection of 2 μg/L for all metals tested. To account for the impact of variations in urine concentrations between individuals, urine metal concentrations were normalized by converting results to urine μg/g creatinine (Keil et al., 2011; Pulscher et al., 2020). Due to quarantine restrictions limiting the movement of urine samples, creatinine concentrations were determined using Reflotron creatinine test strips (Roche, Mannheim, Germany) on a Reflotron IV Chemical Analyzer (Roche, Mannheim, Germany) at Taronga Zoo Wildlife Hospital.

2.4. Urinalysis and serum biochemistry analysis

For urine samples of sufficient volume, urine specific gravity (USG) (n=4) was measured using a handheld refractometer (Bellingham and Stanley, Kent, UK). Urine specific gravity was classified as hyposthenuric (USG < 1.008), isosthenuric (USG 1.008–1.012), weakly concentrated (USG 1.012–1.034), and hypersthenuric (USG > 1.035), as previously adopted for flying-fox species (Edson et al., 2018; Olsson and Woods, 2008). Urinary ketones (n=4), glucose (n=5), and protein (n= 5) concentrations were determined using Combur 9 Test Strips (Roche Diagnostics, Mannheim, Germany). In individuals where ketonuria, glucosuria and proteinuria were present and in one euthanized individual (n=4) serum creatinine (umol/L), blood urea nitrogen (BUN) (mmol/L) and glucose (mmol/L) concentrations were determined on an Abaxis VetScan VS2 (Abaxis Inc., Union City, California, USA) and compared against published reference values for the species (Hall et al., 2014).

2.5. Statistical analysis

The concentrations of metals in fur (n = 54), kidney (n = 2), and liver (n = 2) were expressed in μg/g dry weight and urine (n=10) in μg/g creatinine. For the purposes of statistical analysis, metals with results below the detection limit (n = 1 urine Pb) were replaced with a value equivalent to half of the detection limit. This method has been suggested previously to reduce bias compared to when results below the detection limit are reported as zero (Helsel et al., 2020). The means of duplicate fur samples for each individual were used for all statistical analyses. Normality of data was assessed by the Shapiro-Wilk test and visual examination of Q-Q plots and histograms. Apart from Zn concentrations in fur, all data were non-normally distributed. Natural log transformation was attempted but normality was still not achieved for all metals. For this reason, and due to sample sizes for fur and urine, median and 90% confidence intervals for fur were calculated using the robust method (Friedrichs et al., 2012), and median and observed range of values are provided for urine. To establish 90% confidence intervals for fur, data were tested for outliers by the assessment of histograms and Tukey's interquartile fences (Friedrichs et al., 2012 and references therein). Outliers for the concentrations of Cd (n = 1), Cu (n = 2), Fe (n = 6), Hg (n = 3), and Pb (n = 2) in fur were identified and removed prior to the calculation of 90% confidence intervals. Confidence intervals were calculated using Reference Value Advisor freeware (Geffré et al., 2011).

A Wilcoxon rank sum test was used for interspecies comparisons of metal concentrations in fur (n = 54) and urine (n = 10). Christmas Island flying-fox metal concentrations in fur and urine were compared to data on free-living black flying-fox (*P. alecto*) fur (n = 9) and grey-headed flying-fox (*P. poliocephalus*) fur (n=11) and urine (n=5) from a recent study (Pulscher et al., 2020). Due to the small sample size for kidney and liver concentrations, the observed range of values are reported. All statistical analyses were performed in R version 3.6.0 for Windows. Statistical significance was considered at $\alpha = 0.05$.

