Faculty of Science and Engineering

School of Molecular and Life Sciences

Tiger snakes, *Notechis scutatus*, as a Bioindicator of Wetland Health across the urban matrix of Perth, Western Australia

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of

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). Chapters 3-7 received animal ethics approval from the Curtin University Animal Ethics Committee, Approval number ARE2018-23 and the Western Australian Department of Parks and Wildlife, Approval numbers 08-002624-1.

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Signature:

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Statement of Contribution by Others

Chapters 2-7 have been prepared as manuscripts for peer-reviewed publication in the scientific literature. These chapters are reproductions of submitted and published manuscripts, reformatted to be consistent with the thesis. Signed author statements can be found in Appendix 1. I have obtained permission from the copyright owners to use any third-party copyright material reproduced in this thesis, and to use any of my own published work in which the copyright is held by another party.

The study presented in Chapter 2 was accepted in the peer-reviewed journal '*International Journal of Parasitology: Parasites and Wildlife*' on 28 November 2019.

Lettoof, D. C., von Takach, B., Bateman, P. W., Gagnon, M. M., Aubret, F. (2020). Investigating the role of urbanisation, wetlands and climatic conditions in nematode parasitism in a large Australian elapid snake. *Int J Parasitol Parasites Wildl*. 11:32-9. doi: 10.1016/j.ijppaw.2019.11.006.

DCL, BvT and FA conceived the idea and designed the methodology. I collected the data; I analysed the data with the guidance from BvT and FA; I wrote the manuscript and all authors contributed to the revisions of the manuscript.

The study presented in Chapter 3 was accepted in the peer-reviewed journal '*Archives of Environmental Contamination and Toxicology*' on 17 February 2020.

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DCL, and MMG conceived the idea and designed the methodology. I collected the data; I analysed the data; I wrote the manuscript and all authors contributed to the revisions of the manuscript.

The study presented in Chapter 4 was accepted in the peer-reviewed journal '*Journal of Wildlife Diseases*' on 14 October 2020.

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All authors conceived the idea and designed the methodology. I collected the data; I analysed the data with guidance from FS; I wrote the manuscript and all authors contributed to the revisions of the manuscript.

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DCL, KR, NE and MMG conceived the idea and designed the methodology. DCL and KR collected the data; I analysed the data; I wrote the manuscript and all authors contributed to the revisions of the manuscript.

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Lettoof, D. C., Thomson, V. A., Cornelis, J., Aubret, F., Bateman, P. W., Gagnon, M. M., von Takach, B. (2021). Top predator snake shows genomic signatures of natural and anthropogenic barriers to gene flow. *PLoS One*, 16: e0259124. doi: 10.1371/journal.pone.0259124.

DCL and BvT conceived the idea and designed the methodology. DCL, VAT, JC and FA collected the data; I analysed the data with guidance of BvT; I wrote the manuscript and all authors contributed to the revisions of the manuscript.

The study presented in Chapter 7 was accepted in the peer-reviewed journal '*Environmental Pollution*' on 10 December 2021.

Lettoof, D. C., Cornelis, J., Jolly, C., Aubret, F., Gagnon, M. M., Hyndman, T., Barton, D., Bateman, P. W. (2021). Metal(loid) pollution, not urbanisation nor parasites predicts low body condition in a wetland bioindicator snake. *Environ Pollut*, 118674. doi: 10.1016/j.envpol.2021.118674.

I conceived the idea and designed the methodology. DCL, JC and TH collected the data; I analysed the data with guidance from CJ; I wrote the manuscript and all authors contributed to the revisions of the manuscript.

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Dedicated to the 67 tiger snakes
who lost their lives for this research,
may your sacrifice better the conservation
of your species and ecosystem.

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Acknowledgement of Country

We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

I would specifically like to acknowledge and thank the Whadjuk, Yued and Gnaala Karla Booja people, the First Nations peoples whose land my research was conducted on.

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Writing this thesis acknowledgements really allowed me to stop looking ever-forward and reflect on the last three and half years. It's eye-opening to realise how many people contributed both directly and indirectly to my research and PhD journey.

I'll start with my three supervisors. From the beginning, the focus of my PhD rapidly changed to ecotoxicology (of which I knew nothing). This inevitably led to the brunt of supervision coming from the ecotoxicologist on the team. I am forever grateful to Monique Gagnon for her enthusiasm and generous support of my project – both intellectually and financially. I would not have been able to achieve this without you, thank you for introducing me to ecotoxicology, a research field that allowed me to take my pre-existing knowledge of snake ecology and challenge myself with the steep learning curve of chemistry, biochemistry and reptile physiology, ultimately stimulating my growth as a scientist. For this input I'll always consider you my academic mother.

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Next, and of equal value, I would like to thank the office administration and technical staff at Curtin University: Carey Ryken-Rapp, Sophia Clark-Ioannou, Liliana Rejon Torres, Lydia Kupsky and Peta Beach. At any notice this team would go above and beyond to help me navigate the university bureaucracy and get the equipment I needed. It would have been a nightmare to try and manage all of that myself! In addition, I'm really grateful for the Curtin media team for helping put a voice to my research, and for generating a surprising amount of interest in a topic that is usually met with disdain.

Matt Greenlees and Rick Shine – my former supervisors – deserve recognition and thanks; for taking me as a cheeky little Masters student, inspiring me, and teaching me the ins-and-outs of science so I could hit the ground running when I started this PhD.

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Whenever one plans a PhD, they usually develop a bunch of side-projects they never have time to do. I was extremely lucky that Jari Cornelis wanted to do a Masters on snakes and had the field skills to do it properly; not only answering the questions I had no time to, but taking the project well beyond what I could have achieved myself. Not only was he a great 'first student' of mine, but he contributed months of his time to assisting my field work and datasets in this thesis. He also taught me how to cook fancy. Thanks for taking on a significant portion of the responsibility, and legend, of #TeamTiger!

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I was lucky to conduct this PhD with a substantial amount of prior knowledge on snake ecology and fieldwork, which was obtained from a decade of volunteering, working intermittently and living off minimal income. My mother, Leesa, endlessly encouraged, supported and enabled this lifestyle. At the time I was oblivious to how valuable this was, but now I realise I would not have achieved this PhD without her. Moreover, throughout the PhD she has provided incredible emotional and financial support from the other side of the country. Although you may not understand a lot of content in this thesis, I owe it all to you. I'm forever grateful for you, thanks Mumsy.

All research costs money, and ecological research is usually bottom of the barrel for funding support. I commend and thank the Ecological Society of Australia, and the Holsworth Wildlife Research Endowment for generously funding ecology PhD projects – including my own research on one of Australia's most vilified residents.

In conclusion, I would like to emphasize how much I have enjoyed this last three and a half years. It began with me driving across the country with everything I owned to start a new life in Perth. At this point in time all I wanted to do was a PhD studying snakes, preferably large and venomous. I knew the difficulties of working with snakes, the risks working on a system with next-to-no baseline data, and the harsh trials of learning experimental design and statistically analysis. Despite not being naturally 'academic', I had a substantial support – and collaborative – network so I could hit the ground running. And when I approached an obstacle I knew exactly who to ask, and who I could rely on so I could charge on through. This network, in conjunction with motivation and passion, was critical to my positive experience.

So for those who aren't naturally 'academic' I offer my formula for a happy, successful PhD:

- Become emotionally invested in your project. Everyone always says "do a project you want to" and I can't stress this enough! There will inevitably be times where everything feels completely cooked; and you will almost always do a chapter/project that was a supervisor's idea that you don't really care about it. Find an aspect of it to care about, and make it your project.
- Maintain healthy work boundaries; treat the PhD like a full-time job. Go to your office five days a week, work 9-to-5, and take holidays. Sometimes you have to spend long times in the field or the lab to get the job done, do these and then take a break after. Do not let the work consume you.
- No one knows how to do everything, so don't be afraid to ask for help. And if you aren't satisfied with the answers or efforts of your immediate research team then go further afield. Someone will always have the answers and be happy to contribute!

And finally, to any reader who is considering doing a PhD: I hated homework from about the age of five, I barely committed to studying throughout high school, or during my bachelor degree, and was so-so during my Masters. My grades, thus my measure of 'academic potential' was comparable to a brown tree snake (*Boiga irregularis*). This was largely due to being too enthralled with the superficial highs of living life. What this PhD, and my support network, has taught me is that no matter how you lived your past, you always have the power to change your future. So I'll end with the wise words of Christopher Wallace, a.k.a The Notorious B.I.G. "Damn right I like the life I live, coz I went from negative to positive".

