Title: Snake scales record environmental metal(loid) contamination

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Abstract: Wetland snakes, as top predators, are becoming globally recognised as bioindicators of wetland contamination. Livers are the traditional test organ for contaminant exposure in organisms, but research is moving towards a preference for non-lethal tissue sampling. Snake scales can be used as an indicator of exposure, as many metals bind to the keratin. We used laser ablation with inductively coupled plasma-atomic emission spectroscopy and mass spectrometry (LA-ICP-MS) to quantify the concentrations of 19 metals and metalloids (collectively referred to 'metals' hereafter) in Western tiger snake (*Notechis scutatus occidentalis*) scales from four wetlands along an urban gradient, and compared them to concentrations measured in captive tiger snake scales. We conducted repeat measures to determine the concentration accuracy of each metal using LA-ICP-MS. Concentrations in wild Western tiger snake scales were significantly higher than in reference tiger snake scales for most metals analysed, suggesting accumulation from environmental exposure. We compared the scale concentrations to sediment concentrations of sampled wetlands, and found inter-site differences between mean concentrations of metals in scales parallel patterns recorded from sediment. Four metals (Mn, As, Se, Sb) had strong positive correlations with liver tissue contents suggesting scale concentrations can be used to infer internal concentrations. By screening for a larger suite of metals than we could using traditional digestive methods, we identified additional metals (Ti, V, Sr, Cs, Tl, Th, U) that may be accumulating to levels of concern in tiger snakes in Perth, Western Australia. This research has progressed the use of LA-ICP-MS for quantifying a suite of metals available in snake scales, and highlights the significance of using wetland snake scales as a non-lethal indicator of environmental contamination.

Summary: Laser ablation-ICP-MS can be used to accurately quantify 19 metal(loid)s in snakes scales, and can be used to monitor environmental contamination.

Keywords: LA-ICP-MS; urbanisation; bioindicator; non-lethal; pollution

Introduction:

Aquatic and wetland reptiles are becoming globally recognised as reliable indicators of environmental contamination (Campbell et al. 2005; Haskins et al. 2019; Lemaire et al. 2021; Quintela et al. 2019). Long-lived taxa such as turtles and crocodilians are particularly suitable bioindicators due to their longevity and affiliation with water (Buah-Kwofie et al. 2018; Rowe 2008; Slimani et al. 2018); however, the use of high trophic tier snakes is becoming increasingly common (Haskins et al. 2019; Hopkins et al. 2004; Lettoof et al. 2020b; Liu et al. 2019; Schwabenlander et al. 2019). Reptiles respond to contaminant exposure differently to other taxa such as birds and mammals in several ways: they can ingest and accumulate high concentrations of contaminants that would be fatal to other taxa (Hopkins et al. 2005; Weir et al. 2015); they are generally more resistant to the toxicological effects of contaminants (Chin et al. 2013; Finger et al. 2016; Mauldin et al. 2019); and their slower energy expenditure results in longer contaminant depuration times (Linder et al. 2010; Rueda et al. 2016). Consequently, reptile ecotoxicological responses may effectively reflect the more pernicious effects of chronic environmental contamination.

Livers have been the primary target test organs for ecotoxicological studies as they may retain a significant portion of the contaminants to which an animal is exposed (Frossard et al. 2019; Hinton et al. 2001), particularly lipophilic organic pesticides and metals. Testing the liver reflects a life history of exposure, yet there are some limitations: The animal (usually) has to be dead and sampled either after euthanasia or through dissection of opportunistic carcasses, which can impose limits to systematic surveying and assessment of protected species. In snakes, testing blood and scale samples for contaminants can be non-lethal alternatives to bioaccumulation assessment, although blood concentrations usually only reflect recent exposure (Burger et al. 2005; Burger et al. 2017; Hopkins et al. 2001; Lemaire et al. 2018). Several studies have shown the value of testing snake scales for contaminants. Mercury binds to keratin (Hopkins et al. 2013), while Co, Mn, Ni, Pb and Zn preferentially accumulate in the melanin of sea snake (*Emydocephalus annulatus*) scales (Goiran et al. (2017). Arsenic, Cd, Cr, Pb, Hg and Se levels in pine snake (*Pituophis melanoleucus*) scales correlate with the metal and metalloid (hereafter referred to as ‘metals’ for brevity)content in internal tissues suggesting that these metals bind to keratin and sequester in scales in abundances that are proportional to accumulation in tissue (Burger et al. 2017). Furthermore, measuring the unique isotopic and elemental signatures of snake skins can be used to differentiate the diet and source population of snakes (Natusch et al. 2017).