3. Results

3.1. Metal concentrations in Christmas Island flying-fox fur and urine compared to grey-headed and black flying-foxes

Christmas Island flying-foxes had significantly higher fur Cd (W = 11, $p < 0.001$) and significantly lower fur Hg (W = 436, $p = 0.02$) and Pb (W = 530, $p < 0.01$) concentrations compared to urban mainland Australian grey-headed flying-foxes (Table 1). Similarly, Christmas

Table 1
Median (90% CI) and observed range of values for metal concentrations in Christmas Island flying fox (*Pteropus natalis*) fur ($n = 53$ for Cd; $n = 52$ for Cu; $n = 51$ for Hg; $n = 48$ for Fe; n = 52 for Pb; $n = 54$ for Zn; μg/g dry weight for 90% CI; n = 54 for observed range; μg/g dry weight) samples collected between 2016 and 2018 and other *Pteropus* spp. and frugivorous bats for comparison. A Wilcoxon rank sum test was used to evaluate statistical differences between metals in Christmas Island flying-fox, free-living grey-headed (*P. poliocephalus*; $n = 11$) and black (*P. alecto*; $n = 9$) flying-fox fur samples.

	Fur metal concentrations (μg/g dry weight)				
	Christmas Island flying-fox		Fur metal concentrations reported in other *Pteropus* and frugivorous bat *species*		
	Median (90% CI)	Observed range	Grey-headed flying-fox (observed range)[a]	Black flying-fox (observed range)[a]	Other *Pteropus* and frugivorous species
Cd	0.07 (0.00–0.19)	0.02–0.32	0.01 (<0.01–0.04)*	0.02 (0.01–0.03)*	
Cu	1.61 (0.79–2.43)	1.17–11.4	2.02 (0.89–9.30)	2.83 (1.30–8.63)*	
Fe	13.6 (0.00–46.5)	4.99–332	14.5 (2.67–51.1)	36.5 (16.5–67.5)*	
Hg	0.04 (0.00–0.14)	0.02–0.58	0.12 (0.03–0.44)*	0.06 (0.03–0.25)	0.04[b,c], 0.26[d], 0.01–0.02[c,e]
Pb	0.10 (0.01–0.19)	0.04–0.62	0.34 (0.09–1.35) *	1.61 (0.72–3.75)*	0.85[f], 5.82–20.8[g]
Zn	24.5 (16.7–32.2)	14.6–35.1	17.4 (9.11–68.0)	33.3 (17.3–47.9)*	

[a] Pulscher et al., 2020.
[b] Becker et al., 2018.
[c] Mean concentrations reported.
[d] Moreno-Brush et al., 2018.
[e] Mean range reported for three frugivorous species. Syaripuddin et al., 2014.
[f] Mean fur concentrations reported for *Pteropus* spp. in non-urban locations. Hariono et al., 1993.
[g] Mean range reported for *Pteropus* spp. in urban locations. Hariono et al., 1993.
* *P*-value ≤ 0.05.

Island flying-foxes had significantly higher fur Cd ($W = 4$, $p < 0.01$) but significantly lower fur Cu ($W = 366$, $p = 0.02$), Fe ($W = 371$, $p = 0.01$), Pb ($W = 486$, $p < 0.01$), and Zn ($W = 367$, $p = 0.02$) concentrations compared to black flying-foxes (Table 1). Christmas Island flying-foxes had significantly lower urine Hg ($W = 50$, $p < 0.01$) and Pb ($W = 42$, $p = 0.04$) concentrations, compared to grey-headed flying-foxes (Table 2).

3.2. Metal concentrations in tissues from two Christmas Island flying-foxes and histopathological findings

Tissue metal concentrations were determined for one adult and one subadult female Christmas Island flying-fox (Table 3). The subadult female Christmas Island flying-fox was estimated to be one year of age based on morphometric measurements and sexual characteristics (Todd et al., 2018). Cementum tooth aging of the adult female Christmas Island flying-fox estimated this individual's age to be between 11 and 13 years. Histopathological examination of the proximal femur revealed thin subchondral and cortical bone, with sparse and thin medullary trabeculae within the epiphyseal region (Fig. 2). Thin cortices were also evident throughout the diaphysis, which contained no visible medullary trabeculae; with the medullary cavity containing only adipose and small clusters of haematopoietic cells. The articular cartilage appeared to be mildly thin, but this was difficult to interpret due to artefactual fragmentation, and the lack of age matched controls. Examination of vertebral bodies in the lumbar spine identified similar markedly thin cortices and a reduced number and volume of trabeculae. Unmineralised osteoid was not evident within any of the bony sections examined. The periosteum was diffusely thin and mature. The uterus contained a small product of conception, with no visible skeletal elements. A mature corpora luteum was present in one ovary, supporting the finding of pregnancy. Multifocally random groups of hepatocytes had vacuolated cytoplasm. No further abnormalities were detected during microscopic examination of a full range of tissues.