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“So what are you boys doing with them, baggin’ and taggin’?”

- Old love, Herdsman Lake

General Abstract

Urbanisation is the anthropogenic transformation of natural ecosystems via the growth of cities. Wetlands are particularly sensitive to urbanisation which can change hydrology, water chemistry and habitat structure, as well as introducing a suite of environmental contaminants. Measuring the health and function of wetlands often requires substantial resources and multifaceted data; however, the monitoring of bioindicator species – organisms that exhibits a response to an environmental stressor – is a practical alternative. Top predators are susceptible to bioaccumulating toxicants that move throughout the food web, and a toxicant-induced change in predator health can determine the severity of pollution in the ecosystem. The life-history traits and bioaccumulation susceptibility supports the use of top predator snakes as bioindicators of wetland health.

In this thesis I investigated how the health (e.g. parasitism, body condition, injuries) of a wetland top predator, the Western tiger snake (*Notechis scutatus occidentalis*), differs across wetlands in Perth, Western Australia, that vary in degree of urbanisation and environmental contamination. I used museum specimens to explore spatial and temporal patterns of gastric nematode parasitism, with a focus on urbanisation. I then conducted a broad-scale screening of contaminants in wetland sediments, and wild snake livers and scales. To identify geographic isolation, heterozygosity-fitness correlations and adaptive potential in populations around Perth, I assessed the genomic population structure. Finally, I compared plasma biochemical profiles and eight parameters of health in wild snake populations and modelled their associations between contemporary urbanisation and contamination.

Wetland sediments, and snake populations therein, were contaminated with varying levels of metal(loid)s, but not organochlorine pesticides nor polycyclic aromatic hydrocarbons. Surprisingly, the most natural wetland was the second most contaminated, thus wetland contamination was independent of surrounding urbanisation. Snake populations exhibit higher signals of inbreeding and genetic drift in wetlands isolated by urbanisation, but genomic diversity did not correlate with body condition. The average liver pollution index of the population, however, showed strong negative associations with population body condition. Patterns of parasitism were complex, but abundance and intensity was usually lower in more urbanised

wetland populations, and higher in snakes with better body condition. Other health parameters differed among populations and are likely associated with an unmeasured site effect. Interestingly, no health parameters were strongly associated with landscape urbanisation. Urban wetlands provide refuge 'islands' of habitat for many species; however, these ecosystems can be polluted with a cocktail of toxicants. Tiger snakes – a wetland top predator – persist in some urban wetlands, were they are exposed to, and bioaccumulate, a suite of contaminants. I found populations of tiger snakes demonstrated a negative association between population body condition and population metal(loid) pollution, which may be a precursor indication of reduced survival and population decline/extirpation. Tiger snakes are an excellent bioindicator of wetland pollution, and their health indices may be appropriate measures of assessing wetland health. Future urban wetland management strategies (especially in Australia) should include routine environmental contamination monitoring, and incorporate the use of top predator reptile species as bioindicators of wetland health and function.

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Glossary

Bioaccumulation: the gradual accumulation of substances in an organism, occurring when the organism is exposed to the substance at a faster rate than it can catabolise or expel.

Contaminant: a biological, chemical, physical or radiological substance that is either introduced, or exists naturally at a higher than 'normal' concentration but may not cause harmful or adverse effects in organisms. E.g. zinc (Zn) and other trace elements, low concentrations of pesticides.

Pollutant/pollution: An introduced contaminant that causes harmful or adverse effects. E.g. lead (Pb) waste, artificial light and noise, pesticides.

Toxicant: a substance – introduced or naturally existing – that causes toxicity in organisms. E.g. pesticides, mercury (Hg).

Xenobiotic: a substance (typically a synthetic chemical) that is foreign to an organism's body. E.g. accumulated heavy metals or pesticides in an organism.

Topographic wetness index (TWI): a measure of landscape 'wetness' calculated from a combination of upslope water supply, slope gradient, flow width and total area of a GIS layer cell.

Chapter 1. General introduction

1.1 Introduction

Urbanisation is the expansion of urban infrastructure into natural landscapes, in conjunction with increasing human population growth (Moll et al. 2019). Consequently, urbanisation introduces a myriad of novel conditions to the ecosystem such as structural habitat (MacGregor-Fors and Schondube 2011; Newbery and Jones 2007), climate (Camilloni and Barros 1997; Zhang et al. 2014), dynamic resources (Santana and Armstrong 2017; Widdows and Downs 2015), chemical pollution (Müller et al. 2020; Serieys et al. 2018), constant anthropogenic disturbance (Doherty et al. 2021) and ultimately reduces species diversity. Patches of, often degraded, remnant natural habitat can persist as 'islands' in a sea of urbanisation (Bryant et al. 2017), and provide refuge for isolated populations of many species (Fusco et al. 2021; Ives et al. 2016; Soanes and Lentini 2019).

Wetlands, generally located at the lowest point in the landscape and collecting the natural flow of water, are common 'island' urban ecosystems. Wetlands perform critical environmental functions such as the recycling of nutrients, the storage and supply of water, and the production of living matter (Novitski et al., 1996); yet they are particularly sensitive ecosystems, which have suffered from anthropogenic degradation and destruction (>50% global surface loss (Faulkner 2004; Sievers et al. 2018)). Urbanisation specifically impacts wetlands by disturbing and altering hydrology (White and Greer 2006), introducing nutrient enrichment, pollution and salinization (Adams et al. 2020; Davis and Froend 1999; Van Meter et al. 2011) and homogenising vegetation/habitat structure (Lougheed et al. 2008), all of which can disrupt biodiversity and food-webs (Eagles-Smith et al. 2018; McKinney 2008). Measuring and assessing the health and integrity of these complex ecosystems is, therefore, often difficult and resource intensive.

To circumvent these problems, the use of indicator species has become increasingly common (Burger 2006). An ecological indicator is defined as an organism that reacts to changes occurring in a landscape (Paoletti and Sommaggio 1996). A good indicator species is easily detectable, exhibits a change in response to an environmental stressor, but is not so sensitive that minor or biologically unimportant alterations stimulate a

change (Burger et al. 2007; O'Connor and Dewling 1986). These changes should be easily measurable, clearly attributable to a specific stressor, and can provide insight on the impairment of other populations and species within the same ecosystem (Linthurst et al. 1995).

Plants and invertebrates are predominantly used as indicators. A review by Siddig et al. (2016) found 31% of more than 1900 papers used vertebrates to measure the trophic effects of environmental health. Of this 31% there was a noticeable bias among the vertebrate taxa, which was dominated by fish (51%), birds (32%), mammals (13%) and finally herpetofauna (4%). Of the herpetofauna studies, anurans have received most of the attention – owing to their trophic link, aquatic habits and well-known sensitivity to environmental change (Simon et al. 2011) – followed by turtles and crocodilians (Hopkins et al. 2001; Manolis et al. 2002), and the least of all lizards and snakes.

Snakes, however, demonstrate great potential for use as a bioindicator (Bauerle et al. 1975; Haskins et al. 2021a; Stafford et al. 1977). As a taxon, snakes can fill most trophic tiers in an ecosystem, and the larger growing species fill the roles of secondary, tertiary or top predators (Campbell and Campbell 2001; Shine 1995). For example, snakes start life very small (4 – 30cm) and usually suffer high predation from birds, predatory mammals, large fish and frogs, and even invertebrate predators (Greene 1997). At small sizes, snakes are limited by the prey items they can consume – typically small lizards, fish and frogs – and represent a low trophic level in the ecosystem. As surviving individuals grow, they consume larger prey items (Cipriani et al. 2017; Hampton 2018) and are less vulnerable to predation (Mushinsky and Miller 1993), consequently climbing the trophic ranks until larger species can climax at a top tier. As with most top predators, reaching this trophic highpoint comes with a cost – a lifelong exposure to parasites and bioaccumulation of xenobiotics. Wetland snakes, in particular, have potential to be more accurate bioindicators than wetland birds as they are more sedentary, and likely express high site fidelity (Campbell and Campbell 2001).

