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Portable X-ray fluorescence for bone lead measurements of Australian eagles

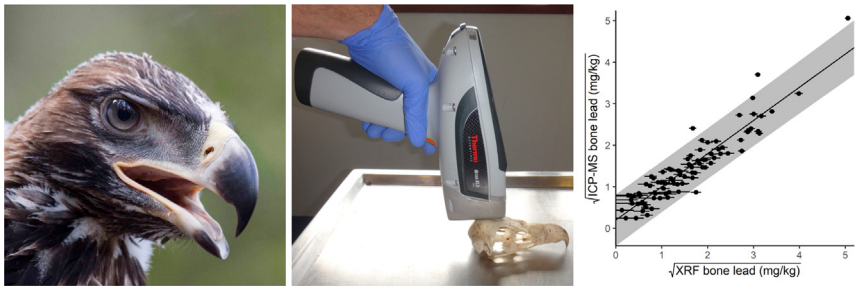
Jordan O. Hampton [a,b,*], Aaron J. Specht [c], James M. Pay [d], Mark A. Pokras [e], Andrew J. Bengsen [f]

[a] Game Management Authority, Melbourne, Victoria 3000, Australia
[b] School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia
[c] Harvard T. H. Chan School of Public Health, Boston, MA, United States
[d] University of Tasmania, Churchill Ave, Hobart, Tasmania 7005, Australia
[e] Cummings School of Veterinary Medicine, Tufts University, N. Grafton, MA, United States
[f] Vertebrate Pest Research Unit, NSW Department of Primary Industries, Orange, NSW 2800, Australia

HIGHLIGHTS

- Portable XRF and ICP-MS bone lead measurements shared strong correlations in Australian wedge-tailed eagles.
- Wedge-tailed eagles from south-eastern mainland Australia have extensive chronic exposure to lead reflected in their bone.
- Portable XRF allows non-destructive and inexpensive measurement of bone lead in archived specimens from Australian raptors.

GRAPHICAL ABSTRACT



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ABSTRACT

Lead (Pb) toxicity from ammunition has been shown to be a threat to scavenging birds across the globe. Toxic levels of lead have recently been found in Australia's largest bird of prey, the wedge-tailed eagle (*Aquila audax*), through inductively coupled plasma mass spectrometry (ICP-MS) analysis of liver and bone samples. However, ICP-MS is consumptive (causing damage to archived specimens), time-consuming, and expensive. For these reasons, portable X-ray fluorescence (XRF) devices have been optimized to measure bone lead in North American avian species, humans, and other environmental samples. In this study, we assessed portable XRF for bone lead measurement in Australian raptors in two parts. First, we validated the method using tissues from wedge-tailed eagles from Tasmania (*A. a. fleayi*), analysing bone samples taken from sites on the femur immediately adjacent to sites for which we had ICP-MS data ($n = 89$). Second, we measured lead via portable XRF in the skulls of wedge-tailed eagles from south-eastern mainland Australia (*A. a. audax*) collected during a criminal prosecution ($n = 92$). Portable XRF bone lead measurement demonstrated an excellent correlation with ICP-MS results using root-transformed regression ($R^2 = 0.88$). Calculated equivalent ICP-MS values revealed that greater than 50% of the eagles from mainland Australia had elevated lead levels (>10 mg/kg) and 13% had severe lead exposure (>20 mg/kg). Our results support previous studies of North American avian species and suggest that portable XRF could be a useful and inexpensive option for measurement of bone lead in Australian scavenger species.

* Corresponding author at: Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria 3052, Australia.
E-mail address: jordan.hampton@unimelb.edu.au (J.O. Hampton).

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1. Introduction

There is growing worldwide recognition of the threat posed by toxic lead-based ammunition for scavenging wildlife (Plaza and Lambertucci, 2019). Lead exposure can cause harm to animals through lethal effects (mortality) and nonlethal effects (morbidity) such as inhibited spatial movements (Ecke et al., 2017). Harmful lead levels have been found in a multitude of scavenging species associated with lead-based ammunition, with avian species particularly impacted (Bassi et al., 2021; Descalzo et al., 2021). Notably affected species include iconic and critically threatened raptors such as Californian condors (*Gymnogyps californianus*) (Bakker et al., 2017). This is a worldwide phenomenon, with harmful lead exposure from bullet-derived lead having been reported from numerous scavenging bird species in North America, Europe, Asia, South America and Africa (Pain et al., 2019). However, until the last three years, little attention had been devoted to this issue in Australia (Hampton et al., 2018).