Table 2
Median (observed range of values) metal concentrations in Christmas Island flying-fox (*Pteropus natalis*) urine ($n = 10$; μg/g creatinine) collected between 2016 and 2018. A Wilcoxon rank sum test was used to evaluate statistical differences between metals in Christmas Island flying-fox and free-living grey-headed flying-fox (*P. poliocephalus*) urine samples (n = 5; μg/g creatinine).

	Urine metal concentrations (μg/g creatinine)	
	Christmas Island flying-fox	Grey-headed flying-fox[a]
	Median (observed range)	Median (observed range)
Cd	14.2 (3.65–1500)	23.9 (8.93–169)
Cu	399 (54–3723)	109 (0–6418)
Fe	564 (39.0–4505)	256 (176–3235)
Hg	7.68 (1.07–189)	541 (437–2001)*
Pb	1.49 (0.03–341.2)	8.60 (3.50–418)*
Zn	1264 (249–16,566)	1040 (592–40,787)

[a] Pulscher et al., 2020.
* P-value ≤ 0.05.

3.3. Urinalysis and serum biochemical analysis

The urine samples were hyposthenuric ($n = 1$), isosthenuric ($n = 2$), and weakly concentrated (n = 1) (Table 4). One adult male Christmas Island flying-fox with weakly concentrated urine had ketonuria, glucosuria, and proteinuria and one juvenile female Christmas Island flying-fox had glucosuria and proteinuria. Serum creatinine ($n = 4$) concentrations were within the published reference ranges for Christmas Island flying-foxes (Hall et al., 2014). One Christmas Island flying-fox had slightly elevated BUN (3.9 mmol/L) and serum glucose was elevated above the reference interval for another (9.7 mmol/L); the remaining BUN and serum glucose concentrations were within normal limits for the species (Hall et al., 2014).

4. Discussion

This study sought to determine metal concentrations of Cd, Cu, Fe, Hg, Pb, and Zn in the Christmas Island flying-fox population and determine possible implications for the health of the population. A significant finding from this study was the elevated levels of Cd in Christmas Island flying-fox fur, urine, liver and kidney which are consistent with the

Table 3
Kidney and liver metal concentrations from an adult female (approximately 11–13 years of age) and subadult female (approximately 1 year of age) Christmas Island flying-fox (*Pteropus natalis*) collected in September 2017 and July 2016, respectively.

Age	Tissue	Tissue metal concentrations (μg/g dry weight)					
		Cd	Cu	Fe	Hg	Pb	Zn
Adult	Kidney	391	27.6	333	0.09	0.14	165
	Liver	24.8	27.6	447	0.13	0.11	125
Subadult	Kidney	3.80	10.1	324	0.13	0.10	67.0
	Liver	0.86	8.47	282	0.26	0.10	51.8