Up to 23 organic and 11 inorganic (heavy metal) contaminants have been detected in snake tissues (Campbell and Campbell 2001), including the retention of DDE in snake tissue nearly a decade after the pesticide use was banned (Ford and Hill 1991). Of

these, research has rarely quantified the impacts of these contaminants on snake health. Notable studies have identified mercury (Hg) can reduce motivation to feed and strike efficiently (Chin et al. 2013), and suppress immune-responses (Haskins et al. 2021b). Biota rarely have the luxury of single-contaminant exposure, especially in urban ecosystems. Instead they are often exposed to a cocktail of chronic environmental contaminants, which may have additive or synergistic effects on host toxicity (Singh et al. 2017) and warrant *in situ* ecotoxicological studies that consider a broad range of contaminants. In snakes, a suite of metal(loid)s was shown to alter the concentration and activity of multiple blood biochemical analytes (Gavric et al. 2015), and increase the standard metabolic rate (Hopkins et al. 1999). Besides these few studies, knowledge on the influence of contaminants – as a by-product of urbanisation – on snake health is deficient, and non-existent in Australia (Death et al. 2019).

A subtle consequence of urbanisation and environmental contamination is a change in parasite diversity and abundance. Such changes can be attributed to habitat fragmentation and modification increasing host density or disrupting complex parasite lifecycles (Koprivnikar and Redfern 2012; Mbora and McPeek 2009; Puttker et al. 2008), and/or contaminant-induced immunosuppression in host species (Haskins et al. 2021b; Linzey et al. 2003). As natural parasitic infections are generally benign (Mayer et al. 2015) – due to a long co-evolutionary history with hosts – high parasitism can often indicate a healthy host *and* ecosystem (Hudson et al. 2006; Sanchez et al. 2018). Therefore, considering changes in parasitism and quantifying their influence on host health is important when assessing the impacts of urbanisation and contaminants.

1.2 Thesis overview

This thesis aims to assess how the health of Western tiger snakes (*Notechis scutatus occidentalis*) changes throughout wetlands in an urban matrix, and what measures of health reflect the condition of their wetland (Fig 1.1). I begin this thesis (Chapter 2) by conducting a landscape assessment of gastric nematodes in tiger snakes, and model how parasitism responds to urbanisation and climatic factors. In Chapter 3, I screen my study sites and tiger snakes for 52 contaminants to document the qualitative and quantitative extent of contamination and explore the accumulation of xenobiotics in the snakes, as well as conduct a mini-review of contamination history and potential point-sources. In Chapter 4, I compare the plasma biochemical profiles of tiger snakes

from a natural wetland and a highly-urbanised wetland, and again after snakes had been kept in stress-reduced captive conditions for six months. In Chapter 5, I implement a novel method of elemental analysis on snake scales (laser-ablation with inductively coupled plasma-atomic emission spectroscopy and mass spectrometry) to further identify environmental metal(loid) exposure in tiger snakes using non-lethal sampling. In Chapter 6, I explore the genomic structure of tiger snake populations throughout Perth, and assess the relationship between population isolation and fitness. And finally, in Chapter 7, I model and determine how urbanisation and pollution impact a suite of tiger snake health parameters across my study sites, and justify their use as a bioindicator of wetland health.

This thesis has five primary research aims:

1. Explore the prevalence of a potentially pathogenic helminth in tiger snakes across space and time, with focus on urbanisation and climate;
2. Identify the contaminants of concern and their abundance in wetlands across an urban matrix, then identify which contaminants are bioaccumulating in tiger snake populations, and assess the use of tiger snakes as bioindicators of their ecosystem;
3. Investigate the plasma biochemistry profiles of tiger snake populations at polar ends of the urban matrix, and how plasma profiles change after a period of stress-reduction;
4. Explore the genomic structure of tiger snake populations across an urban matrix, and assess the relationship between genetic diversity and snake fitness; and
5. Determine how urbanisation and contaminant pollution impact the health of tiger snakes.

Figure 1.1 Conceptual flow diagram of thesis research aims and output. All chapters and appendices are published manuscripts.

1.3 Study area and species

This research was conducted around the city of Perth, Western Australia (31°56' S, 115°51' E). Perth is built on the Swan Coastal Plain bioregion, an ecosystem characterised by sand dunes interlaced with groundwater-connected wetlands, *Banksia* or Tuart woodlands and coastal heath (Mitchell et al. 2002). Perth began as a small colonial town in 1829 and has now become one of the most sprawling cities in the world (Kelobonye et al. 2019). As a result of urbanisation, the Swan Coastal Plain and Perth has lost an estimated 70% of wetlands (Halse 1989), and most remnant wetlands have suffered some degree of degradation (Davis and Froend 1999). For the majority of this thesis I focus on four wetlands: Herdsman Lake, Bibra Lake, Lake Joondalup and Loch McNess, the latter within Yanchep National Park (see Chapter 3.3.1 & 3.4.1 and Fig 3.1 & 5.1 for more details). Wetland sites were selected based on their difference in historical and contemporary urbanisation, but ultimately the on presence and abundance of tiger snakes.

The tiger snake, *Notechis scutatus*, is a large (~ 90 – 120cm SVL), dangerously venomous Australian elapid (Aubret 2015; Fearn et al. 2012). They are commonly found in wetlands, wet forests and coastal heaths in the cooler, wetter parts of Australia (Wilson and Swan 2017); however, many isolated populations exist on off-shore islands, where they live amongst atypical habitat and prey species which has resulted in remarkable demonstrations of morphological change and adaptive plasticity (Aubret 2012). The polymorphism, geographical range and discontinuity of tiger snake populations has led to several taxonomic classifications – two species *N. scutatus* and *N. ater*, and six subspecies (Rawlinson 1991) – but assessment of mtDNA found minimal divergence across their range which suggests that all tiger snakes comprise a single polymorphic species with the maximum genetic divergence existing between the Western and Eastern clades (Keogh et al. 2005). Considering the ecological flexibly of tiger snake populations, the geographic isolation of Western and Eastern populations, and emerging criticisms of the use of mtDNA for phylogenetic assessment (Balloux 2010; Rubinoff and Holland 2005), I consider my study species the Western tiger snake, *Notechis scutatus occidentalis*. Mainland Western tiger snakes are predominantly anurophagous (frog-eaters), but occasionally eat small birds, rodents, and lizards (Aubret et al. 2004; Shine 1987). I have also recorded predation

of a young Quenda bandicoot, *Isoodon fusciventer* (Lettoof et al. 2020), and hatchling oblong turtle, *Chelodina oblonga* (Lettoof et al. 2021).

1.4 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Chapter 2. Investigating the role of urbanisation, wetlands and climatic conditions in nematode parasitism in a large Australian elapid snake

The study presented in Chapter 2 was accepted in the peer-reviewed journal '*International Journal of Parasitology: Parasites and Wildlife*' on 28 November 2019, and is an exact reproduction of the copyright paper reformatted for this thesis.

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2.1 Abstract

Tiger snakes (Notechis scutatus) in wetlands of South-West Western Australia (SW WA) are commonly parasitised by the nematode *Ophidascaris pyrrhus*. Host-parasite interactions are complex and can potentially be impacted by factors such as urbanisation or climate. We assessed whether urbanisation, distance to wetland sites, and climatic factors have influenced parasitism in tiger snakes from specimens collected over the last century. We dissected 91 museum specimens of tiger snakes across SW WA and counted gastrointestinal nematodes. Binomial generalised linear modelling, with presence/absence of nematodes as a response variable, was used to determine which factors were driving infection. Model selection using AICc values showed that proximity to wetlands, rainfall and topographic wetness were most strongly associated with the probability of infection of snakes by nematodes. We also found a slight positive correlation between nematode abundance and annual mean maximum temperature. We found no significant influence of distance to urban centre on nematode burdens; however, our results suggest that water-related variables are a key driver of nematode parasitism in tiger snakes in SW WA. We also suggest that urbanisation is still of interest as its role in wetland and climate modification may increase parasitism in wetland snakes.

2.2 Introduction

The modification and degradation of ecosystems through urbanisation has been well-documented for decades (Faulkner 2004; Mcdonnell and Pickett 1990; Santiago-Alarcon et al. 2018). Such modification has resulted in a plethora of impacts on wildlife; however, the first and most detectable change is often a reduction in species richness with some taxa unable to persist in urban areas, while others seem able to adapt or benefit (Bateman and Fleming 2012; McKinney 2008). Further research has included evaluation of the health of these taxa that persist in urban areas—urban utilizers/dwellers (Fischer et al. 2015) or urban adapters *sensu* McKinney (2008)—as a reflection, and thus indicator, of ecosystem health (Carignan and Villard 2002; Siddig et al. 2016; van der Oost et al. 2003). One such measure of organism health is assessing the parasite abundance of target organisms, a typically sub-lethal impact that may have pernicious effects on individual fitness and thus long-term population persistence (Bower et al. 2019; Davis et al. 2012; Sanchez et al. 2018).