Recent studies have demonstrated that harmful lead exposure can affect captive wildlife and mammalian scavengers in Australia such as Tasmanian devils (*Sarcophilus harrisii*) exposed to lead-based bullet fragments in breeding programs (Hivert et al., 2018). There is anecdotal historical awareness that some Australian raptors may die directly from lead poisoning (Mooney, 1986), but lead may also increase the susceptibility of individuals to other causes of mortality, such as collisions with anthropogenic structures (e.g. wind farm turbines) and vehicles (Ecke et al., 2017). We are aware of only two published studies on lead exposure in wild Australian raptor species, and both focused on the wedge-tailed eagle (*Aquila audax*), Australia's largest raptor (Olsen, 2005), on the continental mainland (*A. a. audax*) (Lohr et al., 2020), and on the island of Tasmania (*A. a. fleayi*) (Pay et al., 2021). Both studies used inductively coupled plasma mass spectrometry (ICP-MS) in a commercial laboratory to quantify lead concentrations, a costly and time-consuming process. An alternative method for measuring lead concentrations in bone is X-ray fluorescence (XRF), which has been used for decades in human medicine (Specht et al., 2019a; Specht et al., 2021). This device uses a low energy X-ray tube (50 kV) source to quantify elemental content of samples through the fluorescence produced from the X-ray. XRF is being trialled for an increasing number of applications in wildlife research. Among these have been forensic use to measure heavy metal concentrations in the teeth and antlers of wild ungulates (Buddhachat et al., 2016), detection of provenance in the illegal wildlife trade for monotreme species (Brandis et al., 2018), and for calcium (Ca) analysis in waterfowl eggshells (Śliwiński et al., 2020). Portable XRF is also widely used in multiple applications outside of animal biology, e.g. soil science (Hu et al., 2017).

A recent technological advance has seen the wide availability of commercially available portable XRF units. The portable XRF has the advantage of being handheld and battery-powered, with only a 3-min measurement time for most applications, making it inexpensive and convenient for measurements in the field, as well as allowing non-destructive sampling of archived specimens. Portable XRF has been validated for use in measuring bone lead in vivo in humans (Specht et al., 2016) and in vitro for bone lead measurement in Californian condors (Specht et al., 2018b) and common loons (*Gavia immer*) (Specht et al., 2019b), which both demonstrated good correlations with ICP-MS results. To our knowledge, portable XRF has not been validated for bone lead measurement in any avian species outside of North America.

In this study, we validated the use of portable XRF to measure bone lead in bone samples from Australian eagles, and compared the values to those obtained using ICP-MS. Our study species was the wedge-tailed eagle. Our hypotheses were that: 1) bone lead concentrations measured via portable XRF in wedge-tailed eagles are comparable to ICP-MS results, and 2) portable XRF can provide insights into long-term lead exposure levels in unstudied raptor populations through non-destructive and inexpensive analysis of archived bone samples.

2. Materials and methods

2.1. Study species

Wedge-tailed eagles are distributed throughout mainland Australia and Tasmania. Weighing 3–5.5 kg and with a wingspan of 1.9–2.3 m, the wedge-tailed eagle is the largest bird of prey throughout its range, serving an important ecological function as an apex predator (Olsen, 2005). The species is known to scavenge extensively, both on roadkill as well as shot wildlife (Brooker and Ridpath, 1980; Olsen, 2005). Wedge-tailed eagles have been observed to scavenge on several species of shot wildlife including native marsupials (Read and Wilson, 2004; Spencer et al., 2021), introduced deer (Woodford et al., 2020), and smaller introduced mammalian species such as European rabbits (*Oryctolagus cuniculus*) (Peisley et al., 2017).