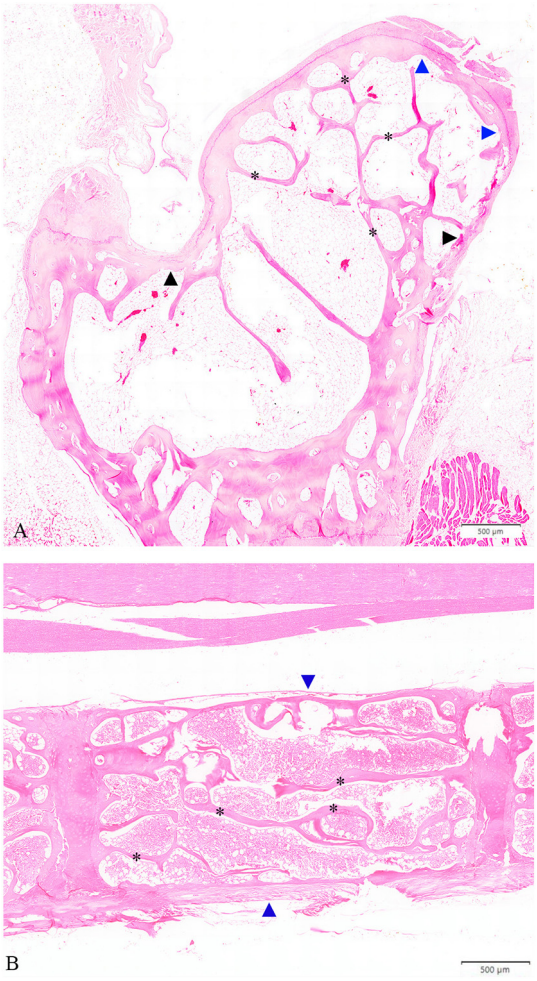

Fig. 2. Photomicrographs of the head of femur with greater trochanter (top) 1A, and vertebral body (bottom) 1B of an aged female Christmas Island flying-fox (*Pteropus natalis*) collected in 2017, illustrating thin cortices (blue arrowheads), thin subchondral bone (black arrowheads) and thin and sparse trabeculae (asterix).

hypothesis that the Christmas Island flying-fox population is exposed to environmental Cd. A recent study identified high Cd concentrations in grey-headed and black flying-foxes from mainland Australia (Pulscher et al., 2020). Compared to flying-foxes on mainland Australia, Cd concentrations in the fur (median 0.07 μg/g dry weight; observed range 0.02–0.32) of Christmas Island flying-foxes were nearly seven times higher (Pulscher et al., 2020). Although median Cd concentrations in urine were not significantly different between species, 30% of the Christmas Island flying-foxes sampled had urine Cd concentrations equivalent to or greater than the highest urine Cd concentration reported in grey-headed flying-foxes in Sydney (>169 μg/g creatinine), suggesting significant Cd exposure (Pulscher et al., 2020). Furthermore, 70% of the Christmas Island flying-foxes sampled had urine Cd concentrations considered to be diagnostic for toxic exposure in humans (≥5 μg/g creatinine; Keil et al., 2011) suggesting that flying-foxes in this study have high and likely chronic exposure to Cd. In addition, an aged female Christmas Island flying-fox had nearly fourfold higher kidney Cd concentrations than the toxic threshold (≥ 100 μg/g) reported for domestic animals (Nordberg et al., 2014; Degernes, 2008) and 13-fold higher than median concentrations reported for mainland flying-foxes (Pulscher et al., 2020). The fur, urine and tissue data suggest chronic Cd exposure at high concentrations.

