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Investigation into the utility of flying foxes as bioindicators for environmental metal pollution reveals evidence of diminished lead but significant cadmium exposure.

Laura A. Pulscher [a, *], Rachael Gray [a], Robert McQuilty [b], Karrie Rose [c, d], Justin Welbergen [e], David N. Phalen [a]

[a] Sydney School of Veterinary Science, Faculty of Science, University of Sydney, Sydney, NSW, Australia
[b] Department of Chemical Pathology, Royal Prince Alfred Hospital, Level 7 Building 65, Missenden Rd, Camperdown, NSW, Australia
[c] Australian Registry of Wildlife Health, Taronga Conservation Society Australia, Bradleys Head Road, Mosman, NSW, 2088, Australia
[d] College of Public Health, Medical and Veterinary Sciences, James Cook University, James Cook Drive, Townsville, Queensland 4814, Australia
[e] Hawksbury Institute for the Environment, Western Sydney University, Richmond, NSW, 2753 Australia

HIGHLIGHTS

- The utility of flying-foxes as bioindicators of metal pollution was explored.
- Tissue lead concentrations were reduced compared to flying-foxes sampled in the 1990s.
- Evidence of chronic cadmium exposure was found.
- Urine concentrations of lead and cadmium reflected those found in tissues.

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ABSTRACT

Due to their large range across diverse habitats, flying-foxes are potential bioindicator species for environmental metal exposure. To test this hypothesis, blood spots, urine, fur, liver and kidney samples were collected from grey-headed flying-foxes (*Pteropus poliocephalus*) and black flying-foxes (*P. alecto*) from the Sydney basin, Australia. Concentrations of arsenic, cadmium, copper, lead, mercury and zinc and 11 other trace metals were determined using inductively coupled plasma mass spectrometry. As predicted, kidney and fur lead concentrations were lower compared to concentrations found in flying-foxes in the early 1990's, due to reduced environmental lead emissions. Tissue cadmium concentrations in flying-foxes were higher compared to previous studies of flying-foxes and other bat species, suggesting that flying-foxes were exposed to unrecognized cadmium sources. Identification of these sources should be a focus of future research. Urine concentrations of arsenic, cadmium, mercury, and lead were proportional to kidney concentrations. Given that urine can be collected from flying-foxes without handling, this demonstrates that many flying-foxes can be assessed for metal exposure with relative ease. The analysis of blood spots was not viable because of variable metal concentrations in the filter paper used. Fur concentrations of metals correlated poorly with tissue concentrations at the low levels of metals found in this study, but fur could still be a useful sample if flying-foxes are exposed to high levels of metals. Lastly, heat inactivation had minimal impact on metal concentrations in kidney and liver samples and should be considered as a tool to protect personnel working with biohazardous samples.

1. Introduction

Environmental contamination with heavy metals, such as arsenic (As), lead (Pb), cadmium (Cd), and mercury (Hg) and trace elements such as copper (Cu) and zinc (Zn) (subsequently referred to as metals), has serious consequences for humans, other animals,

* Corresponding author.
E-mail addresses: laura.pulscher@sydney.edu.au (L.A. Pulscher), rachael.gray@sydney.edu.au (R. Gray), Robert.McQuilty@health.nsw.gov.au (R. McQuilty), krose@zoo.nsw.gov.au (K. Rose), j.welbergen@westernsydney.edu.au (J. Welbergen), david.phalen@sydney.edu.au (D.N. Phalen).

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and the environment (Callan et al., 2012; DECWA, 2007; Gulson et al., 2012; Mackay et al., 2013; Martley et al., 2004; Zhijia and Morrison, 2001; SA Health, 2013). The impact of exposure to metals has been best studied in rodent models and humans where serious detrimental outcomes have been demonstrated from both acute high-level and chronic low-level exposure. Based on these studies, it is known that toxic concentrations of metals can interfere with hepatic, renal and central nervous system function through physiological processes and irreversible cellular changes resulting in necrosis (Godt et al., 2006; Kapaj et al., 2006; Keil et al., 2011; Liu et al., 2013; Needleman, 2004; Plum et al., 2010; Uriu-Adams and Keen, 2005; Zahir et al., 2005). Higher levels of these metals can also alter the function of multiple aspects of innate and acquired immune function, interfere with fecundity, cause endocrine disruption, and some metals, for example As, Cd, Hg, and Pb, are carcinogens (Godt et al., 2006; Kapaj et al., 2006; Keil et al., 2011; Liu et al., 2013; Needleman, 2004; Plum et al., 2010; Uriu-Adams and Keen, 2005; Zahir et al., 2005).

Metal contamination of the Australian environment has been, and continues to be, a problem for animal, human and environmental health (Berger et al., 2019; Callan et al., 2012; DECWA, 2007; Gulson et al., 2012; Mackay et al., 2013; Martley et al., 2004; SA Health, 2013; Zhijia and Morrison, 2001). The sources of environmental metal contamination are many and are both focal and diffuse. Australia is one of the largest producers of Pb and other toxic metals. Significant local and regional environmental contamination has occurred at mining and processing sites as well as during transport to processing sites (Berger et al., 2019; Callan et al., 2012; DECWA, 2007; Gulson et al., 2012; Mackay et al., 2013; Martley et al., 2004; SA Health, 2013; Zhijia and Morrison, 2001). Australia also has a large agricultural sector that relies heavily on phosphate fertilizers and agricultural products that may be contaminated with Cd and other heavy metals (Williams and David, 1976). In addition, urbanisation has resulted in metal contamination of the environment, in particular, through historical burning of leaded petrol and the use of lead-based paint (Kayhanian, 2012; Needleman, 2004). Lastly, Australia has been and is still largely dependent on burning fossil fuels to generate the majority of its energy needs, and Hg and Cd are known to be released from coal-fired power plants (Peralta-Videa et al., 2009).