The most common methods for quantifying contaminant concentrations in animal tissue are inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and mass spectrometry (ICP-MS) on acid digested samples (Lettoof et al. 2020a; Quintela et al. 2019; Schwabenlander et al. 2019); however, the sample preparation and acid digestion process can be both expensive and time consuming, and often require large amounts of tissue, e.g. ~100mg or more per analyte (Jackson et al. 2003). Consequently, using these methods for resource-limited ecotoxicological studies can reduce the sample size and the suite of contaminants analysed, hindering the ability to infer strong conclusions. Laser ablation (LA), in combination with ICP-MS has the benefits of including wide elemental coverage, fine-scale limits of detection and mapping, minimal sample preparation, small analytical volume, and the ability to measure many samples in a single analytical session. It is, therefore, becoming an increasingly common method for quantifying metals in biological tissues, especially keratin (Limbeck et al. 2015). To date, LA-ICP-MS has only been used to quantify metals in reptile tissues in two studies: Jackson et al. (2003) used LA-ICP-MS to determine As, Se and Sr contamination in tail-tips of banded water snakes (*Nerodia fasciata*), and Seltzer and Berry (2005) used LA-ICP-MS to semi-quantitatively determine a suite of metal concentrations and potential uptake in desert tortoise (*Gopherus agassizii*) carapace. The progression of this analytical technique offers a novel approach to environmental monitoring; however, more rigorous testing of *in situ* LA-ICP-MS on biological tissues like keratin is needed.

In Perth, Western Australia, Western tiger snakes (*Notechis scutatus occidentalis*) persist as top-predators in a minority of wetlands located between the city centre and bordering national parks. Sediments from these wetlands and livers from resident tiger snakes have been shown to accumulate a range of contaminants, suggesting tiger snakes are useful bioindicators at these sites (Lettoof et al. 2020a). This preliminary study screened four sediment and five liver samples at each of the four wetlands for 17 metals, offering a snapshot, and highlighting differences, of contamination levels between wetlands along an urban gradient. The present study aimed to advance the application and reliability of LA-ICP-MS in measuring metals in keratin, thereby quantifying a broader suite of metals in a larger sample size of individuals. By using wetland top predator snakes as a model indicator species, this study further justifies the use of scales (or keratinous structures on other species) as a non-lethal gauge of environmental contamination. While the data presented in this study only offers a more detailed insight into Perth wetlands and tiger snakes, the techniques can be globally applied to other bioindicator species to further inform management of contaminants.

To achieve these aims, firstly 26 metals were analysed in keratin standards to determine which return reliable concentrations. Secondly, concentrations of wild-caught snakes from different sites were compared to multi-generation captive tiger snakes to identify metals that are likely accumulated from the environment; furthermore, the inter-site differences of metal concentrations in scales were compared against that of known sediment concentrations. Finally, the metal concentrations in scales were compared to the liver metal concentrations in the same individual to determine which metals in scales correlate with the liver metal burden. Results from this study offer a novel technique for environmental monitoring of metal contamination using keratinous tissues like snake scales.

Materials and methods:

Sites

This study was conducted across four Perth wetland sites: Herdsman Lake (31° 55′ 12 S, 115° 48′ 19 E), Bibra Lake (32° 5′ 32 S, 115° 49′ 27 E), Lake Joondalup (31° 45′ 34 S, 115° 47′ 33 E) and Loch McNess (31° 32′ 44 S, 115° 40′ 50 E), the latter being located within Yanchep National Park. These wetlands were once partially inter-connected (prior to urbanisation) and share similar climatic conditions, yet differ in degree of anthropogenic disturbance and contamination (Davis and Froend 1999; Lettoof et al. 2020a). Figure 1 shows the wetland sites and the 2016 land use for Perth, prepared by the Australian Collaborative Land Use and Management Program Partners (ABARES 2016). Based on current land use and historic modification (discussed in detail in Lettoof et al. (2020a)), the sites are considered most-to-least urbanised in the following order: Herdsman Lake > Bibra Lake > Lake

Joondalup > Yanchep. Despite this gradient, individual contaminant concentrations varied between wetlands and the collective concentrations of contaminants by site was Herdsman Lake > Yanchep = Bibra Lake > Lake Joondalup. Sediment samples exceeded the Australian government quality guidelines for: As, Cu, Pb and Zn at Herdsman Lake, Se at Bibra Lake, Hg at Lake Joondalup and Hg and Se at Yanchep. Currently, point sources of contamination are unknown and contaminant inputs into these wetlands are likely from diffuse sources (e.g. a complex combination of storm water run-off and drainage, historical dumping, contaminated connected groundwater and naturally high element content in sediments).