Urbanisation can either increase, decrease or have no detectable impact on host parasite infection (Ancillotto et al. 2018; Giraudeau et al. 2014). An increased level of parasite infection in urban areas has commonly been attributed to a reduced host immunocompetence from external stressors; for example, increased stress levels and/or host exposure to contaminants can result in a suppressed immune system in anurans (Linzey et al. 2003; Rohr et al. 2008) and reptiles (Day 2003). The fragmentation of habitat through urbanisation can result in an increase of host density and in contact rates, thus facilitating horizontal transmission for parasites (Puttker et al. 2008); however, urban habitat fragmentation can also disrupt a complex parasite life cycle or transmission frequency between hosts resulting in decreased parasite abundance (Barbosa et al. 2005; Dugarov et al. 2018; Resasco et al. 2019; Santiago-Alarcon et al. 2018). Assessing the change of parasite abundance in top predators, such as larger snake species, can be an important indicator of the impact of urbanisation on ecosystems (Davis et al. 2012).

The tiger snake (*Notechis scutatus*) is a large (mean 1 m snout-vent length [SVL] on the mainland) polymorphic Australian elapid (Aubret 2012; Shine 1995). It occurs in disjunct populations across most of the southern parts of the country, and varies in both diet and habitat across its range (Aubret et al. 2004; Aubret et al. 2006). In

mainland South-West Western Australia (SW WA) it is abundant in wetlands and, despite the rapid expanse of urbanisation degrading and heavily modifying wetlands in SW WA (particularly Perth and its surrounds) (Davis and Froend 1999), the tiger snake has remained an abundant top reptile predator in many urban wetlands (Aubret 2005). In mainland SW WA, the tiger snake is primarily anurophagous (Aubret et al. 2006; Shine 1987a), and like most other frog-eating snakes it has been recorded as having high abundance of parasitic worms (Fantham and Porter 1954; Mayer et al. 2015; Yildirimhan et al. 2007).

The tiger snake is commonly infected with the ascaridoid nematode *Ophidascaris pyrrhus*, the life history of which is unknown (Jones 1980; Watharow 1997). Other species of *Ophidascaris* and ascaridoid nematodes often develop indirectly in reptiles, amphibians or mammals (Ash and Beaver 1962; Sprent 1970). Larvae of the first and third stage infect intermediate host's (reptile, amphibian or mammal) via egg consumption and encyst the lungs, muscles and liver. If this host is then consumed by a snake the adult nematodes parasitise the posterior stomach wall (Sprent 1955). Adult female nematodes lay eggs in the digestive tract of the snake from where they pass into the environment with faeces. Eggs are then consumed by an invertebrate or vertebrate host (Anderson 1988; Sprent 1954). As nematode infection is common in tiger snakes (Jones 1980), and frogs are known to host a variety of nematode species larvae (Kelehear and Jones 2010; Lettoof et al. 2013; Mayer et al. 2015), it is likely that *O. pyrrhus* uses frogs as an intermediate host (Jones 1980). Adult *Ophidascaris* feed on the host's digested prey (Elbihari and Hussein 1973; Jones 1980; Sprent 1988). Most individual nematodes thread the middle of their bodies into the stomach wall creating deep lesions of calcified material and necrotic debris (DCL pers. obs.). Although the impact of these lesions on the host snake's health is unknown, the intensity of nematode infection is more concerning. A large burden of gastric nematodes can cause malnutrition from substantial loss of nutrients to the parasites (Hlaing et al. 1991), or intestinal blockages (de Silva et al. 1997). Observations of wild tiger snakes in poor body condition frequently identify a high intensity of *O. pyrrhus* infection (DCL and FA pers. obs.).

Most natural wetlands of SW WA are ephemeral, with water levels peaking in spring (October) and at their lowest in autumn (April) (Davis and Froend 1999). However, the seasonal filling of wetlands is under pressure from regional climate shift and

urbanisation. Since the 1960s, remnant wetlands have received less water as mean annual rainfall has decreased by 100-150 mm and mean annual temperature has increased by about 1^{o}C (Bureau of Meteorology 2019). Even so, water levels in several urban wetlands have risen due to increased surface run off, storm water drainage networks and elevated groundwater levels (Clarke et al. 1990; Lund 1992). Urban altered wetlands that are now isolated and permanent may influence nematode abundance in several ways: (a) nematode eggs persist longer in water and thus increase in prevalence, abundance and transmission year round (Marcogliese 2008); (b) reduced and isolated habitat for both frogs and snakes increases host densities and thus parasite exposure and transmission frequency (Mugabo et al. 2015); and (c) host frogs and snakes are potentially immunocompromised by contaminants in urban wetlands (Linzey et al. 2003; Martin et al. 2010).

By inspecting the stomach contents of tiger snake museum specimens collected across SW WA we aimed to investigate the impact of urbanisation on nematode burdens over 100 years of collection and across the entire range of SW WA tiger snakes. Specifically, we aimed to identify what factors might influence parasitism in tiger snakes including proximity to urban centres and wetlands, and climatic variables. Considering the influence urbanisation can have on parasitism we predicted that parasite prevalence and abundance in tiger snakes would be higher closer to urban centres and wetlands, and in areas with a wetter, warmer climate. The results of this study provide insight into the influence of urbanisation and climate on host-parasite interactions of snakes, and highlight the importance of museum specimens in assessing spatial and temporal changes in urban ecology.

2.3 Methods

2.3.1 Snake morphology, diet and nematode parasitism

We examined the tiger snake records from the Western Australian Museum (WAM) to determine when and where specimens were collected across SW WA (specimens were collected between 1917 and 2018). From this we excluded specimens collected from offshore islands as the island ecology and diet of these tiger snakes differs from mainland wetland conspecifics (Aubret et al. 2006). Where possible, we attempted to examine similar numbers of specimens from urban and non-urbanised locations, as well as across collection dates. If multiple snakes were collected from the same

location on similar dates (indicating a collection survey), two were randomly selected for examination.

Specimens were partially dissected to allow inspection of the stomach. Since sampling involved destructive modification of the specimens, we were limited by museum policy in the number of specimens we could sample (n = 91). Of the 91 dissected tiger snakes, we obtained the SVL, wet mass (after draining of excess preservative liquid), location and year of collection. A number of specimens (n = 27) had no year of collection attached, so we estimated year of collection based on the registration identification number relative to other registered reptile specimens. Upon dissection all nematodes and prey items (identified to lowest possible taxonomic level) were carefully removed, counted and stored in separate collection jars. Nematodes were identified as *O. pyrrhus* (Sprent 1988). Thirty five tiger snake specimens (including 11 juveniles) could not be sexed with confidence. Four specimens consisted solely of viscera and the original body could not be located; however, these could still be dissected for nematode counts and prey identification.

2.3.2 Classifying urbanisation

Classifying 'urbanisation' is ambiguous and is often based on human densities (Madsen et al. 2010). Our research is based on the hypothesis that environmental degradation from urbanisation would be driving parasite prevalence and abundance, hence our measure of urbanisation is calculated from degree of urban infrastructure. We could not accurately classify the historical outlines of the urban centres, as detailed records and aerial photographs are not available for all locations prior to the 1960s. We also decided not to classify areas based on government census human density data, as Local Government Areas (LGAs) in SW WA have historically been quite large and the urban settlements occupied a small portion of each LGA. Thus, human densities calculated over this large area do not accurately represent if the snake was collected from an 'urban' area. We therefore calculated a distance to urban centre (DUC) for each specimen.

2.3.3 Spatial analysis and climate data

The location of each specimen was determined using the GPS coordinates provided by WAM records, and plotted using QGIS (QGIS 2018). Each specimen was colour-coded to indicate nematode abundance (total number of nematodes; Fig. 2.1). DUC

was calculated by measuring the distance between the specimen and the closest urban centre, which we consider to be a good proxy measure of urbanisation across the spatial and temporal scales of the study. The point of urban centre was the middle of the CBD of the five largest cities in SW WA. These cities were selected by inspecting census data of 1911 (ABS 1911) for human population confirming they have been the largest since specimen collection began. These centre points are located in the City of Perth, City of Busselton, City of Collie, City of Albany, and City of Esperance.