In humans and other mammals, even low levels of chronic Cd exposure (as low as 1–2 μg/g creatinine Cd in urine) can lead to renal dysfunction, bone disease, and reduced reproductive success (Järup et al., 1998; Johri et al., 2010; Noda and Kitagawa, 1990; Satarug, 2018; Trzcinka-Ochocka et al., 2010; Umemura and Wako, 2006). When ingested, Cd is absorbed by the liver, where it is bound to metallothionein which detoxifies Cd. The metallothionein bound Cd is then released into the blood, filtered through the glomerulus, and reabsorbed by the proximal tubules (reviewed in Järup et al., 1998; Nordberg et al., 2014; Satarug, 2018). After the proximal tubules uptake Cd, lysosomes catabolize metallothionein, releasing the toxic un-bound form of Cd (reviewed in Nordberg et al., 2014; Satarug, 2018). One of the first indications of low-level environmental Cd exposure is urinary excretion of low molecular weight proteins and glucose (Chakraborty et al., 2013; Johri et al., 2010; Nogawa et al., 1975; Satarug, 2018). Chronically elevated Cd exposure causes nephrotoxicity resulting in increased concentrations of glucose, amino acids, phosphate, calcium and bicarbonate (Fanconi syndrome) in the urine even in absence of hyperglycaemia (Chakraborty et al., 2013; Johri et al., 2010; Satarug, 2018). Histopathological changes associated with Cd exposure in the kidneys and liver of experimental animals identified focal proximal tubule degeneration and glomerular swelling in addition to hepatocellular swelling, vacuolation and inflammation (Bonnell et al., 1960; Salinska et al., 2012). In this study, glucosuria and proteinuria, with normoglycaemia were detected in an adult male and juvenile female Christmas Island flying-fox. Although data are limited, these results suggest renal dysfunction. However, histopathological evidence supporting this was not observed in the aged female Christmas Island flying-fox with high kidney Cd concentrations (391 μg/g dry weight).

Table 4
Urine (n=10; μg/g creatinine) and kidney (*n*=2; μg/g dry weight) Cd concentrations and urinalysis and serum biochemistry results for Christmas Island flying-foxes (*Pteropus natalis*) collected between July 2016 and March 2019. Neg = negative.

Age	Sex	Urine Cd concentration	Kidney Cd concentration	Urinalysis				Serum biochemistry		
				USG	Ketones (mmol/L)	Glucose (mmol/L)	Protein (mg/dL)	Creatinine (umol/L)	Urea (mmol/L)	Glucose (mmol/L)
Juvenile	M	1500	–	–				–		
Juvenile	M	389	–	–				–		
Adult	F	164	–	–				–		
Juvenile	F	15.4	–	1.004	Neg	<1.4	Neg	31	<0.7	9.7
Juvenile	F	15.0	–	–				–		
Juvenile	M	13.4	–	1.010	Neg	<1.4	Neg	–		
Adult	M	8.85	–	1.021	15	17	30	<18	2.6	4.5
Juvenile	F	4.81	–	–	–	5.5	30	26	1.5	1.1
Juvenile	F	4.35	–	–				–		
Juvenile	F	3.65	–	1.009	Neg	<1.4	Neg	–		
Subadult	F	–	3.80	–				–		
Adult	F	–	391	–				<18	3.9	3.8

Metabolic bone disease has also been associated with chronic Cd exposure in humans and other animals (Itokawa et al., 1974; Noda and Kitagawa, 1990; Trzcinka-Ochocka et al., 2010; Umemura and Wako, 2006). It is not entirely clear whether metabolic bone disease is secondary to Cd-induced renal failure or whether excess Cd has a direct impact on bone (Trzcinka-Ochocka et al., 2010), although both processes could play a role. A classic example of metabolic bone disease associated with chronic Cd exposure is in Itai-Itai disease, which was first described in Japanese women exposed to toxic concentrations of Cd from a Zn mine (Nogawa et al., 1975). The disease is characterised by severe bone pain, multiple bone fractures and skeletal deformities, excessive bone mass reduction, osteomalacia and less commonly osteoporosis (Kazantis, 2004; Noda and Kitagawa, 1990; Nogawa et al., 1975; Uriu-Adams and Keen, 2005). Histological changes in bones of Itai-Itai patients include thinned trabeculae, reduced bone mass, and increased unmineralised osteoid (Noda and Kitagawa, 1990). Experimental studies in laboratory animals to mimic this disorder have not always been successful (see Umemura and Wako, 2006), and in some studies, but not all, the described changes included thinned cortical and epiphyseal bone, and thinned trabeculae. Studies with laboratory animals suggest that unmineralised osteoid occurs when Cd intoxication is accompanied by a calcium deficient diet, but is not seen in animals fed a diet with sufficient calcium (Itokawa et al., 1974). The microscopic bone lesions seen in the aged female Christmas Island flying-fox in this study were consistent with lesions seen in laboratory animals with chronic Cd intoxication fed a diet containing adequate calcium. Other possible causes for the observed bone lesions include dietary vitamin D deficiency, calcium deficiency or elevated dietary phosphorus. It is not entirely clear how bats meet their vitamin D requirements, however studies of frugivorous bats reported vitamin D can be synthesized from exposure to sunlight (Southworth et al., 2013) or through consumption of fruit skins (Cavaleros et al., 2003). It is therefore unlikely that the Christmas Island flying-fox would be deficient in vitamin D as it is constantly exposed to sunlight while roosting during the day, however further studies are necessary to confirm this. Furthermore, fibrous osteodystrophy, a characteristic lesion of calcium deficiency and dietary phosphorus excess, was not observed suggesting the bone pathology was not a result of these imbalances (Anderson et al., 1977; Shah et al., 1966). Combined, the high concentration of Cd found in this female flying-fox and the characteristic bone lesions observed are suggestive of Cd intoxication. However, our results are limited to one individual, therefore further investigations are necessary to confirm an association with Cd and metabolic bone disease in the Christmas Island flying-fox.