Scale sampling

Snake scales are composed of beta-keratin, which forms the hard corneous exposed part of the scale, and alpha-keratin which forms the softer, more cellular-complex inner part of the scale (Alibardi and Toni 2006; Toni et al. 2007). From the hinge region of a ventral scale, we cut approximately 10 mm length of scale and stored each sample individually in a sterile 1.5ml Eppendorf tube. To remove potential surface contamination we sonicated and rinsed each scale in ultrapure Milli-Q water for one minute, then pressed the scales flat between two clean glass slides and dried them in an oven for 48h at $40^{o}C$. During September - October 2019, we took scale clips from 30 Herdsman Lake, 28 Bibra Lake, 29 Lake Joondalup and 26 Yanchep wild Western tiger snakes. We also took scale clips from 9 captive adult tiger snakes. These snakes were multi-generation captive bred, fed laboratory-grown mice and had been housed in individual enclosures lined with clean paper. These snakes were considered ‘reference’ snakes.

Scale LA-ICPMS Analysis

For LA-ICP-MS analysis in the GeoHistory Facility, John de Laeter Centre, Curtin University, cleaned scale clips were mounted on double-sided tape on a glass microscope slide, alpha-keratin side up, with 20 scales mounted per slide. We chose to run the laser over the alpha-keratin as it has the most complex cellular structure and was therefore more likely to provide host sites for metals. In addition, the underside of the scale was also less likely to be in direct contact with sediment, although thorough cleaning prior to analysis precluded adherence of sediment which could have contributed surface contamination.

Ablations used an ASI RESOlution-SE 193 nm excimer laser controlled by GeoStar μGIS™ software. Laser fluence was calibrated above the sample cell using a hand-held energy meter, and subsequent analyses were performed in constant energy mode. The Laurin Technic S155 sample cell was flushed by ultrahigh purity He (320 mL min^{-1}) and N_2 (1.2 mL min^{-1}), both of which were passed through inline gold sand Hg traps. High purity Ar was used as the ICP-MS carrier gas (flow rate ~1 L min^{-1}). Standards and samples were ablated under the same conditions using line scans. Laser parameters were set to 50 µm beam diameter, 20 µm s^{-1} scan speed, 5 Hz laser repetition rate, and on-sample laser energy of 2 J cm^{-2}. Each sample was analysed in duplicate (two 700 µm lines), and the mean result for each isotope was used for statistical analysis.

All measurements were performed using an Agilent 8900 QQQ quadrupole ICP-MS operated in single quad mode. Each analytical session consisted of initial gas flow and ICP-MS ion lens tuning for sensitivity and robust plasma conditions ($^{238}U/^{232}Th$ ~1; $^{206}Pb/^{238}U$ ~ 0.2; and $^{238}UO/^{238}U$ <0.004). Pulse-analog (P/A) conversion factors were determined on NIST 610 reference glass by varying laser spot sizes and/or laser repetition rate to yield 1-2 Mcps per element. The primary reference material used in this study for the determination of trace element concentrations in snake scales was a pressed powder pellet of human hair CRM GBW07601a (National Institute of Metrology, China). For determination of trace element concentrations in snake scales, primary and secondary standards (human hair CRM GBW09101b, silicate glass NIST 612) were interspersed with the unknown samples in the analytical sequence in a ratio of about 1:10. ^{25}Mg, ^{27}Al, ^{29}Si, ^{31}P, ^{43}Ca, ^{45}Sc, ^{47}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{77}Se, ^{88}Sr, ^{95}Mo, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{133}Cs, ^{137}Ba, ^{201}Hg, ^{205}Tl, ^{208}Pb, ^{209}Bi, ^{232}Th, and ^{238}U were collected with a dwell time of 20 ms each during ablation after 40 s of baseline acquisition with the laser off. The time-resolved mass spectra were then reduced using the 'Trace Elements' data reduction scheme in Iolite 4.3 (Paton et al. 2011). Sulphur content was measured on a representative snake scale using a Sercon EA-IRMS at the UWA Biogeochemistry Centre (Skrzypek 2013). The resultant S = 1.8 ± 0.4 (2s) wt% was used as our internal reference for all samples.