The impact of metals on humans and Australia's ecosystems have at times been catastrophic. In Port Pirie, South Australia, 120 years of Pb smelting resulted in widespread environmental contamination and human exposure with the highest Pb levels detected in children (SA Health, 2013). Community and ecosystem-wide contamination with Pb also occurred in Esperance, Western Australia as a result of lead carbonate dust being released during transport operations (Callan et al., 2012; Gulson et al., 2012). In this instance, hundreds of nectar feeding birds died from foraging on Pb contaminated plants, and significant human exposure was documented. In Port Kembla, New South Wales (NSW) multiple industries were implicated in the release of metals into Port Kembla Harbor resulting in a marked decrease in diversity of both marine animals and plants, and extensive soil contamination (Zhijia and Morrison, 2001). In response to these and other contamination events, there has been increased regulation to reduce metal exposure in the environment (Callan et al., 2012; Zhijia and Morrison, 2001).

Ongoing monitoring of environmental metal loads is required to determine if regulatory efforts to reduce emissions are successful and to identify new or unrecognized sources of environmental metal pollution. One means of monitoring environmental metal loads is to measure metal concentrations in specimens (tissues, blood, fur, faeces, urine) from bioindicator species. An ideal bioindicator species would be one that ranges across the area of interest and is readily sampled using minimally-invasive methods, preferably ones that do not require capture or restraint of the bioindicator species. The potential use of bats as bioindicator species for environmental pollution, including metal pollution, has been investigated in many parts of the world (Zukal et al., 2015). In Australia, two species of bat, the grey-headed flying-fox (*Pteropus poliocephalus*) and black flying-fox (*P. alecto*), have the potential to fill a bioindicator role as both species have a wide distribution, which combined covers approximately 60% of the coastal regions of Australia, and they have a varied diet sourced from natural and modified rural and urban habitats representing a broad cross section of the Australian environment (Eby, 1991; Palmer and Woinarski, 1999). Additionally, these flying-foxes roost in large camps across their range, creating opportunities to obtain samples from many animals from different locations in a short period of time, either by capture and sampling (blood, fur, and urine) or collection of urine onto plastic sheets placed under bat roosts. Previous studies have also determined Pb and Cd levels in free-living flying-fox tissues in Australia providing data for comparison. Most notably, Hariono et al. (1993) found up to 30% of flying-foxes that they sampled in the Brisbane urban area had Pb levels indicative of Pb toxicosis. It was hypothesised that these bats were exposed by ingesting Pb containing dust while grooming. The Pb in the dust would have most likely come from the combustion of leaded petrol (Kristensen, 2015). Another small study of three black flying-foxes with neurological signs found one individual with extremely high levels of Pb, and while there was some exposure to Cd in one animal, tissue concentrations were below the toxic threshold (Skerratt et al., 1998).

Determining concentrations of metals in wild flying-foxes is challenging and has previously relied on the analysis of tissue samples, most commonly liver and kidney, from deceased individuals (Hariono et al., 1993; Skerratt et al., 1998). In addition to the logistical and animal welfare complexities of handling flying-foxes and their fluids or tissues, the biosecurity risks are a considerable impediment, given that flying-foxes are known reservoirs of multiple zoonotic viruses (Calisher et al., 2006). Thus, there is a need to identify minimally-invasive samples that can be obtained from live animals where metal concentrations will reflect those found in tissues. There is also an imperative to identify decontamination methods that can apply to all flying-fox sample types, and which do not interfere with the results of metal analysis.

Fur, blood, and urine can all be obtained minimally-invasively. Fur has been used extensively to determine metal concentrations in wildlife, including bats (Becker et al., 2017; Flache et al., 2015; Hariono et al., 1993; Hernout et al., 2016; Little et al., 2015; Mina et al., 2019). However, while some studies have found a correlation between fur, liver and kidney metal concentrations others have not; the strength of these correlations, when identified, was low to moderate (Hernout et al., 2016; Mina et al., 2019). Additionally, the use of fur for metal analysis requires knowledge of the fur growth and moult cycle, to determine the time period in which the bat has been exposed, however, this is not well understood in bats (Eizadi Mood, 2007). Blood spots (blood dried and stored on filter paper) and urine are alternative sample types that have routinely been used to screen for, and determine, the extent of exposure to metals in humans (urine and blood spots) and wildlife (blood spots) (Hansen et al., 2014; Keil et al., 2011; Lehner et al., 2013; McHuron et al., 2019). Blood spots are advantageous as they require a small blood volume (100 μL), do not require the use of a cold chain, and once dried, the samples are not considered infectious. Urine also requires a small volume (100 μL) and is easy to collect under roosts without the need for animal capture and restraint. However, the utility of blood spots and urine for metal screening in bats has not been assessed and validated.

To reduce the biosecurity risks associated with analysing tissues and fluids from flying-foxes, samples are routinely formalin- or ethanol-fixed to eliminate viral infectivity; however, it is known that many metals leach out of the tissues into these fixatives, which can bias results (Gellein et al., 2008). Another method recommended by the World Health Organization for reducing biosecurity risks is heat inactivation of a sample at 60 °C for 1 h (WHO, 2014). However, the impacts of heat inactivation on metal concentrations in tissue or other biological samples are not well understood.