Reproductive effects, including testicular necrosis, decreased testosterone production and reproductive capacity, disrupted oocyte development and ovulation, reduced foetal weight, tissue and skeletal development, and in extreme cases foetal death have also been reported in humans and laboratory animals exposed to Cd (see Järup and Akesson, 2009; Thompson and Bannigan, 2008). The impacts of Cd on foetal and juvenile development needs to be further investigated in the Christmas Island flying-fox. Microscopic examination of the aged Christmas Island flying-fox in this study determined this female was pregnant; however, due to the early stage of pregnancy it was not possible to assess foetal bone and tissue development. Skeletal deformities that would be indicative of in utero Cd intoxication have not been observed in captured juvenile Christmas Island flying-foxes but further investigations are required to fully understand developmental and geriatric impacts of Cd in the population.

It is not clear how the Christmas Island flying-fox is exposed to Cd, in humans the most significant route of Cd exposure is the ingestion of Cd concentrating plants, notably tubers and leafy vegetables (Ali and Khan, 2018; Järup and Akesson, 2009; Keil et al., 2011; Peralta-Videa et al., 2009). Studies of legumes, grasses, and maize grown on post-mined areas of Christmas Island, found that some of these plant species had Cd levels above World Health Organization recommended levels (Howieson et al., 2016; Ruthrof et al., 2018). However, Christmas Island flying-foxes have a dietary predilection for pollen, nectar, fruits and native leaves (Tidemann, 1985a; Todd, 2020) which were not assessed in these studies, therefore further testing is required. Another possible route of Cd ingestion for the Christmas Island flying-fox is Cd containing phosphate dust contamination of food plants or via grooming. Phosphate dust generated from Christmas Island is rich in Cd due to the naturally Cd rich soil (see David et al., 1978; Loganathan et al., 2003; Ruthrof et al., 2018). Mined phosphate dust is transported across the island in uncovered trucks creating a film of dust on vegetation lining the island's roadways and mining facilities, areas where Christmas Island flying-foxes are known to forage. Similar instances of heavy metal poisoning occurred in Esperance, Western Australia where hundreds of nectar feeding birds died from foraging on plants contaminated with lead carbonate containing dust (DECWA, 2007). Analysis of Cd concentrations in and on plants and on fur is necessary to better understand the route of ingestion of Cd in the Christmas Island flying-fox.