Statistical analyses

For statistical analysis, we ran duplicate lines along the scale alpha-keratin and used the mean to represent the concentration of each metal. All data were non-normally distributed (Shapiro-Wilk test), thus we used a non-parametric Kruskal-Wallis test to determine significant differences (at $\alpha < 0.05$) between element concentrations in reference snakes and wild-caught snakes at each wetland. Furthermore, we used a Dunn post hoc test to identify pairs of sites that were significantly different, and adjusted *p* values using the Benjamini-Hochberg method. All lines below detectable limits (BDL) were given half the detection limit to facilitate statistical analysis (Zeghnoun et al. 2007).

A subset of 20 snakes had both scale clips and livers sampled. The latter were analysed for a suite of metal concentrations by acid digestion ICP-AES and ICP-MS (Table 4; Lettoof et al. 2020a). We compared the relationship between snake liver and scale concentrations for using Spearman rank correlations on log-transformed data. Mixed effects models were used to identify if snout-vent length (SVL), sex or weight (with site as a random factor) influenced reliable metal concentrations.

Results and Discussion:

Precision and accuracy of keratin analyses

In order to evaluate standard homogeneity and elemental reproducibility we investigated precision and accuracy for all certified elemental abundances in secondary standard CRM GBW09101b, measured against primary standard GBW07601a, over the course of six analytical sessions (Table 1). Compared to analyses of silicate glasses standards, reproducibility of metal abundance in GBW09101b is less-precise, with an average uncertainty of about 64%. We ascribe this to residual heterogeneity of the pressed hair powder pellets, and concentrations in the hair standards that are often close to, or at the detection limit of the method. Further grinding of the standard materials to sub-micron size might help to alleviate this problem if contamination during the grinding process can be excluded. The low S content in NIST 612, and the lack of a suitable matrix match to the unknowns, precluded the use of this material as a reliable primary or secondary standard.

Comparing the accuracy of measured concentrations in GBW09101b to the certified values led to divide the data set into three categories of elements (Table 1): a) those that yield results which overlap within error with the certified value (‘high accuracy’: Ca, Ti, V, Se, Sb); b) those that deviate from the certified value by no more than 100% (‘low accuracy’: Mn, Zn, As, Sr, Cd, Ba, Hg, Pb); and c) those that were not certified in the secondary standard, but which yielded consistent results between runs (‘indicative’: Sn, Cs, Tl, Bi, Th, U). Elements that showed large deviation from the certified value (Mg, Al, Cr, Co, Ni, Cu, and Mo) were not considered further (classified as ‘rejected’ in Table 1). Discrepancies between measured and certified data may arise from unresolved polyatomic interferences on the analyte of interest, or batch heterogeneity of either the primary or secondary hair standard. Further wet chemical analyses of finely ground and homogenized samples of the specific standards used in this study are needed to evaluate these remaining analytical uncertainties.

Choice of an internal standard

In general, the accuracy of quantitative laser ablation analysis depends on the standards used for calibration (e.g. Jochum et al. (2007)). Analytical performance is further improved by use of an internal standard (IS), whereby trace element abundances are measured as a ratio to a major element of known concentration (typically Si or Ca in silicate mineral analyses). In this way, temporal signal variations from changing ablation efficiencies and/or transport conditions effectively cancel out. For organic sample matrices such as keratin, ^{13}C (Jackson et al. 2003) or ^{34}S (Luo et al. 2017; Seltzer and Berry 2005) may serve as internal standardisation nuclides, and their concentrations can readily be quantified via combustion analysis. Whereas ICP-MS determinations of ^{13}C are afflicted with uncertainties from atmospheric CO_2 entrained in the plasma, ^{34}S may be compromised by entrained atmospheric SO_2 and/or polyatomic interferences from $^{18}O^{16}O$ and ^{33}SH. Our initial testing showed that ^{34}S provided a superior signal-to-noise ratio and signal stability, and for this reason was chosen for normalizing trace element data. Snake scales consist of layers of alpha- and beta-keratin (Klein and Gorb 2012). Both have a high content of cystine (~11% and 8% of keratin amino acids, respectively), which distinguishes keratin from other biopolymers as a high-sulphur protein (Wang et al. 2016).