2.2. Tasmanian wedge-tailed eagle bone samples

Femurs from Tasmanian wedge-tailed eagles were collected from archived collections, as described in Pay et al. (2021). Femur samples were first analysed via ICP-MS, as described in Pay et al. (2021), and we then used the remainder of the same femur samples for XRF measurements to minimize sources of variation in lead concentration within the bones (Specht et al., 2019a). XRF readings were taken from sites on the femur immediately adjacent to sites for which we had ICP-MS data.

2.3. Mainland wedge-tailed eagle bone samples

We accessed skulls of wedge-tailed eagles from south-eastern mainland Australia collected during a criminal prosecution into malicious killing of a protected species (Lazzaro, 2018). Skulls rather than femurs were used as these were the only bones available from the majority of animals for which skeletal remains were archived. The same site was used for portable XRF measurement each skull, namely the midline of the frontal bone directly dorsal to the center of the orbit (Fig. 1). Skull should be reflective of primarily cortical bone, similar to the femur. All skin, feathers and dirt were removed from each skull prior to measurement. These samples were not dried. The curators of these specimens could not allow destructive sampling, hence ICP-MS analysis could not be utilized.

2.4. ICP-MS measurements

Bone samples were freeze-dried and underwent acid digestion. Briefly, this entailed samples being digested for 15 min in a Multiwave GO microwave digestion system (Anton Paar, Graz, Austria) set to 150 °C (see Pay et al. (2021) for detailed sample preparation methods). Lead concentrations were determined via ICP–MS using an iCAP Q ICP-MS (Thermo-Fisher Scientific, Omaha, USA) coupled to an ASX-520 AutoSampler (Agilent Technologies, Santa Clara, USA). Data acquisition, element quantitation, and isotope percentage analyses were carried out using Qtegra (Thermo-Fisher Scientific, Omaha, USA). Bone Ash Standard Reference Material 1400 (National Institute of Standards and Technology) was used as a positive control. Accuracy of CRM ICP–MS readings averaged 96.7%. Lead concentrations were measured as mg/kg dry weight.

2.5. Portable XRF bone lead measurement system

The portable XRF instrument used for the bone lead measurements in this study was the Niton XL3t GOLDD+ (Thermo-Fisher Scientific, Omaha, USA), as per past studies of bone lead measurement in wild birds (Specht et al., 2019b; Specht et al., 2018b). The device uses a thermoelectric cooled silicon drift detector with 25 mm^2 area and 1 mm thickness. The commercial device allowed for customization, which we used to optimize X-ray tube settings and filtration to be used for in vivo

Fig. 1. Methods used to measure bone lead concentrations via X-ray fluorescence (XRF) in bare skulls of wedge-tailed eagles (*Aquila audax audax*) from mainland Australia.

measurements. An optimized setting of 50 kV, 40 μA, and filter combination of silver and iron were selected for bone lead measurements as per previous studies (Specht et al., 2019b; Specht et al., 2018b). The device was not calibrated using lead-doped bone phantoms as per previous work (Specht et al., 2019b; Specht et al., 2018b) due to the unavailability of these products in Australia at the time, but instead the device was calibrated against the known ICP-MS measures for the Tasmanian wedge-tailed eagle femur fragments in order to determine lead concentrations in the eagle skulls. Each sample was analysed using a 3-min (180 s) read. Only one measurement was performed per sample.

The Tasmanian wedge-tailed eagle femur fragments were placed on top of the XRF with the device in its stand, as per Specht et al. (2018b), so that the samples could be laid as flat as possible against the beam aperture and detector. Due to the shape of eagle skulls, these were measured with the skulls sitting upright on a bench top and the XRF positioned above them (Fig. 1). The measurements were analysed using the standard procedure of previous studies, namely, a peak fitting through MATLAB was used to identify the net counts of the lead peak and corresponding Compton scattering peak associated with the silver X-ray tube anode. The silver Compton Scattering peak was used to normalize the geometry differences between samples of different sizes, which has been shown to be an effective process for abnormal shaped samples (Specht et al., 2018a). The femur samples with corresponding ICP-MS results were used as calibration standards to derive units of mg/kg in skull samples (Table 2).