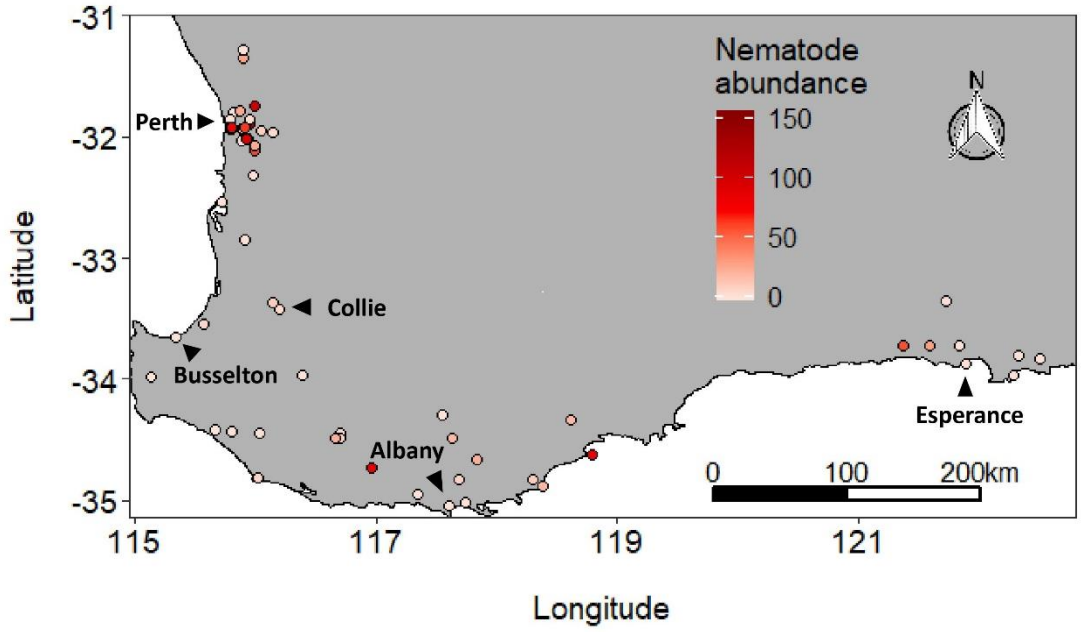

Fig. 2.1 The abundance of nematode infection for each tiger snake specimen in South-West Western Australia. Arrows indicate major cities used as urban centres. Colour indicates the number of worms (intensity) found in the stomach of each specimen.

As nematodes are probably acquired through predation on frogs we also compared distance to wetlands for each specimen, as well as basic climatic factors that may influence the parasite's lifecycle. To determine distance to wetlands we used regional surface water hydrology GIS shape files (representing major natural and man-made features including lakes, reservoirs and farm dams) generated in from Geoscience Australia (Geoscience Australia 2019) and measured the distance of each specimen from the closest water body. While contemporary mapping is not appropriate for calculating urbanisation categories in this study, we suggest that the number of wetlands and the boundaries of those wetlands are typically more stable components of the environment and are thus less likely to have experienced major differences over

the past century. It is possible that a small number of wetlands have been lost or created over this time, however we believe that these are unlikely to have a substantial impact on the results of the study. The location (coordinates in decimal degrees) of all 80 adult snakes were uploaded into the Atlas of Living Australia's (ALA) Spatial Portal (https://spatial.ala.org.au/#), and five environmental variables at each point were extracted: mean annual precipitation (RAIN), topographic wetness index (TWI), mean annual maximum temperature (MAXTEMP), mean annual temperature (MEANTEMP) and mean annual minimum temperature (MINTEMP) (von Takach Dukai et al. 2019). Variables extracted from the ALA have been derived from various sources, and are based on an average of 50 years of climate data centred on 1990 (Williams et al. 2010; Xu and Hutchinson 2013).

2.3.4 Correlations and logistic regression

All analyses were conducted in R Studio version 3.5.2 (R Core Team 2021). We conducted a preliminary investigation into correlations between the specimen nematode abundance and various spatial and climatic factors, using Spearman's rank correlation coefficient. Any samples without complete data were removed. We then used a binomial generalised linear model (GLM) to assess the influence of time, distance to nearest urban centre, distance to nearest wetland, and climatic variables on nematode prevalence. To account for increasing urbanisation through time we included an interaction between DUC and year. We first scaled and centred all predictor variables to improve model fitting, and then removed correlated variables to reduce multicollinearity (retaining variables with variance inflation factors <5 and pairwise correlations <0.7). We then ran a binomial GLM (using the *glm* function), and checked the model fit of the global model using pseudo-R-squared values calculated in the glmmADMB package (Fournier et al. 2012). Model selection was performed using the *dredge* function and all sub-models were ranked according to AICc values (i.e. corrected for small sample sizes). All models with ΔAICc <2 were considered useful for inference. We checked for signatures of spatial autocorrelation using a variogram of the model residuals, created using the geoR package (Ribeiro and Diggle 2018), and found no strong evidence of spatial autocorrelation. Results were visualised and the results were plotted using the ggplot2 package (Wickham 2016).

2.4 Results

2.4.1 Snake morphology and diet

A total of 88 tiger snakes with complete carcasses were dissected (Table 2.1), with three additional viscera-only samples. The majority of snakes were collected between 1930 and 1989 (n = 46). A body condition index could not be calculated due to the varying degree of damage and drainage capabilities of the specimens. However, based on absence of body fat and muscle mass only five (5.6%) of the 88 specimens appeared to be in very poor body condition (more in section 3.2). Prey items were found in the stomach of 38 (41.8%) of the 91 specimens (whole snakes plus viscera specimens) and 11 specimens contained more than one prey item. The majority of tiger snake prey items were frogs (86.8%; Table 2.2).

Table 2.1: Length and body mass measurements for 88 complete tiger snake specimens held at the Western Australian Museum.

Sex (*n*)	**mean SVL ± SE (range), cm**	**Mean wet mass ± SE (range), g**
Male (33)	78.6 ± 28.6 (49.9 – 113)	364.4 ± 36.6 (50 – 900)
Female (21)	79.3 ± 25.8 (60.2 - 100)	316.0 ± 22.1 (100 - 600)
Undetermined sex (23)	68.5 ± 24.2 (50.1 – 90.0)	22.6 ± 23.9 (100 – 450)
Juvenile (11)	36.1 ± 17.2 (27 – 45.6)	45.5 ± 6.7 (25 – 100)

Table 2.2: Prey items observed in the digestive tracts of 91 tiger snake specimens and viscera from the Western Australian Museum.

Taxon	**No. of individuals**	**Percentage of prey**
Frogs		86.8%
Limnodynastes dorsalis	11	
Litoria sp.	9	
Pseudophryne sp.	3	
Heleioporus sp.	3	
Unidentified	20	
Mammals		5.7%
Mus musculus	1	
Unidentified rodent	2	
Reptiles		1.8%
Actritoscincus trilineatus	1	
Birds		5.7%
Unidentified	3	
Fish		1.8%
Unidentified	1	

2.4.2 Abundance and intensity of nematode infection

Nematodes were found in the stomachs of 74% of the adult specimens, with the mean intensity of infected adult snakes being 31 nematodes (range = 1 to 152, SE = 4.87). The five snakes considered to be in poor body condition had a nematode intensity of 72 – 152 individuals. Only four other snakes had intensities within that range (79 – 136) and were considered in normal body condition. Nematodes were detected in three (27%) of the 11 juvenile specimens, with the smallest infected specimen measuring 31.9 cm (SVL). Five (5.7%) of 88 whole body specimens contained infections of 40+ individuals of unidentified helminths encysted throughout the muscle wall. Nematode abundance varied greatly both spatially (Fig. 2.1) and temporally (Fig. 2.2). We found no significant correlations between nematode abundance and distance to urban centre, year of collection, distance to wetland or any climatic variables except for mean maximum temperature ($r = 0.257$, $p = 0.002$).