Choice of a suitable standard material

Laser ablation analysis relies to a large extent on the availability of suitable, matrix-matched standards, preferably certified reference materials (CRM) such as the NIST glasses. This is especially critical for the evaluation of trace elements in organic matrices, where a plethora of polyatomic interferences from abundant H, C, N, O, and S (+Ar) may lead to erroneous quantification. Several CRMs are available for the quantification of elemental abundances in human hair, including two CRMs from China used in this study (GBW07601a, GBW09101b), one from the European Commission (ERM-DB001), two from the IAEA (IAEA-085, IAEA-086), and one from Japan (NIES CRM No. 13). Human hair essentially consists of alpha-keratin (besides other proteins, lipids and water), with a total S content of ~5 wt% (Hilterhaus-Bong and Zahn 1987), and thus represents a suitable matrix-matched standard material for the determination of trace elements in snake scales. Because CRM GBW07601a has the largest range of certified elements (see table 1), and the accuracy of the certified data has been confirmed independently (Rodushkin and Axelsson 2000), a pressed powder pellet of GBW07601a was used as the primary reference material to check analytical performance in this study. CRM GBW09101b was treated as a secondary standard throughout the study.

Data evaluation

In order to ensure analytical accuracy, several difficulties arising from ablation of an organic sample matrix need to be addressed. First, ablation efficiency is poor for organic and water-rich samples such as keratin (Vogel and Venugopalan 2003). High ablation rates in conjunction with relatively thin samples (~500 µm for terrestrial snake scales; Shine et al. (2019)) could potentially lead to laser drill-through into the adhesive substrate and glass slide holding the sample. We assessed this problem via repeated ablation tests of typical snake scales and subsequent microscopic inspection of the ablated samples. Ablation conditions that avoided drill-throughs involve scanning the laser beam over the sample at 20 µm per second, and using a low laser energy of 2 J cm^{-2}. An additional LA analysis of the adhesive tape used to fix the samples did not yield significant analytical signals above detection limit for any of the elements investigated. We noted, however, that compound snake scales tended to disintegrate if exposed to the adhesive tape solvents for days. Keratin contains a considerable amount of intercellular water (e.g., 8-22% in human nail (Barba et al. 2009)) depending on ambient humidity.

Because keratin dehydration may cause snake scales to deform and detach from the glass slides when subjected to dry Argon in the laser cell, it was helpful to dry and flatten the scales between two glass slides prior to laser analysis.

Scale surface contamination

Seltzer and Berry (2005) used LA-ICMS to determine metal concentrations in different layers of desert tortoise (*Gopherus agassizzi*) scutes. They found that cleaning the exterior of the shell was not sufficient enough to remove residual surface contamination, and including the surface in analysis may impede determining the true abundance of metals in the bulk of the scute. Similarly, Ek et al. (2004) found surface contamination of several metals in bird feathers collected in contaminated urban areas. Ultrasonic bathing was used as a cleaning technique to minimise tissue damage, as the samples were small and delicate (Zhu et al. 2017). We also attempted to minimise analysing surface contamination by running the laser over the alpha-keratin surface of the scale, which is closer to the body and rarely in contact with the sediment. We therefore believe surface contamination to be minimal in the present study, but cannot entirely exclude it.

Comparison of metal content in wild snake and reference snake scales

The concentrations of Ca, Ti, V, Se, Sn, Sb, Cs, Tl, Bi, Th and U in reference snake scales were significantly lower ($p < 0.05$) than in wild-caught snake scales from at least one site for all metals except Bi and Th (Table 2). The Mn, As, Sr, Hg, and Pb concentrations in reference snake scales was significantly lower than in wild-caught snake scales from at least one site. Scale metal concentrations were normalised by dividing the mean concentration obtained at each site by the mean concentration in reference scales. Generally, scales from Herdsman Lake snakes contained the highest metal concentration, followed by Yanchep, then Bibra Lake, then Lake Joondalup (Fig. 1).

There was no significant difference between Zn, Cd, and Th contents in the scales of reference snakes and wild snakes. This suggests that either the range of concentrations determined reflects those naturally present in tiger snake scales, or that these metals do not accumulate particularly well in snake keratin. Zinc is a bioessential element and is naturally abundant in organic tissues. In the short sea snake

(*Lapemis curtus*), the concentration of Zn was lower in scales relative to other tissues (Heydari Sereshk and Riyahi Bakhtiari 2015); however, we found higher mean Zn contents in snake scales from Herdsman Lake (which had significantly higher Zn in the sediment relative to other sites), which could suggest accumulative properties or reflect some degree of remnant surface contamination on snakes collected from this site. Wild Burmese pythons (*Python bivittatus*) skins contain higher levels of Zn compared to captive python skins (Natusch et al. 2017), which does suggest this metal can accumulate to some degree.