The primary objective of this study was to investigate the potential utility of black and grey-headed flying-foxes as bioindicator species for environmental metal (As, Cu, Cd, Pb, Hg, and Zn) exposure using traditional methods of screening liver and kidney samples for metal concentrations. It was predicted that as the result of mitigation efforts that have significantly reduced Pb emissions into the environment, tissue concentrations of Pb in recently sampled flying-foxes would be lower compared to tissue concentrations determined from flying-foxes prior to the institution of these mitigation efforts. Using these data, the secondary objective was to determine if minimally-invasive samples (blood spots, fur, and urine) could be used to predict the level of metal exposure in tissues. Lastly, the impact of heat inactivation on metal concentrations in tissue and other biological samples was investigated, to better understand the effect of this sterilization method on flying-fox metal concentrations. During the course of this investigation, tissue, urine, and fur concentrations of 11 additional metals were determined and reported.

2. Materials and methods

2.1. Sample collection

Samples were collected from individual black flying-foxes (n = 9) and grey-headed flying-foxes (n = 25) that died naturally or were presented to carers and veterinarians in the greater Sydney basin, NSW, Australia in January 2013 (black flying-foxes (n = 8)) and between November 2017–September 2018 (black flying-foxes (n = 1) and grey headed flying-foxes (n = 25)). Animals were euthanised if the extent of their injuries precluded rehabilitation. To account for the possible impact of long-term care on the results of the study, flying-foxes were split into two categories, those that had been in care for less than three weeks, hereafter referred to as free-living flying-foxes (black flying-fox; n = 9 and grey-headed flying-fox; n = 11), and those that had been in care for longer than three weeks, hereafter referred to as captive flying-foxes (grey-headed flying-fox; n = 14). All animals were sampled under a NSW Office of Environment and Heritage Licence to Rehabilitate Injured, Sick or Orphaned Protected Wildlife (#MWL000100542).

Live flying-foxes were anaesthetised with 2% isofluorane in 1 L/min oxygen (Isoflurane 100%, Zoetis, Australia). Whole blood was collected from the uropatagial vein and placed on duplicate Nobotu filter papers (Advantec, Toyo Roshi Kaisha, Ltd., Japan) that were dried at room temperature and stored in individual paper envelopes. Urine was collected from free-living grey-headed flying-foxes (n = 5) and captive grey-headed flying-foxes (n = 7) into cryovials (Greiner Bio-One International, Austria) and immediately frozen at −80 °C until analysis. Tissue samples were collected after natural death or after euthanasia via intravenous overdose of pentobarbitone sodium (Lethabarb Euthanasia Injection, Virbac, Sydney, NSW, Australia). Prior to necropsy, body mass (g), sex, forearm length (mm), and age were recorded, and a general physical examination of the flying-fox was conducted noting any external signs of injury or disease. Approximately 50–100 mg of fur was collected from the nucha (nape) of the neck and stored at room temperature until further analysis. During necropsy, gross pathological findings were noted and one half of each kidney and liver was collected, stored in cryovials, and frozen at −80 °C until further analysis. Samples were collected under the Opportunistic Sample Collection Program of the Taronga Animal Ethics Committee (#R17B252), and under scientific licenses #SL10469 and SL100104 issued by the NSW Office of Environment and Heritage.

2.2. Impact of heat inactivation, a sterilization method, on metal concentrations in chicken liver and pig kidney samples

Chicken (*Gallus gallus*) liver and pig (*Sus scrofa*) kidney samples were used as a proxy to determine the impact of heat inactivation, a sterilization method, on metal concentrations in flying-fox tissue samples. Tissues from chicken and pigs were selected for this purpose due to the restrictions of workers health and safety when working with untreated flying-fox samples. Four pig kidneys and four chicken livers were sectioned into eight replicates of 5–20 mg each using stainless steel scalpel blades that were cleaned between each sample, placed into pre-weighed sterile 1.5 mL tubes and weighed to four decimal places (Mettler AE100, Mettler Toledo, Port Melbourne, VIC, Australia). Four replicates of each kidney and liver sample (n = 16 total replicates for each sample type and treatment) were then heat inactivated at 60 °C for 1 h and replicates were kept frozen until analysis. Samples were then freeze-dried (Edwards High Vacuum Ltd, Model #EF03, Manor Royal, Crawley, Sussex) for 1 h at 760 mm mercury and re-weighed to obtain a dry body mass. Concentrated nitric acid (Suprapure Nitric Acid 65% Merck, Darmstadt, Germany) was added to the tube and the sample was digested in a hot oil bath at 60 °C for 1 h. After digestion, the sample was diluted with 400 μL double distilled water. The sample was then vortexed and centrifuged at 10,900 rpm for 3 min to check for sediment or undigested sample. Samples were then stored at −4 °C until analysis with inductively coupled plasma mass spectroscopy (ICP-MS; Agilent 7500Ce ICP MS, Santa Clara, California, USA).