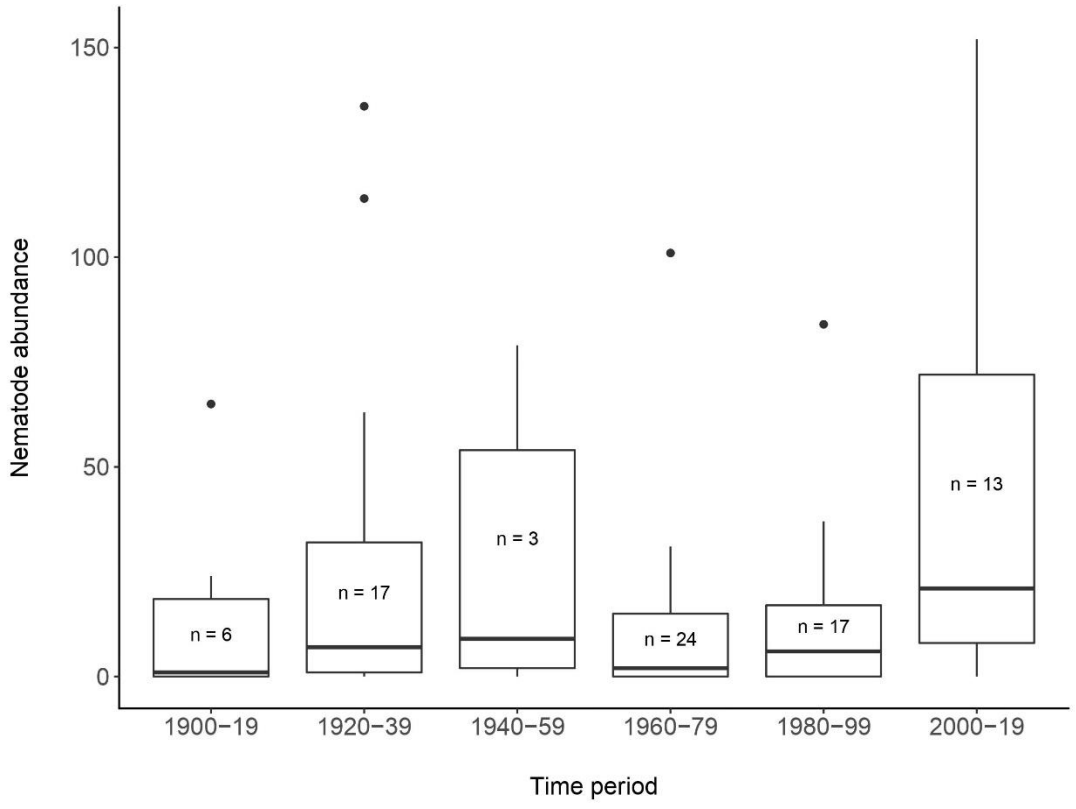

Fig. 2.2 Abundance (mean number nematodes per snake) of stomach nematodes in adult SW WA tiger snakes based on specimen collection time period. Bars represent standard errors and dots represent outliers, *n* = sample size for each period.

2.4.3 Probability of nematode infection

MAXTEMP and MINTEMP highly correlated with MEANTEMP (r = 0.825, p < 0.001 and r = 0.903, p < 0.001 respectively), and were excluded from the global model. The global model included the variables: DUC, distance to wetland, year (of collection), DUC with year as an interaction (D*Y), MEANTEMP, RAIN, TWI and SVL. Seven top models (ΔAICc < 2) were produced, which frequently identified the variables distance to wetland, mean annual precipitation and TWI (Table 2.3 & 2.4). DUC, D*Y and MEANTEMP were not present in any of the top models. The three water-related variables (distance to wetland, RAIN and TWI) were the strongest predictors of nematode infection, as they occurred in many of the top models whereas year and SVL were only in one or two top sub-models. We found an inverse relationship between probability of nematode infection and the three water-based variables (Fig 2.3). The probability of nematode infection decreased (from over 0.8 to below 0.3) for snakes collected away from wetlands; however, nematode infection increases in areas with lower rainfall (infection probability of 0.9 in areas with mean annual precipitation around 400 mm dropping to 0.3 when precipitation is over 1100 mm), and TWI (infection probability dropping from 0.9 to 0.3 with increasing TWI).

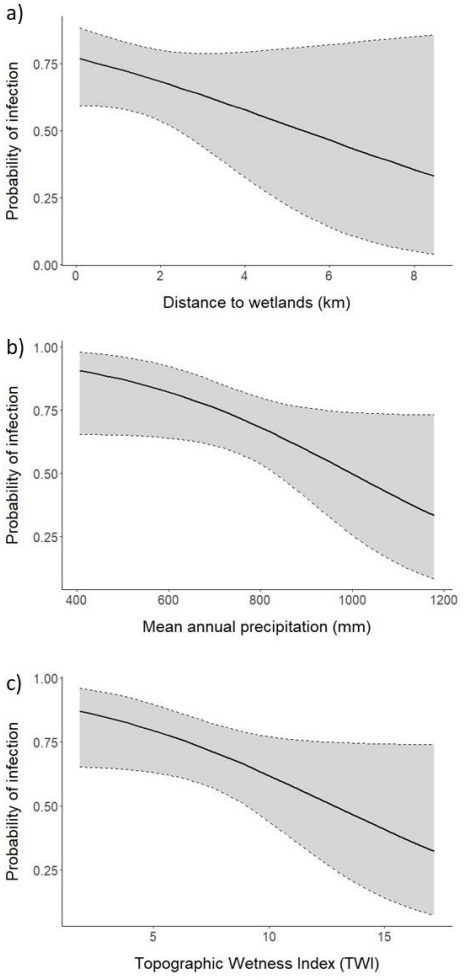

Fig. 2.3 Probability of tiger snake stomach nematode infection in relation to a) distance to wetlands, b) mean annual precipitation and c) topographic wetness index (TWI). Shaded areas represents 95% confidence intervals.

Table 2.3 Seven top models and the null model identifying the strongest predictor variables of nematode infection based on ΔAICc and weight (Dis.W = Distance to wetland, D*Y = interaction between DUC and year and M.Temp = mean annual temperature).

	DUC	Dis.W	D*Y	M.Temp	RAIN	SVL	TWI	Year	logLik	AICc	Δ	weight
Mod1	-	♦	-	-	♦	-	♦	-	-40.38	89.31	0	0.057
Mod2	-	♦	-	-	♦	-	-	-	-41.94	90.21	0.90	0.036
Mod3	-	♦	-	-	♦	-	♦	♦	-39.85	90.54	1.23	0.031
Mod4	-		-	-	♦	-	-	-	-43.31	90.79	1.48	0.027
Mod5	-	♦	-	-	♦	♦	♦	-	-40.03	90.90	1.59	0.026
Mod6	-		-	-	♦	-	♦	-	-42.44	91.21	1.90	0.022
Mod7	-	♦	-	-	♦	♦	-	-	-41.37	91.29	1.98	0.021
Null	-	-	-	-	-	-	-	-	-45.12	92.29	2.98	0.013

Table 2.4 Standard errors for the strongest predictor variables for the top seven models. Reported as estimate (standard error).

	Intercept	Dis.W	RAIN	SVL	TWI	Year
Mod1	1.12 (0.29)	-0.56 (0.27)	-0.61 (0.29)	-	-0.47 (0.27)	-
Mod2	1.07 (0.27)	-0.43 (0.26)	-0.60 (0.28)	-	-	-
Mod3	1.13 (0.29)	-0.61 (0.28)	-0.62 (0.28)	-	-0.57 (0.29)	-0.31 (0.30)
Mod4	1.03 (0.27)	-	-0.49 (0.27)	-	-	-
Mod5	1.13 (0.29)	-0.54 (0.27)	-0.64 (0.29)	0.22 (0.27)	-0.44 (0.27)	-
Mod6	1.06 (0.27)	-	-0.47 (0.27)	-	-0.34 (0.25)	-
Mod7	1.09 (0.28)	-0.43 (0.26)	-0.64 (0.29)	-0.29 (0.27)	-	-

2.5 Discussion

We used natural history collections to investigate the spatial and temporal influence that multiple climatic, environmental and urbanisation variables have on the prevalence and abundance of parasitic nematodes in a large Australian elapid snake. We found that the probability of tiger snake infection with nematodes increased with

proximity to wetland sites but declined with increasing rainfall and topographic wetness. Thus, our prediction that tiger snake nematode prevalence and abundance would be higher closer to wetlands was supported. Interestingly, we did not detect any influence of distance to urban centres on the probability of infection.