Cadmium was found in lower concentrations in the skin of pine snakes (*Pituophis melanoleucus*), short sea snakes (*L. curtus*) and water snakes (*N. sipedon*) relative to other tissues, which suggests it does not accumulate in high abundance in keratin (Burger et al. 2007; Burger et al. 2017; Campbell et al. 2005; Heydari Sereshk and Riyahi Bakhtiari 2015). Cadmium was below the limit of detection in many LA-ICP-MS analyses, but where abundances above the limit of detection were observed, higher mean Cd concentrations were detected in snake scales collected from sites with higher concentrations of Cd in sediments. Similarly, Hopkins et al. (2001) found the shed skins of *N. fasciata* had higher concentrations of Cd from contaminated sites compared to reference sites. This suggests Cd may not accumulate particularly well in snake scales but will to a degree if snakes are exposed to high Cd levels in the environment. Thorium was detected at significantly lower concentrations in reference snake scales compared to scales from snakes captured at Herdsman Lake (p = 0.07) and Yanchep (p = 0.09). There is no Th sediment data with which to compare our scale concentrations, nor has Th content been reported in reptile tissue in the scientific literature.

Metals in wild snake scales vs. sediment

The inter-site differences of metal concentrations in sediment and snake scales were visually compared using Fig. 2, noting that only four sediment samples were taken from each site as opposed to 26 - 30 scales. Mean sediment concentrations of each wetland were obtained from Lettoof et al. (2020a) and compared with mean scale Mn, Zn, As, Se, Cd, Sn, Sb, Ba, Hg and Pb concentrations. We chose to compare scale and sediment concentrations as scale and liver contents for most metals did not correlate, likely reflecting a difference in metal sequestration between the two biological matrices. Mn, Zn, Sb and Pb show near identical inter-site patterns between sediment and scale concentrations (i.e., higher sediment metal concentrations were associated with higher scale metal concentrations), while As, Se, Cd and Ba also reflect sediment concentrations albeit to a lesser extent (Fig. 2).

The inter-site Sn concentrations was different between snake scales and sediment, with Hg showing an inverse inter-site pattern between sediment and scales (Fig. 2). The inter-site pattern of scale Hg concentrations measured in this study, however, was very similar to the Hg concentrations in livers (Yanchep > Herdsman Lake > Lake Joondalup > Bibra Lake; liver data from Lettoof et al. 2020a). As Sn and Hg are known to accumulate in snake keratin (Burger et al. 2017; Jones and Holladay 2006; Natusch et al. 2017), our results suggests that a larger sample size of ~30 scales per site provides a more accurate picture of metals in the local environment than a limited sampling of sediment. This study demonstrates the potential application of LA-ICP-MS to screen a suite of metals in snake scales collected for biological impact monitoring, and compliments existing approaches to environmental monitoring using snakes the suite of biological parameters already used in snakes (Goiran et al. 2017; Haskins et al. 2020; Soliman et al. 2019), which are recognised as important indicators of environmental degradation and local contamination (Beaupre and Douglas 2009; Haskins et al. 2019; Stafford et al. 1977).

Scales as an indicator of internal accumulation

Scale concentrations could be compared to liver concentrations (from Lettoof et al. 2020a) for Mn, Zn, Se, Cd, Sn, Sb, Ba, Hg and Pb. Scale and liver concentrations were positively correlated for As (rho 0.46, $p = 0.04$), Se (rho 0.62, $p = 0.004$) and Sb (rho 0.61, $p = 0.004$), while Mn approached significance (rho 0.38, $p = 0.1$). These relationships suggest that these four metals sequester in tiger snake scales at a similar proportion to liver tissue, and that measuring these metals in scales should reflect internal concentrations. Similar positive correlations between snake liver and scale tissue has been reported for As, Cd, Pb, Mn, Hg and Se (Burger et al. 2005; Burger et al. 2007). The present study did not establish a clear correlation between scale and liver metal concentrations for all of these metals nevertheless, the absence of correlations does not mean scales cannot be used to indicate metal exposure and accumulation in snakes.

Most metals appear to accumulate in a lower concentration in scale tissue compared to internal organs (Burger 1992; Burger et al. 2005; Burger et al. 2017; Heydari Sereshk and Riyahi Bakhtiari 2015). This could be a product of different chemical partitioning in the tissues, as well as the depuration of metals whenever a snake sheds its skin. Corn snakes (*Elaphe guttata*) that were experimentally fed metals were, over three sheds, only able to eliminate 0.035%, 0.121%, and 0.06% respectively of the Pb, Cd and Hg they *ingested* (Jones and Holladay 2006). Although the aforementioned research did not test concentrations in the scales before and after shedding to determine the relative abundance of metal in the scales between sheds, it is apparent that shedding is not an effective metal depuration mechanism and that scales may record the 'tip of the iceberg' metal content of the individual.