2.3. Comparison of metal concentrations in Australian sea lion whole blood and blood spots

Due to the restrictions of working with flying-fox samples for workers health and safety and to increase sample numbers, Australian sea lion (*Neophoca cinerea*) blood samples were opportunistically sampled to determine if there was any difference between metal concentrations in filter paper samples and those in whole blood. Whole blood samples (n = 28) were collected into metal-free tubes (Vacutainer Trace Element Tube, BD) and placed on matching Nobotu filter papers (n = 28). Samples collected from Australian sea lions were collected under the University of Sydney's Animal Ethics Committee's approval #2017–1260. Whole blood was stored frozen at −20 °C in the field, then frozen at −80 °C prior to analysis. Nuboto filter papers were air dried and stored in envelopes at room temperature until analysis. Blood spots collected on filter paper from sea lions and blank filter paper controls were sectioned using new or cleaned scalpel blades and placed into clean pre-weighed 10 mL tubes and re-weighed to obtain a dry mass. Samples were digested with 500 μL 65% nitric acid in a hot sand bath at 85 °C for 2 h. Digested material was diluted with 1 mL of double distilled water, vortexed and centrifuged at 10,900 rpm to remove residual particles and stored at −4 °C until ICP-MS analysis.

2.4. Comparison of metal concentrations in fur, urine, blood stored on filter paper, liver and kidney in flying-foxes

To remove external metal contamination of the fur, flying-fox fur samples (n = 34) were placed into 10 mL tubes, washed with

agitation for 10 min with double distilled water, followed by three washes with reagent grade acetone (Acetone ACS reagent, ≥99.5%, Sigma-Aldrich, Australia), and finished with a final wash of double distilled water (Gray et al., 2008). Fur samples were then freeze-dried at 760 mm mercury over a 10-h period until the fur was determined to be moisture-free. Ten milligram duplicates of washed and freeze-dried fur were placed into clean pre-weighed 1.5 mL tubes. Eighty microliters of concentrated nitric acid were added to each tube and digested in a hot oil bath at 95 °C for 2 h. Digested material was then diluted with 320 μL of double distilled water, vortexed and centrifuged at 10,900 rpm for 3 min to check for sediment or undigested sample and stored at −4 °C until ICP-MS analysis.

Flying-fox urine samples (n = 12) were heat inactivated at 60 °C for 1 h and stored at −80 °C until analysis. Flying-fox heat inactivated urine samples, flying-fox blood spots (n = 24), and duplicate heat inactivated flying-fox liver (n = 34) and kidney (n = 34) samples were processed as described in sections 2.2 and 2.3 and stored at −4 °C until ICP-MS analysis. To account for the impact of varied urine concentration of each animal, metal concentrations in urine were normalized by converting results to urine μg g^{-1} creatinine (Keil et al., 2011). However, due to workers health and safety requirements, creatinine could not be measured in a standard laboratory setting and therefore was measured using Reflotron creatinine test strips (Roche, Mannheim, Germany) on a Reflotron IV Chemical Analyzer (Roche, Mannheim, Germany).

2.5. ICP-MS analysis

Urine and whole blood, and digested tissues, filter papers, and fur 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-Octylphenoxypoly-ethoxyethanol 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 on an ICP-MS. Concentrations of magnesium (Mg), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), Cu, Zn, As, selenium (Se), Cd, tin (Sn), antimony (Sb), Hg, thallium (Tl), Pb, and bismuth (Bi) were determined in all samples. The limit of detection was 2 μg L^{-1} for all metals tested. Excepting As, Cd, Cu, Hg, Pb, and Zn concentrations, results for all other metals are provided as supplementary material (Supplementary Tables 1–5).

2.6. Statistical analysis

Concentrations of metals in liver, kidney, and fur were expressed in μg g^{-1} of dried tissue. Concentrations of metals in whole blood and blood spots were expressed as μg g^{-1} of whole blood, and concentrations of urine were expressed as μg g^{-1} creatinine. To enable comparison of whole blood to blood spots, metal concentrations in blood spots were converted to an equivalent μg g^{-1} wet weight based on filter papers containing 100 μL of whole blood. The same calculation was used for control filter paper. Prior to analysis, all metals with results below the detection limit were replaced with an estimated value equal to half of the detection limit. The mean of duplicates for flying-fox digested tissues and fur was used for all statistical analysis. Prior to analysis normality of data was determined using a Shapiro-Wilk test and assessment of Q-Q plots and histograms. Apart from control filter papers, all data were determined to be non-normally distributed. All statistical analysis was performed in R version 3.4.3 for Windows. Statistical significance was considered at p-value ≤ 0.05.

An inter-class correlation coefficient (ICC) was computed to assess agreement between heat inactivated and frozen pig kidney and chicken liver samples. To meet normality assumptions, all data were log-transformed prior to ICC analysis. ICC's were computed with the 'irr' package (version 0.84.1) in R using a two-way random effect model and single rater unit. The two treatments were classified as having poor (K < 0.50), moderate (0.50 < K < 0.75), good (0.75 < K < 0.90), or excellent (K > 0.90) agreement (Koo and Li, 2016). If a negative ICC value was obtained it was reported as K = 0 as it was conservatively estimated that there was little to no agreement between the two methods in this case. Given that multiple fold increases in tissue concentrations would be expected in flying-foxes exposed to toxic concentrations of metals, we would expect that heat inactivated material metal concentrations which differed by less than 25% from frozen material would not be clinically significant. Therefore, heat inactivated and non-heat inactivated pig kidney and chicken liver tissues were determined to have clinically significant differences in metal concentrations if: i) there was a poor agreement between the two methods (k < 0.50), and ii) the concentration of a metal in the heat inactivated material differed more than 25% from the concentration in frozen material. The same procedure described above for heat inactivated material was used to compare filter paper methodology to paired whole blood samples. In addition, mean and standard deviation were determined for control filter paper metal concentrations.