2.6. Statistical analysis

We used regression analysis to estimate the relationship between bone lead concentrations estimated using ICP-MS and portable XRF. Both variables were square-root transformed to remove heteroscedasticity in the residuals:

$$\sqrt{ICP_i} = \beta_0 + \beta_1\left(\sqrt{XRF_i}\right) + \epsilon_i$$

where β0 is the intercept, β1 is the slope and ϵ_i is the error term, as per convention. The uncertainty values for the portable XRF measurements were determined as in previous studies (conducted using this same equipment), using counting statistics in the fitted area for lead. The uncertainty (σ) of each measurement was calculated using the equation:

$$\sigma = \frac{c \times \sqrt{\frac{BKG}{t}}}{NET}$$

where c is the concentration, BKG is the background counts as estimated by our fitting, *t* is measurement time, and Net is the net lead counts from the Gaussian function in our fitting (Zhang et al., 2021). This produced one negative XRF point estimate (−0.16, σ = −0.44) which was excluded from the regression analysis because it could not be square root transformed. The predictive ability of the regression model was assessed using 10-fold cross validation repeated three times. Models were fitted and evaluated using the caret package (Kuhn, 2020) in the R statistical environment (R Core Team, 2020).

We calculated the proportion of mainland wedge-tailed eagles displaying evidence of different categories of lead exposure. As per Pay et al. (2021), we used an exposure threshold of bone lead concentrations <10 mg/kg as indicative of low exposure (reviewed in Franson and Pain (2011)), 10–20 mg/kg as elevated, and concentrations >20 mg/kg as severe. We report proportions of specimens in each category with 95% confidence intervals.

3. Results

3.1. Validation of portable XRF for femur samples

Fig. 2 and Table 1 show the correlation between portable XRF and ICP-MS bone lead measurements ($n = 88$). Expected XRF bone lead concentration estimates were slightly higher than corresponding ICP-MS estimates. The regression model predicted a square root ICP-MS bone lead concentration estimate of 0.20 (SE = 0.06) for an XRF estimate of zero, and predicted square root ICP-MS values increased by 0.80 (SE = 0.03) mg/kg for every unit increase in the square root of

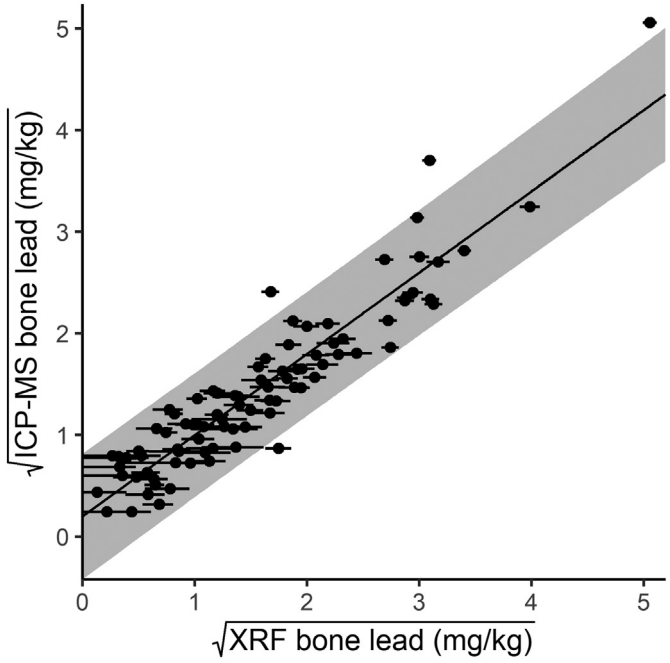

Fig. 2. Correlation between portable XRF and ICP-MS bone lead concentration measurements (mg/kg) in bare femur bone samples from Tasmanian wedge-tailed eagles (*Aquila audax fleayi*) from Tasmania, Australia. Shaded polygon represents the 95% prediction interval.

the XRF estimate ($R^2 = 0.88$, $t_{88} = 23.43$, $p < 0.001$). Hence, expected ICP-MS values were predicted using the equation:

$$ICPMS = \left(\beta_0 + \beta_1\left(\sqrt{XRF}\right)\right)^2$$

where $\beta_0 = 0.20$ and $\beta_1 = 0.80$. The root mean squared error estimated using ten-fold cross validation was 0.30.