Melanin could be an influencing factor when quantifying metals in snake scales. Calcium, Cu, Mg and Zn distributions are related to melanin distribution in bird feathers (Hanć et al. 2017); furthermore, Goiran et al. (2017) found evidence to suggest that Zn, Mn, Ni, Pb and Co probably bind to melanin in sea snake (*E. annulatus*) scales, resulting in increased melanism and more frequent shedding in sea snake populations found around urban-industrial areas. Western tiger snakes have a large degree of pattern and melanism variation within populations, and the potential for melanin to influence metal distributions and abundances could also account for variation in the scale metal concentrations. The posterior ventral scales of Western tiger snakes are primarily dark and therefore we believe melanin should not have influenced these results; however, an investigation into metal distribution across different coloured scales, in relation to melanin distribution, warrants further research in order to increase knowledge on the value of snake scales in environmental monitoring.

There was no significant difference in scale concentrations between sexes, nor an influence of SVL, apart from a positive relationship with V ($F_1 = 3.96$, $p = 0.049$). Some studies have found Hg concentrations in snake scales increase with SVL and thereby reflect biomagnification (Burger et al. 2017; Lemaire et al. 2018); however, other studies have found a lack of relationship between body size and level of metals in the scales (Burger et al. 2007; Campbell et al. 2005). The lack of a positive correlation between scale metal concentration and SVL (with SVL being associated to age of the snakes) observed in this work suggests that these metals are accumulating but not biomagnifying in snake scales, and that exposure to metals may fluctuate throughout the snake's lifetime. This is supported by the lack of correlation between most liver metal concentrations and SVL reported in our previous study (Lettoof et al. 2020a).

Metal abundances of environmental significance

Snake scale metal concentrations were noticeably higher at certain sites relative to the metal concentration in reference snake scales (Fig. 1). Specifically, scales from Herdsman Lake snakes showed enrichment of the following metals where the value in brackets is the enrichment factor: As (22.5 times higher than in reference snakes), Ba (13.7), Pb (8.2), U (8.3), Sr (3.5), Cd (2.0), Sb (5.1) and Th (4.9); Yanchep snakes had more Ti (6.5), As (32.6), Sn (14), Tl (8.3), Se (3.2) and Cs (5.2) relative to reference snakes, and Bibra Lake snakes had 20.2 times more V than the reference snakes. Generally, metals in Lake Joondalup snake scales were the lowest of all the sites investigated. The snake scale metal content between sites strongly reflects the cargo of metal in sediment and snake livers at these sites, with the overall level of metal enrichment in snake scales being Herdsman Lake > Yanchep > Bibra Lake > Lake Joondalup (Lettoof et al. 2020a). Many of these metals exist naturally in the sediment so the anthropogenic contribution cannot be assessed; however, it is suspected that the higher metal abundances in highly urbanised wetlands (Herdsman and Bibra Lake) is influenced by proximity to industrial and residential areas subject to storm water run-off, inflow from drainage, and historic dumping (Lettoof et al. 2020a).

Although Loch McNess is surrounded by Yanchep National Park, it is worth noting the high concentration of many metals at this site. The sediment in the wetlands of the Swan Coastal Plain are rich in iron pyrite (Prakongkep et al. 2010), which is naturally enriched by local metals (Ljung et al. 2009). The wetlands of Yanchep National Park receive most of their water from the groundwater system, yet the groundwater levels is suffering a significant decline due to excessive draining (Department of Water 2011). As a result, previously submerged sediments are now exposed in the warmer months and dry out, leading to oxidation of pyritic sediment and increased acidification of the wetland waters (Sommer and Horwitz 2009). The oxidation of pyritic sediment can release and mobilise metals which have accumulated from either natural occurrence in the sediment or from contaminated groundwater (Ljung et al. 2009). Thus, it is likely that the abundance of metals in Yanchep National Park wetland sediment and in the region's tiger snake scales may be an indirect result of anthropogenic disturbance.

Advantages, limitations and future directions for using LA-ICP-MS for keratin analysis

The traditional use of acid digestion to quantify metal concentrations in tissue is limited by the quantity of tissue required to achieve a detectable target metal concentration and the time-consuming nature of digestion procedures. This can incur a high financial cost. For example, using LA-ICP-MS to analyse 80 snake scales for 19 metals cost approximately 30% of what it would cost to analyse 20 tiger snake livers between 4-10g for 17 metals. Laser ablation-ICP-MS analysis can also be completed in a shorter timeframe. Hence, LA-ICP-MS offers a faster, inexpensive and more efficient alternative for quantifying a broader suite of metals in a very small quantity of tissue. By screening as many metals as possible, patterns of contamination can be detected that might not be targeted in traditional studies. Furthermore, a larger sample size of individual animals can be used, which is often a limiting factor in ecotoxicological research.