Median and range for kidney, liver, urine, and fur metals were calculated for all samples. To determine if there was a correlation between grey-headed flying-fox fur, urine, kidney, and liver samples, a Spearman's rank order correlation coefficient was calculated to determine if the correlation was significant. Interspecies differences for free-living black and grey-headed flying-foxes, and differences among free-living and captive grey-headed flying-foxes, were compared for kidney, liver, fur, and urine samples using a Wilcoxon signed rank test.

3. Results

3.1. Impact of heat inactivation on metal concentrations in chicken liver and pig kidney samples

There was good agreement between the heat inactivated and frozen chicken liver samples for Hg (K = 0.81) but poor or no agreement for Cu (K = 0.33), Zn (K = 0), As (K = 0), Cd (K = 0), and Pb (K = 0.08) (Supplementary Table 1). For the pig kidney, there was excellent agreement between the heat inactivated and frozen tissues for Hg (K = 0.90), good agreement for Cu (K = 0.82), moderate agreement for Zn (K = 0.73) and Cd (K = 0.68) but poor agreement for As (K = 0.36) and Pb (K = 0.20) (Supplementary Table 1). While there was poor agreement between some heat inactivated and frozen tissue samples, the differences between the median values obtained in the heat inactivated groups were less than 25% (Supplementary Table 1).

3.2. Comparison of metal concentrations in Australian sea lion whole blood and blood spots

There was poor or no agreement between Australian sea lion blood spots and whole blood samples for all metal concentrations (Supplementary Table 2). Heavy metal concentrations in the control filter paper samples were highly variable and in the case of Cd, Hg, and Pb, concentrations were 13-fold, 12-fold, and 2-fold higher than what was seen in paired filter paper samples with whole blood present, respectively (Supplementary Table 2).

Table 1
Tissue metal concentrations for free-living grey-headed flying-foxes (*Pteropus poliocephalus*) (n = 11) and captive grey-headed flying-foxes (n = 14) collected in the Sydney basin, Australia from November 2017–September 2018. Median (observed range) metal concentrations are presented for heat treated kidney (free-living n = 11, captive n = 14; μg g^{-1} dry weight), heat treated liver (free-living n = 11, captive n = 14; μg g^{-1} dry weight), fur (free-living n = 11, captive n = 14; μg g^{-1} dry weight), and heat treated urine (free-living n = 5, captive n = 7; μg g^{-1} creatinine). A Wilcoxon signed rank test was used to evaluate statistical differences between metals in free-living grey-headed flying-fox and captive grey-headed flying-fox samples. *P-value ≤ 0.05

	Free-living Grey-headed flying-fox (captive < 3-weeks)			
	Kidney (n = 11)	**Liver (n = 11)**	**Fur (n = 11)**	**Urine (n = 5)**
Cu	14.0 (7.80–18.7)	8.91 (5.92–79.0)	2.02 (0.89–9.30)	109 (0–6418)
Zn	92.2 (68.5–201)	58.1 (44.4–242)	17.4 (9.11–68.0)	1040 (592–40787)
As	0.11 (0.06–0.18)	0.07 (0.05–0.16)	0.11 (0.04–0.25)	53.5 (21.0–114)
Cd	29.8 (0.27–219)	1.45 (0.02–12.6)	0.01 (<0.01–0.04)	23.9 (8.93–169)
Hg	0.85 (0.11–2.62)	0.38 (<0.01–4.47)	0.12 (0.03–0.44)	541 (437–2001)
Pb	0.59 (0.19–1.86)	0.62 (0.14–3.39)	0.34 (0.09–1.35)	8.60 (3.50–418)
	Captive Grey-headed flying-fox (captive > 3 weeks)			
	Kidney (n = 14)	**Liver (n = 14)**	**Fur (n = 14)**	**Urine (n = 7)**
Cu	22.4 (10.8–37.2)*	6.82 (2.18–16.3)*	2.03 (1.10–25.4)	63.5 (4.92–2259)
Zn	229 (97.6–1318)*	68.2 (33.5–395)	32.7 (10.1–1477)	1010 (530–13473)
As	0.09 (0.05–0.19)	0.06 (0.03–0.11)	0.12 (0.03–0.25)	26.8 (6.43–34.4)
Cd	10.9 (0.59–62.8)	0.32 (0.01–1.06)*	0.01 (0.00–0.02)	13.5 (6.92–34.6)
Hg	0.40 (0.20–6.68)	0.70 (<0.01–2.31)	0.21 (0.03–0.86)	96.3 (38.0–999)
Pb	1.24 (0.14–13.2)	0.24 (0.03–2.11)	0.23 (0.06–2.73)	4.61 (1.79–157)

3.3. Comparison of metal concentrations in tissues of free-living grey-headed flying-foxes and captive grey-headed flying-foxes

Captive grey-headed flying-foxes had significantly higher kidney Cu (W = 105, p < 0.001) and Zn (W = 105, p < 0.001) concentrations in comparison to free-living grey-headed flying-foxes (Table 1). Captive grey-headed flying-foxes also had significantly lower liver Cu (W = 105, p < 0.001) and Cd (W = 105, p < 0.001) concentrations in comparison to free-living grey-headed flying-foxes (Table 1).

3.4. Comparison of metal concentrations in fur, urine, liver and kidney in grey-headed flying-foxes

Significant moderate to strong positive correlations were identified for kidney and urine As (p = 0.05), Cd (p = 0.03), Pb (p = 0.01) and Hg (p = 0.01) concentrations, liver and fur Pb (p = 0.01) concentrations, and liver and urine Pb (p < 0.01) concentrations (Table 2).