In addition to high Cd concentrations, high Fe concentrations (median 564 μg/g creatinine; range 39.0–4505 μg/g creatinine) were detected in the urine of flying-foxes in this study. Urinary Fe concentrations were twofold higher than those reported for grey-headed flying-foxes (Pulscher et al., 2020). Iron storage disease has been reported in the Egyptian fruit bat (*Rousettus aegyptiacus*) with liver concentrations of >12,000 μg/g (Crawshaw et al., 1995; Leone et al., 2016; Stasiak et al., 2018). Iron concentrations in the liver of two Christmas Island flying-foxes in the current study were considerably lower and hepatic pathology, as described for the Egyptian fruit bat, was not observed (Leone et al., 2016). Excessive iron storage has been previously documented in flying-foxes but in the absence of associated clinical disease (Crawshaw et al., 1995). Stasiak et al. (2018) recently assessed susceptibility of bat species to iron storage disease by exploring the effect of hepcidin, an iron regulating hormone, in the Egyptian fruit bat to a less susceptible Pteropodid, the straw-coloured fruit bat (*Eidolon helvum*), and an obligate sanguivore the common vampire bat (*Desmodus rotundus*). When challenged with Fe, the Egyptian fruit bat and common vampire bat had increased hepcidin expression, but no hepcidin was expressed in the straw-coloured fruit bat suggesting that this species could have other mechanisms to excrete or control body Fe, making them less susceptible to iron storage disease (Stasiak et al., 2018). In humans and other mammals, Fe is primarily lost through menstruation and faecal excretion (2015; Green et al., 1968; Hunt et al., 2009). High urinary Fe concentrations were seen in this study (nine times higher than in human urine; Rodríguez and Díaz, 1995) and in grey-headed flying-fox urine (Pulscher et al., 2020) suggesting a novel excretory route for excess Fe. Christmas Island flying-foxes may require an additional mechanism to excrete Fe due to increased concentrations of Fe in native fruit (L. Pulscher, unpublished data). Further investigation of urinary Fe concentrations and Fe metabolism in this, and other flying-fox species in the wild, is warranted to better understand these proposed pathways of intake and excretion.

One Christmas Island flying-fox had high urine Pb concentrations (341 μg/g creatinine) and the highest urinary concentrations of Cd, Zn, Fe, and Hg. While the reason for the elevated metal concentrations in this animal are not known, one possibility is exposure of this individual to metal contamination through foreign substance ingestion. Possible sources of Pb include motor vehicle emissions and transportation services, construction material, and other urban sources (National Pollutant Inventory, 2020). Lead poisoning is commonly reported in domestic and free ranging animals and can be ingested incidentally or in some cases purposefully (De Francisco et al., 2003; McLelland et al., 2010). Further studies monitoring the Christmas Island flying-fox population for metal contamination are necessary to understand sources of metal exposure. Of the remaining metals investigated, the fur, urine, and tissue concentrations were similar or significantly lower than those reported in grey-headed and black flying-foxes sampled in Sydney and in other frugivorous bat species (Becker et al., 2018;

Hoenerhoff and Williams, 2004; Pulscher et al., 2020; Syaripuddin et al., 2014). The exposure of Christmas Island flying-foxes to lower concentrations of other metals is unsurprising considering that, with the exception of phosphate mining and minimal human settlement, Christmas Island is a pristine environment.

5. Conclusion

Findings from this study provide evidence that Christmas Island flying-foxes are exposed to chronic levels of Cd but not Cu, Fe, Hg, Pb, and Zn. While direct associations with disease were not possible, evidence for both renal dysfunction and altered bone metabolism are reported. In addition to identifying high Cd in the Christmas Island flying-fox, the developed reference values have utility for ongoing monitoring of metal exposure in this species, an integral first step in evaluating the role of heavy metals in the species' decline. Given the high concentrations of Cd in flying-foxes, investigating Cd exposure in other endemic fauna and in humans, particularly children, on Christmas Island is recommended.

CRediT authorship contribution statement

Laura A. Pulscher: Conceptualization, Data curation, Funding acquisition, Formal analysis, Investigation, Methodology, Writing - original draft. **Rachael Gray:** Supervision, Data curation, Methodology, Writing - review & editing. **Robert McQuilty:** Resources, Supervision, Methodology, Writing - review & editing. **Karrie Rose:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing. **Justin A. Welbergen:** Data curation, Formal analysis, Supervision, Writing – review & editing. **David N. Phalen:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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