Urbanisation results in a suite of novel selection pressures for native fauna (French et al. 2018; McKinney 2008), influencing the health, fitness, and parasite abundance of many taxa (Bradley and Altizer 2007; French et al. 2008; Giraudeau et al. 2014; Winchell et al. 2019). Here, we found that water availability was more strongly associated with infection than was our metric of urbanisation, suggesting that urbanisation is perhaps less important than biotic environmental variables in determining the relationship between nematode infection and tiger snakes in this region. Importantly, our data suggests that wetlands in drier climate areas are strongly related to nematode parasitism in this species of snake.

Our hypothesis that the relationship between tiger snakes and the complex life cycle of the nematode *O. pyrrhus* would be sensitive to urbanisation (at least for our urbanisation metric: distance to urban centres) was not supported. Tiger snakes that were collected closer to urban centres were not found to have higher infection rates or abundance of infection from nematodes relative to snakes collected in non-urbanised areas; and although the sample size is small we did not detected a change in parasitism over 100 years. Urban wetlands in SW WA have been fragmented, polluted and subjected to modification of structure and hydrology (Davis and Froend 1999; Kobryn 2001; Lund 1992). These wetlands (especially those associated with the Swan Coastal Plain) are naturally ephemeral and seasonal drying may remove (via desiccation) a large proportion of nematode eggs before consumption by a host (Perry 1989; Wharton 1980). Dredging activities have, however, led to some urban wetlands becoming permanently inundated with water, allowing nematodes and their hosts to persist year-round. Permanent water and urban run-off also allows a suite of contaminants to persist which snakes potentially bioaccumulate, putting stress on their immune system, as has been recorded in other taxa (Martin et al. 2010; Patz et al. 2000; Riley et al. 2007). Our findings with respect to urbanisation may be due to a number of factors. The cities of SW WA used in this study are relatively small compared to other cities in Australia. The degree of urbanisation may not have been strong enough to influence parasitism in tiger snakes, and the lack of samples from recent decades following the

increase of Perth's metropolitan area by 45% since 1990 (MacLachlan et al. 2017) may not be adequate to identify more recent changes. Alternatively, it is possible that intensity of parasite infection is more relevant to host health than is the presence/absence of nematodes, as the five snakes we found in poor body condition had some of the highest intensities of infection (72 -152 individuals). While the restrictions on samples sizes meant that we were unable to determine if nematode intensity had changed over time, it is possible that larger sample sizes and a well-designed sampling/collection methodology would allow for a more detailed analysis into whether nematode intensity varies with urbanisation and other environmental variables, and how this impacts snake health.

As frogs are probably the main intermediate host, we also hypothesised that nematode prevalence and infection would be greater if the snakes were collected within or close to wetlands. Our results found higher probability of infection close to wetlands, which suggest a higher frequency of exposure to frogs as an infected food source. The diet and parasite infection of other snakes supports this hypothesis: *O. pyrrhus* has been detected in Western brown snakes (*Pseudonaja mengdeni*), mulga snakes (*Pseudechis australis*) and bardick (*Echiopsis curta*) (Jones 1978, 1980), although most infections have been detected in tiger snakes. All these species are known to predate on frogs (Madsen and Shine 1994; Shine 1987b). Dugites (*Pseudonaja affinis*) are the only other large snake parapatric with tiger snakes and *O. pyrrhus* has not been recorded in them (Ashleigh Wolfe, pers. comm.; Jones, 1978, 1980). Mainland tiger snakes feed heavily on frogs (Aubret et al. 2006; Shine 1987a and Table 2.2) whereas dugites prefer reptiles and mammals (Shine 1989; Wolfe et al. 2018) and thus are not exposed to infection. Additionally, tiger snakes introduced from WA mainland to Carnac Island, WA, a desert island without frogs, are exempt of nematodes (Aubret 2005). Thus, diet offers a convincing explanation as the source of nematodes.

Somewhat counter to our initial predictions, our results also indicate that the probability of nematode infection increases in areas with less rainfall and lower topographic wetness. This suggests that wetlands, particularly more permanent ones in drier areas, are important drivers of infection in tiger snakes. While we can only speculate, one possibility is that tiger snakes may be more likely to disperse from wetlands in areas of higher rainfall. Heavy urbanisation can decrease precipitation and moisture availability in the landscape by replacing vegetated land with infrastructure

(Zhang et al. 2014), as well as funnelling the remaining water into permanent wetlands. Tiger snakes occupying the more northern limit of their range, such as Perth, are at risk from a range of environmental modifications that may increase parasitism such as restriction to isolated wetlands, reduced rainfall and overall drier conditions (McFarlane et al. 2012; Rotstayn et al. 2010).

In addition, we found a slight positive correlation between mean annual maximum temperature and nematode abundance. Due to the limited sample size we interpret this result as a potential trend, and discuss the possible outcomes a changing environment could have on temperature influenced parasitism. Temperature drives activity in both nematodes as poikilotherms and their snake hosts as ectotherms. Exposure to higher maximum temperatures may have an impact on nematode abundance in a number of ways. Regions with higher maximum temperatures allow snakes to reach their preferred body temperature quickly (Schwaner 1989; Shine 1979; Wang et al. 2002). Attaining preferred body temperature earlier in the day gives snakes a longer window of activity each day, including foraging time. Consequently this increases the feeding rate and period of activity for parasitic nematodes (assuming adult *O. pyrrhus* is a snake-specific parasite and has a very similar preferred body temperature). Nematodes operating at their optimum temperature maximise reproductive output (Morgan and van Dijk 2012), and warmer conditions can increase parasite developmental rates in eggs and intermediate life stages (Griffin 1993; Kutz et al. 2004; Kutz et al. 2001; van Dijk et al. 2008).

Urbanisation through infrastructure and deforestation causes an increase in temperature through the urban heat island (UHI) effect (Arnfield 2003), and some parasites and diseases have been shown to be positively influenced as a result (Buczek et al. 2014; Trajer et al. 2014). If the UHI effect were a significant driver of nematode parasitism we would expect to see abundance and intensity increase in specimens collected closer to urban centres, and in more recent years. However, our urban sample size with its lack of recent specimens was too small to accurately detect potential changes. Perth is the largest city of SW WA and yet is still relatively small in comparison to other major cities of the world (Newman 2016), subsequently it still has a low UHI effect (Camilloni and Barros 1997; Earl et al. 2016). Future studies using a more robust dataset of temperatures, with higher sample sizes and urbanisation

scores may be able to accurately detect influences of temperature and the UHI effect on parasitism.

Some limitations in our study should be noted. We were restricted by the number of specimens we were allowed to destructively sample, by juveniles not being used in the statistical analyses, and gaps in the spatial and temporal scale of the natural history collection. The three specimens collected post-2003 were all collected from heavily urbanised wetlands and were all in poor body condition containing a high intensity of nematodes (>79/snake). While this is a small sample size these snakes potentially indicate an increase in the infection rates in urban areas over the last two decades. It is also possible that these specimens were collected as opportunistically found carcasses suffering from poor body condition. This would bias the sample and not be an accurate representation of the nematode intensity of the entire urban snake population. Such limitations are common when using natural history collections for ecological studies (Lister and Climate Change Research Group 2011).

The impact of urbanisation on habitats, climate, organisms and their life cycles is complex and difficult to elucidate fully. While we found a strong influence of water availability on the probability of infection with nematodes, we did not observe any significant influence from our particular urbanisation metric. We suggest that modifications to urban wetland structure and hydrology, and water availability in the landscape is driving parasitic nematode prevalence in tiger snakes, with further study required to examine fine-scale processes. As it is likely that prey items such as frogs are the key infection source for tiger snakes, the effects of urbanisation on frog abundance is also likely to be relevant. This study 1) demonstrates that proximity to wetlands and climatic factors are influencing nematode prevalence, 2) nematode abundance is potentially influenced by warmer temperatures, 3) highlights how these influencing variables are sensitive to a changing environment, specifically urbanisation, and 4) emphasises the importance (as well as limitations) of museums and the systematic collection of specimens for detecting spatial and temporal changes in ecology.

2.6 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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2.7 Chapter 2 addendum

2.3.1 – page 19

Sex was determined by internal examination of gonads, sex could not be determined for damaged specimens. Juveniles (for every chapter mentioning juveniles) were determined from SVL minimum size of maturity (males <56 cm, females <53 cm; Shine 1987a).