3.5. Comparison of metal concentrations in tissues of free-living black flying-foxes compared to free-living grey-headed flying-foxes

Compared to free-living grey-headed flying-foxes, free-living black flying-foxes had significantly higher kidney As (W = 84, p = 0.01) and Pb (W = 99, p < 0.001) concentrations, but significantly lower kidney Hg (W = 23, p < 0.001) concentrations (Table 3). Free-living grey-headed flying-foxes had significantly higher liver Cu (W = 12, p < 0.01) and Zn (W = 10, p < 0.01) concentrations (Table 1). Black flying-foxes were also found to have significantly higher fur As (W = 78.5, p = 0.03) and Pb (W = 90.5, p < 0.01) concentrations in comparison to free-living grey-headed flying-foxes (Table 3).

Table 2
Relationships between metal concentrations in grey-headed flying-fox (*Pteropus poliocephalus*) fur (n = 25), heat treated urine (n = 12), heat treated kidney (n = 25), and heat treated liver (n = 25) collected in the Sydney basin, Australia from November 2017–September 2018. Spearman's correlation coefficients (r_s) were used to evaluate the strength of these associations. *P-value ≤ 0.05

	Kidney vs Fur	Kidney vs Urine	Liver vs Fur	Liver vs Urine
Cu	−0.26	−0.03	−0.17	0.24
Zn	0.25	0.34	−0.12	0.32
As	<0.01	0.59*	0.05	0.23
Cd	0.03	0.65*	0.06	0.40
Hg	0.02	0.74*	0.01	−0.36
Pb	0.29	0.71*	0.50*	0.79*

Table 3
Median (observed range) metal concentrations in black flying-fox (*Pteropus alecto*) heat treated kidney (n = 9; μg g^{-1} dry weight), heat treated liver (n = 9; μg g^{-1} dry weight), and fur (n = 9; μg g^{-1} dry weight) collected from the Sydney basin, Australia in January 2013 (n = 8) and November 2017 (n = 1). A Wilcoxon signed rank test was used to evaluate statistical differences between metals in free-living black flying-fox and grey-headed flying-fox (*P. poliocephalus*) samples. *P-value ≤ 0.05

	Kidney (n = 9)	Liver (n = 9)	Fur (n = 9)
Cu	15.3 (13.5–21.1)	4.61 (3.23–8.39)*	2.83 (1.30–8.63)
Zn	82.6 (57.0–105)	31.4 (18.9–66.2)*	33.3 (17.3–47.9)
As	0.25 (0.09–0.51)*	0.09 (0.02–0.22)	0.16 (0.10–0.45)*
Cd	28.4 (4.08–64.1)	1.44 (0.23–7.58)	0.02 (0.01–0.03)
Hg	0.40 (0.15–0.96)*	0.36 (0.10–1.00)	0.06 (0.03–0.25)
Pb	4.82 (2.56–14.0)*	0.93 (0.60–1.24)	1.61 (0.72–3.75)*

4. Discussion

4.1. Impact of heat inactivation on metal concentrations in pig kidney and chicken liver samples

Flying-foxes are known reservoirs of zoonotic viruses (Calisher et al., 2006) and, as a result, untreated tissues collected from flying-foxes cannot be processed in standard toxicology laboratories. For this reason, the impact of heat inactivation, a sterilization method, on metal concentrations using chicken liver and pig kidney samples was investigated. There was poor agreement between heat inactivated and frozen tissues for five of six metals in liver and two of six metals in kidney. However, the differences between pre- and post-treatment values were relatively small (<25%). Given that multiple fold increases in tissue concentrations would be expected in individuals exposed to toxic concentrations of metals in comparison to those not exposed, we conclude that heat inactivation of tissues would have little impact to the outcomes of studies using liver and kidney for metal screening. Although we were unable to

validate the impact of heat inactivation on flying-fox tissues, processing of samples is based on laboratory techniques used for a variety of species, and from tissues in various media (frozen, fixed, and paraffin embedded) (Davis et al., 2006; Hernout et al., 2016; Keil et al., 2011; Zabka et al., 2006). Therefore, we expect that flying-fox tissues would act similarly under these methodologies as has been demonstrated in other species, however further studies are required to validate this conclusion.

4.2. Utility of blood spots for the detection of metal concentrations in whole blood

Due to workers health and safety requirements, whole blood samples collected from flying-foxes cannot be processed in standard toxicology laboratories. For this reason the utility of blood spots for the detection of metal concentrations utilizing opportunistically collected blood spots and paired whole blood samples collected from Australian sea lions was investigated. There was poor or no agreement between blood spots and paired whole blood samples for all metals assessed in this study. Furthermore, the analysis of the 'control' filter papers themselves determined significant and variable concentrations of the target metals, such that the blood spot method was not considered viable. Similar results for Pb and Cd concentrations have also been reported in filter papers made by another manufacturer (Funk et al., 2013). Based on these studies, if blood is used to assess flying-fox exposure to metals, the preferred sample would be whole blood collected into metal free blood collection tubes.