3.2. Measurement of lead via portable XRF in skulls

Table 2 shows the bone lead measurements and uncertainties derived from portable XRF for eagle skulls from south-eastern mainland Australia ($n = 92$). These data were extrapolated to ICP-MS lead levels using the calibration regression equation calculated above. Lead levels were notably elevated when compared to the Tasmanian samples, with 60% (95%CI 50–70%) of samples showing lead >10 mg/kg when corresponding ICP-MS levels were calculated (Table 2). The proportions of birds showing severe lead exposure (>20 mg/kg) was 13% (95%CI 6–20%) (Table 2).

4. Discussion

To our knowledge, this is the first trial of portable XRF for bone lead measurement in a raptor species outside of North America and the first study to report lead exposure levels in a raptor species from eastern mainland Australia. From our results, there is excellent concordance between portable XRF and the traditional gold standard for bone lead measurement, ICP-MS. These results support the viability of monitoring bone lead levels in other Australian raptor species and taxa using portable XRF. The average uncertainty of 0.4 ± 0.2 mg/kg for portable XRF measurements demonstrate that this technique could be used to effectively quantify bone lead level for Australian raptors and distinguish highly exposed individuals from normal environmental exposures.

There were important limitations to our ability to extrapolate from our results. First, we did not use standard lead-doped bone phantoms to calibrate the portable XRF as per previous work. We tried to reduce error from this by using comparisons to known ICP-MS results to arrive at concentration data. Second, we tested lead concentrations from different types of bones in the validation study (femurs) and assessment of exposure in mainland Australian eagles (skulls). Although both of these are bones with high cortical content, they likely have different turnover values and could have different lead uptake properties when evaluated using the XRF device. However, studies in human cadavers have shown that lead concentrations measured via portable XRF vary non-significantly across different bones within individuals due to XRF only measuring the cortical shell on the surface of bones (Specht et al., 2019a). Third, the sampling methodology for the mainland Australian eagles was not random, but they were birds suspected to have been maliciously poisoned (with a carbamate, not a lead-containing substance) from a restricted geographical area (Lazzaro, 2018). Persecution of wedge-tailed eagles by sheep producers attempting to prevent livestock predation is a long-running and continuing occurrence in Australia (Olsen, 2005).

Our results were not entirely consistent with past studies of portable XRF in raptors. Since we were not able to utilize a standard calibration, the resultant concentrations are derived from the ICP-MS measures we had from femur fragments. This method may introduce higher uncertainty, since the ICP-MS and XRF methodologies were sampling from slightly different portions of the bone. Ideally, the concentration in sampled bone would be homogenous, but previous studies in humans have indicated variations in cortical bones across and within the bone site (Todd et al., 2001). This effect could influence the results, but would likely only impact them all equally, not influencing our group assessments. Notably, the calibration using the ICP-MS measures would have utilized bone core and surface levels, which would bias the comparison with other XRF measures lower, as XRF measures from the cortical shell, with levels shown to be higher in one study (Todd et al., 2001).

Regardless of bias in sample collection in wedge-tailed eagle skulls, the lead levels observed from south-eastern mainland Australia were alarmingly high, with XRF data indicating that 13% of the birds sampled had severe (>20 mg/kg) exposure. In contrast, this level of exposure was found in just 1% of Tasmanian wedge-tailed eagles (Pay et al., 2021) and none of the 11 wedge-tailed eagles sampled in south-western Australia (Lohr et al., 2020). For context, bone lead concentrations >20 mg/kg have been observed after lethal poisonings in raptors (Rodriguez-Ramos Fernandez et al., 2011; Jenni et al., 2015). Future studies could attempt to relate lead exposure to animal age in this species as age relation is likely to be indicative of the severity of toxicity, as in other raptor species (García-Fernández et al., 1997).