The progressive use of LA-ICP-MS to quantify a suite of metals in a keratinous structure for the purpose of environmental monitoring is not without limitations. Isobaric interferences resulting from the CNOHS-rich matrix precludes analysis of some metals e.g. in this study Mg, Al, Cr, Co, Ni, Cu and Mo did not return accurate results. At present, if these metals are present in a study system at toxic concentrations LA-ICP-MS cannot be used to quantify their levels in sampled tissues. Nonetheless, by employing LA-ICP-MS as an inexpensive method to quantify a suite of metals in target organism tissues, future research could more accurately map out metal contaminant dispersal amongst tissues to help determine how they move through organisms and sequester in indicator tissues (such as a reptile scale or bird feather). This knowledge, in conjunction with geochemical modelling, could create a stronger justification for using non-lethal organism tissues as indicators of environmental contamination.

Conclusions

This research successfully demonstrated and progressed the use of LA-ICP-MS for quantifying a suite of metalsin snake scales. Through repeat analysis, 19 of the 26 screened metals were accurately determined. The concentrations of most of these metals were significantly higher in wild Western tiger snake scales than in reference tiger snake scales, suggesting accumulation from environmental exposure. In addition, inter-site differences between mean concentrations of Mn, Zn, As, Se, Cd, Sn, Sb, Ba, Hg and Pb in scales reproduced the patterns recorded in the sediment collected from the same site, further supporting the hypothesis that the concentration in scales represents environmental exposure. Manganese, As, Se, Sb had strong positive correlations with liver tissue metal contents suggesting concentrations in the scale can be used to infer internal liver concentrations. By screening for a larger suite of metals than we could using traditional digestive methods, additional metals (Ti, V, Sr, Cs, Tl, Th, U) were identified that may be accumulating to levels of concern in tiger snakes in Perth, Western Australia. The novelty of these findings highlight the application of LA-ICP-MS as an inexpensive, rapid method to quantify a suite of metals in snake scales, and the further the significance of using wetland snake scales as a non-lethal indicator of environmental contamination. With further development, these methods can be applied to other keratinous structures of commonly used bioindicator species, identifying more fine-scale movements of metal contamination throughout an ecosystem.

Acknowledgments: We thank Jordan Vos, Nathan Dunstan and Luke Allen for supplying scale clips from captive tiger snakes, Jari Cornelis, Serin Subaraj and Kady Grosser for assistance with catching and scale clipping wild snakes, and Pierre Horwitz for his insight into the wetland systems of Yanchep National Park. Stewart McDonald, Jordan Vos and Eddy Cannella helped with GIS mapping. We also thank Alana de Laive for creating our graphical abstract, and the Noongar people, the traditional owners of the land on which the wild snakes were obtained to conduct this research. The quality of this manuscript was improved thanks to two anonymous reviewers. Research in the John de Laeter Centre Geohistory Facility was enabled by AuScope (auscope.org.au) and the Australian Government via the National Collaborative Research Infrastructure Strategy (NCRIS); and was partly funded by the Holsworth Wildlife Research Endowment.

Figure 1. Map of the land uses of the study area, Perth, Western Australia, based on the 2016 Australian Land Use and Management Classification (Version 8). Red dots indicate Western tiger snakes (*Notechis scutatus occidentalis*) sampled for scale analysis. YC = Loch McNess in Yanchep National Park, JL = Lake Joondalup, HL = Herdsman Lake and BL = Bibra Lake.

Figure 2. A comparison of mean inter-site metal concentrations (ppm) in Western tiger snake (*Notechis scutatus occidentalis*) scales normalised to mean concentrations in reference tiger snake scales. Grey dotted line = normalised reference concentration.

Figure 3. A comparison of inter-site metal concentrations (ppm) in Western tiger snake (*Notechis scutatus occidentalis*) scales determined by LA-ICP-MS (this study) and sediments determined by acid digestion ICP-MS (Lettoof et al. 2020a). Left *y* axis represents mean sediment metal concentration and right *y* axis represents mean scale metal concentration, except for Hg which shares the same range. Sample sizes for sediments are four per site, and for scales are 30 for Herdsman Lake, 28 for Bibra Lake, 29 for Lake Joondalup, and 26 for Yanchep. Brown solid bars = sediment; yellow patterned bars = scales. Error bars = SE.

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