4.3. Investigating the utility of black and grey-headed flying-foxes as bioindicator species for metal exposure

Black and grey-headed flying-foxes have large home ranges that cover much of the coastal areas of Australia and overlap major urban, food producing and wilderness areas making them a potentially important bioindicator species for metal exposure. Their utility as a bioindicator species is also enhanced given that they roost in large and consistently used camps allowing sampling of animals with relative ease. As proof of concept that black and grey-headed flying-foxes can serve as indicator species for environmental metal exposure, this study focused on Pb concentrations in kidney and liver samples collected from black and grey-headed flying foxes from the Sydney basin. It was anticipated that Pb concentrations in flying-fox tissues would be reduced when compared to concentrations found in flying-foxes sampled in 1993 in Brisbane (Hariono et al., 1993) considering ambient release of Pb into the environment has been significantly reduced since leaded based petrol and paint were banned in 2002 and 1978, respectively (Kristensen, 2015). This was indeed the case. In the 1993 study, 30% of the flying-foxes tested for Pb exposure had kidney ($\geq$25 μg g^{-1}) or liver ($\geq$10 μg g^{-1}) concentrations that were in the toxic range for domestic animals (Shore and Rattner, 2001). High concentrations of Pb were also found in the fur of these animals (mean 20.2 μg gm^{-1}; range 5.6–42.0 μg gm^{-1}) (Hariono et al., 1993). In contrast, in the current study, only one black flying-fox and one grey-headed flying-fox had toxic concentrations of Pb in kidney while mean fur Pb concentrations in black and grey-headed flying-foxes were only 7.8% and 1.4%, respectively, of the mean fur concentrations found in the Pb exposed flying-foxes sampled in 1993 (Hariono et al., 1993). These findings support the hypothesis that a reduction in Pb contamination of the environment would result in lower Pb concentrations in flying-fox tissues, lending support to the utility of flying-foxes as a bioindicator of Pb concentrations.

Lead released into the Australian environment in the past persists predominately in soil in large cities (Kachenko and Singh, 2006). This Pb can be resuspended in the atmosphere as dust (Laidlaw and Taylor, 2011) and can also be taken up and found in the edible portions of plants (Summer and Reichelt-Brushett, 2018) and as a result of Pb in the soil, children in urban areas are still being exposed to this heavy metal. While it was hypothesised that flying-fox tissue concentrations of Pb would be reduced, Pb exposure, albeit at a lower level is still evident. Although tissue Pb concentrations were 20-fold and 35-fold lower, for kidney and liver, respectively, compared to those of flying-foxes in 1993 (Hariono et al., 1993), all black flying-foxes and 52% of grey-headed flying-foxes were still exposed to potentially high concentrations of Pb with kidney concentrations of $\geq$1 μg g^{-1} found. The higher concentrations of Pb determined in black flying-foxes compared to grey-headed flying-foxes likely reflect differences in the range and dietary preferences of these two species. Black flying-foxes have a smaller annual range and tend to move within a more localized region while grey-headed flying-foxes travel across their much larger range, often thousands of kilometres, over the course of the year (Welbergen & Martin, unpublished results). All flying-foxes in this study were captured in the Sydney basin, therefore it is possible that black flying-foxes spend more time foraging in urban areas in comparison to grey-headed flying-foxes. Differences in Pb exposure could also reflect differences in the trees on which the different species forage. Range differences and dietary differences could also explain higher concentrations of Hg found in grey-headed flying-fox tissues, higher concentrations of As found in black flying-fox tissues, and lower liver concentrations of Cu and Zn in black flying-fox samples. Possible sources of the Hg determined in flying-foxes in this study could include emissions stemming from transportation services, metal mining and refining industries, and coal-fired power plants (National Pollutant Inventory, 2020). While previous studies in Europe found Hg emissions stem primarily from chlor-alkali and coal-fired power plants (Peralta-Videa et al., 2009), more studies need to be conducted in Australia to determine localized sources of contamination.

A potentially important finding in this study was the high concentrations of Cd determined in the flying-foxes examined. Free-living flying-foxes had six-fold higher kidney Cd concentrations and nearly three-fold higher liver Cd concentrations, including one grey-headed flying-fox with kidney Cd concentrations greater than the toxic threshold ($\geq$100 μg g^{-1}), compared to values reported in a previous study (Skerratt et al., 1998). Furthermore, free-living and captive flying-fox urine Cd concentrations were nearly 5-fold and 3-fold higher, respectively, than what is considered to be diagnostic for toxic exposure in humans ($\geq$5 μg g^{-1} creatinine) suggesting that flying-foxes in this study had high and likely chronic Cd exposure (Keil et al., 2011). The lower concentrations of Cd in the liver of captive flying-foxes could be a result of redistribution of Cd from the liver to the kidney as has been described in experimentally treated laboratory animals (Nordberg et al., 2014). Considering the significant negative impacts Cd has on humans, other animals, and the environment, identification of potential Cd sources that resulted in flying-fox exposure should be a priority for future research. In Australia, Cd sources most likely originate from industry, including metal ore facilities and coal and oil burning power plants, and motor vehicle products including lubricating oils, fuel tanks, batteries and petrol (National Pollutant Inventory, 2020). In humans, the most significant route of exposure is through the ingestion of Cd concentrating plants, however it is unlikely that this is the case in flying-foxes as Cd typically accumulates in tubers and leafy vegetables but not fruit (Ali and Khan, 2018; Jarup and Akesson, 2009; Keil et al., 2011; Peralta-Videa et al., 2009). However, further work is needed to determine metal concentrations in food plants consumed by flying-foxes including leaves, nectar and pollen. Other potential routes of

Cd exposure in flying-foxes include inhalation of Cd particles, ingestion of Cd by the consumption of food sources contaminated by dust, or ingestion of Cd containing dust while grooming. Due to their large ranges, flying-foxes provide a novel tool for determining environmental Cd loads over a large geographical area. To localise the sources of Cd pollution, bioindicator species with smaller ranges may be required. One possible indicator species would be the rainbow lorikeet (*Trichoglossus haematodus*), which feeds on the same plants as flying-foxes, but has a much smaller range (Cannon, 1984). The use of stable isotopes may also help to identify the sources of Cd found in these flying-foxes, as different sources of Cd have different isotopic ratios (Wen et al., 2015).