South-eastern mainland Australia is likely to be one of the highest risk places in Australia for exposure to lead from ammunition (Hampton et al., 2018), with year-round recreational deer hunting

Table 1
Bone lead measurements and uncertainties for ICP-MS and portable XRF measurements of 89 bare Tasmanian wedge-tailed eagle (*Aquila audax fleayi*) femurs ($n = 89$ for each measurement technique).

	Mean	Minimum	Maximum	Standard deviation	% < 10 mg/kg	% 10–20 mg/kg	% > 20 mg/kg
ICP-MS (mg/kg)	2.7	0.06	25.6	3.5	96.6	2.2	1.1
Portable XRF (mg/kg)	3.3	−0.2	25.6	4.0	96.6	2.2	1.1
Portable XRF uncertainty (mg/kg)	0.4	−0.4	0.7	0.2	NA	NA	NA

Table 2
Bone lead measurements derived from portable XRF for wedge-tailed eagle (*Aquila audax audax*) skulls and extrapolated ICP-MS lead levels from regression analysis ($n = 92$ for each category).

Sample size	Mean	SD	Minimum	Maximum	% < 10 mg/kg	% 10–20 mg/kg	% > 20 mg/kg
Portable XRF (mg/kg)	17.3	11.6	1.0	65.9	29	40	30
Extrapolated ICP-MS (mg/kg)	12.4	7.9	1.0	44.8	40	47	13

(Moloney and Hampton, 2020), culling of kangaroos (*Macropus* and *Osphranter* spp.) on agricultural land, unregulated culling of designated pest species such as European rabbits (*Oryctolagus cuniculus*), red foxes (*Vulpes vulpes*) and feral pigs (*Sus scrofa*) on private land, and sporadic aerial shooting (Parks Victoria, 2020). Deer hunting is propounded in the area from which the mainland samples were derived: an estimated 34,000–53,000 deer were harvested in the east Gippsland region in 2019 (Moloney and Hampton, 2020) part of an increasing trend (Watter et al., 2020). Aerial shooting has been increasingly used to control deer in south-eastern mainland Australia in recent years (Dickman and McDonald, 2020; Wintle et al., 2020), and may pose a particularly important source of ingestible lead fragments (Hampton et al., 2018), due to animals being shot-to-waste (meat is not harvested), routine use of repeat shooting (typically 2–4 shots per animal) and use of highly frangible bullets (Hampton et al., 2021).

Finally, the hire of a commercial portable XRF unit was comparatively inexpensive for a relatively large sample size (181) of specimens. At the time of this study, the portable XRF unit cost USD$1544 for one-week hire, at USD$9 per sample. For comparison, destructive analysis via digestion and ICP-MS for bone samples from the same species cost USD$24 per sample (J. Pay, unpublished data).

5. Conclusions

XRF analysis is a quick and inexpensive alternative to traditional methods for determining bone lead concentrations that require acid digestion. With hire of a commercial unit and a large sample size, our mean cost was < USD$10 per specimen. Portable XRF allows for generation of larger datasets that might otherwise be cost-prohibitive, while preserving sample material intact (Śliwiński et al., 2020). There was a strong correlation between portable XRF and ICP-MS measurements of lead concentration in bones of wedge-tailed eagles. This method shows great promise for generating baseline information on long-term lead exposure for species restricted to archive (e.g. museum) specimens and destructive sampling is not possible. The preliminary data generated on long-term lead exposure of eagles in one part of south-eastern mainland Australia suggest that further examination of this phenomena should be a high conservation priority.

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CRediT authorship contribution statement

Jordan O. Hampton: Conceptualization, Methodology, Validation, Data Analysis, Investigation, Data Curation, Project Administration, Funding Acquisition, Writing – Original Draft, Review and Editing. Aaron J. Specht: Methodology, Validation, Data Analysis, Writing – Review and Editing. James M. Pay: Sample Acquisition, Writing – Review and Editing. Mark A. Pokras: Conceptualization, Writing – Review and Editing. Andrew J. Bengsen: Validation, Data Analysis.

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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