In the present study, no evidence of toxic exposure to Cu, Hg, As or Zn was found in the flying-fox samples. Liver Cu and liver and kidney Zn concentrations were in the normal range reported for healthy captive flying-foxes (Hoenerhoff and Williams, 2004) and frugivorous microbats (Zukal et al., 2015), respectively. Mercury concentrations in the kidney, although higher in grey-headed flying-foxes compared to black flying-foxes, were 100-fold less than what is considered a critical threshold in other species and kidney and liver As concentrations were twelve times less than previously reported in insectivorous bats in Europe (Walker et al., 2007; Zukal et al., 2015).

4.4. Use of minimally-invasive sample types (fur and urine) to determine metal concentrations in flying-foxes

Monitoring metal concentrations in flying-foxes is challenging as it has traditionally relied on the use of soft tissue samples, particularly liver and kidney, obtained from deceased animals (Hariono et al., 1993). This study aimed to determine if minimally-invasive sample types, including urine and fur, can be used to estimate metal tissue concentrations in flying-foxes. Fur has been suggested to be a useful sample for assessing metal exposure in insectivorous bats in Europe (Hernout et al., 2016; Mina et al., 2019). Hariono et al. (1993) also found substantial correlations between fur Pb concentrations and liver, kidney, and bone Pb concentrations. However, in the current study, a significant correlation was only found between fur and liver Pb concentrations and not between fur and kidney Pb concentrations. Similarly, no correlation between fur and soft tissue concentrations was found for the other metals examined. This is consistent with a previous theory that suggested that metal deposition in fur could remain low and uncorrelated with tissue concentrations until a certain threshold is reached, at which point, deposition of metals in fur would occur at a rate that was linked to tissue concentrations (Mina et al., 2019). Fur and soft tissue concentrations of metals are also impacted by other factors, in addition to the overall environmental exposure to them, including the species of animal under investigation, its sex, its age, and when the animal was exposed relative to when the growth of the fur occurred (Mina et al., 2019). Fur growth cycles in mammals can be seasonal, where most fur is lost and regrown in a short period of time, can occur in waves across the body, or in a mosaic pattern where there is constant low-level loss and regrowth of individual hairs throughout the year (Higgins et al., 2009). The fur growth cycle in flying-foxes is not known. Thus, if metal exposure does not occur at the same time the fur is growing, then differences in fur and soft tissue concentrations would be expected. Based on these observations and considerations, it appears that flying-fox fur metal concentrations would be most representative of metal exposure if the exposure occurred chronically and was at high levels.

Urine is used to screen humans for metal exposure, but has not previously been studied in bats (Keil et al., 2011). In this study, significant positive correlations between urine and kidney As, Cd, Hg, and Pb concentrations and urine and liver Pb concentrations were found. Urine is the preferred sample for determining long-term Cd exposure in humans because it has been found to correlate with kidney Cd concentrations (Keil et al., 2011). Urine has also been used to estimate exposure to As, Pb, and Hg in humans (Keil et al., 2011). Collection of urine for determining metal concentrations has many benefits. Firstly, in humans, urine estimates exposure over a one to three-week period, depending on the metal of interest, which provides an exposure timeline (Keil et al., 2011; Liu et al., 2013; Nieboer et al., 2013). Similar exposure times in urine of flying-foxes would be expected, however, this would need to be validated. Urine can also be collected using under roost sampling methods; a method that is commonly used to study the health of flying-fox populations. This process minimizes stress on individual flying-foxes and is cost and time effective as it doesn't necessitate capture. Urine also has an advantage as it requires less preparation prior to analysis as compared to tissues and fur. For these reasons, urine should be considered as an alternative sample to fur or used in combination with fur when assessing metal exposure in live flying-foxes.

5. Conclusion

Black and grey-headed flying-foxes have the potential to be valuable bioindicator species for environmental metal pollution in Australia because of their large home ranges and their accessibility for sampling. This study provides proof of concept of the utility of these species as bioindicators by demonstrating that tissue Pb concentrations in these two species of flying-foxes have reduced subsequent to the reduction in ambient Pb release into their environment. While Pb lead concentrations in tissues were determined to be lower in this study compared to previous studies, low concentrations were still detected, suggesting that significant reservoirs of Pb, most likely in urban environments persist, a finding that is supported by studies of Pb levels in children living in cities. Perhaps most importantly, determining metal concentrations in black and grey-headed flying foxes revealed exposure to one or more environmental sources of Cd, which requires further investigation to determine if this poses a threat to flying-foxes, humans and other animals. Lastly, this study demonstrated that practical, minimally-invasive sampling methods can be used to safely screen for metal exposure in flying-foxes. Given the extensive geographical distribution of flying-foxes, the findings of this study suggest the use of flying-foxes as bioindicators for metal exposure could have applications outside of Australia.

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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.

CRediT authorship contribution statement

Laura A. Pulscher: Conceptualization, Data curation, Funding acquisition, Formal analysis, Investigation, Methodology, Writing - original draft. **Rachael Gray:** Supervision, 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 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.

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Appendix A. Supplementary data

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