2.4.2 – page 24

There was no significant correlation between nematode abundance and distance to urban centre (r = -0.16, p = 0.35), year of collection (r = 0.21, p = 0.21), distance to wetland (r = -0.02, p = 0.89), rainfall (r = -0.06, p = 0.71), TWI (r = -0.11, p = 0.53), MEANTEMP (r = 0.31, p = 0.06) or MINTEMP (r = -0.30, p = 0.08).

Chapter 3. The broad-scale analysis of metals, trace elements, organochlorine pesticides and polycyclic aromatic hydrocarbons in wetlands along an urban gradient, and the use of a high trophic snake as a bioindicator

The study presented in Chapter 3 was accepted in the peer-reviewed journal '*Archives of Environmental Contamination and Toxicology*' on 17 February 2020, and is an exact reproduction of the copyright paper reformatted for this thesis.

Lettoof, D. C., Bateman, P. W., Aubret, F., Gagnon, M. M. (2020). The Broad-Scale Analysis of Metals, Trace Elements, Organochlorine Pesticides and Polycyclic Aromatic Hydrocarbons in Wetlands along an Urban Gradient, and the Use of a High Trophic Snake as a Bioindicator. *Arch Environ Contam Toxicol*. 78(4):631-45. doi: 10.1007/s00244-020-00724-z.

3.1 Abstract

Wetlands and their biodiversity are constantly threatened by contaminant pollution from urbanisation. Despite evidence suggesting that snakes are good bioindicators of environmental health, the bioaccumulation of contaminants in reptiles is poorly researched in Australia. We conducted the first broad-scale analysis of 17 metals and trace elements, 21 organochlorine pesticides and 14 polycyclic aromatic hydrocarbons in the sediments (four samples per site, December 2018) from four wetlands along an urban gradient in Perth, Western Australia and from the livers (five livers per site, February – April 2019) of Western tiger snakes *Notechis scutatus occidentalis* captured at those sites. All 17 metals and trace elements were detected in the sediments of wetlands as well as 16 in the livers of tiger snakes. Arsenic, Cu, Hg, Pb, Se and Zn were at concentrations exceeding government trigger values in at least one sediment sample. Two organochlorine pesticides and six of seven polycyclic aromatic hydrocarbons were detected in the sediments of a single wetland, all exceeding government trigger values, but were not detected in tiger snakes. Metals and trace elements were generally in higher concentration in sediments and snake livers from more heavily urbanised wetlands. The least urbanised site had some higher

concentrations of metals and trace elements, possibly due to agriculture contaminated groundwater. Concentrations of nine metals and trace elements in snake livers were statistically different between sites. Arsenic, Cd, Co, Hg, Mo, Sb and Se near paralleled the pattern of contamination measured in the wetland sediments; this supports the use of high trophic wetland snakes, such as tiger snakes, as bioindicators of wetland contamination. Contamination sources and impacts on these wetland ecosystems and tiger snakes are discussed herein.

3.2 Introduction

Wetlands are biodiversity hotspots threatened by urbanisation, worldwide. Wetlands are often the only remaining fragments of habitat within an urban landscape (Ehrenfeld 2004; Garden et al. 2006), and provide islands of water storage, ground water replenishment, supply and transformation of nutrients, high biodiversity, and recreational enjoyment for humans (Lee et al. 2006; Novitski et al. 1996; Zedler and Kercher 2005). As land development increases wetland ecosystems degrade through changes in hydrology (Chadwick et al. 2006), structure (Faulkner 2004; Lee et al. 2006), floral and faunal biodiversity (Gibbs 2000; McKinney 2008), and water and sediment chemistry (Brown et al. 2010; Fitzpatrick et al. 2007; Panno et al. 1999). Environmental contamination, as a consequence of rapid urbanisation, industrialisation and poor waste management practices (Nriagu 1990; Rodriguez Martin et al. 2015), is particularly severe for urban wetlands and threatens the health of biological communities (Spurgeon and Hopkin 1999; Zhang et al. 2017). Due to their topography, urban wetlands are susceptible to contamination from several primary source points: urban runoff (Zhang et al. 2012), stormwater drains feeding into wetlands (Clarke et al. 1990), groundwater (Roy and Bickerton 2011), and pest and weed treatment (Gentilli and Bekle 1993).

Perth is the largest city of the West coast of Australia, and is built almost entirely on the Swan Coastal Plain bioregion (Davis and Froend 1999). The Swan Coastal Plain is characterised by sandy soil dunes systems interlaced with a chain of inter-connected ephemeral wetlands and lakes (Simpson and Newsome 2017). For the past near 200 years urbanisation and agriculture has drained or filled in an estimated 70% of the original wetland area of the Swan Coastal Plain, and the remaining wetlands have been subject to structural and hydrological modifications, isolation, biodiversity loss and

pollution (Davis and Froend 1999; Gentilli and Bekle 1993). The remnant wetlands of urban Perth have a history of contamination from primary sources such as inflowing stormwater drains, as well as historical industrial dumping and pest management through pesticides (Clarke et al. 1990; Department of Water 2009; ESRI 1983; Gentilli and Bekle 1993). Some of the larger wetlands in Perth still support populations of Western tiger snakes (*Notechis scutatus occidentalis*), a top predator of these ecosystems. Snakes are an important organism in the ecosystem, providing shifting predator and prey functions as they ascend the trophic tiers throughout their life. Wetland snakes in particular represent an interface between aquatic and terrestrial habitats; thus they are susceptible to the bioaccumulation of contaminants and can be used as bioindicators for environmental pollution (Campbell and Campbell 2001; Drewett et al. 2013; Lemaire et al. 2018).

Despite the historical contamination and close proximity of infrastructure to urban wetlands in Perth, there are little monitoring data available on the contamination of water, sediment or fauna (see table 3.1 for summary). Wetlands can be polluted with a plethora of contaminants, the more frequently assessed contaminants being metals and trace elements, organochloride pesticides (OCPs) and polycyclic aromatic hydrocarbons (PAHs) (Cooper 1993; Gambrell 1994; Haarstad et al. 2012). As wetland structure plays an important role in contaminant retention, permanently enclosed wetlands such as lakes, are more susceptible to the accumulation of contaminants compared to flowing water systems (Burger et al. 2007; Schulz and Peall 2001). As a result, fauna communities restricted to enclosed and isolated urban wetlands might be vulnerable to bioaccumulation from continuous exposure to contaminants. Three permanent and enclosed wetlands that differ in degree of urbanisation and contain abundant populations of tiger snakes persist in Perth. By comparing these wetlands and tiger snake populations with a similar wetland within a national park we are presented with a rare and ideal system to study the contamination of wetlands and a top predator snake along an urban gradient.

This study presents and examines a snapshot of concentrations of 52 contaminants in both sediment and tiger snake livers from four wetlands around Perth, Western Australia. The objective of this study was threefold: (1) quantify contaminants present in the wetlands; (2) measure contaminants that are bioaccumulating in the top predator: tiger snakes; and (3) investigate whether contaminant concentrations parallel

the degree of urbanisation of wetlands. Research on contaminants in snakes is an emerging field (Burger et al. 2017; Drewett et al. 2013; Gavric et al. 2015; Quintela et al. 2019; Schwabenlander et al. 2019) yet as far as we are aware this study analyses the largest range of contaminants in any species of snake, and is only the third study to present data on the contaminants of both the snake tissue and ecosystem they were collected from (Ford and Hill 1991; Soliman et al. 2019). We also present the first published contaminants in Australian snakes in more than 40 years (Beck 1956; Best 1973).

3.3 Methods

3.3.1 Study sites

We examined a suite of contaminants in four wetlands in the Swan Coastal Plain of Western Australia (Fig. 3.1): Herdsman Lake, Bibra Lake, Lake Joondalup and Loch McNess. Loch McNess is located in Yanchep National Park. We selected these wetlands as study sites based upon presence and abundance of tiger snakes. Historically, these wetland lakes were partially linked and ephemeral (Gentilli and Bekle 1993) yet the development of Perth city lead to the draining of some wetlands while others were dredged to become permanent (Halse 1989). These wetlands share similar climatic factors and some degree of hydrological modification from naturally ephemeral to persistently-filled, yet differ in degrees of urbanisation (Davis and Froend 1999). Table 3.1 describes these wetlands and summarises the few studies on contaminants reported at these wetlands. Because there is no recent and detailed contaminant data for these wetlands, we initially tested the sediments for a suite of contaminants including those listed in Chapter 3 supplementary material.

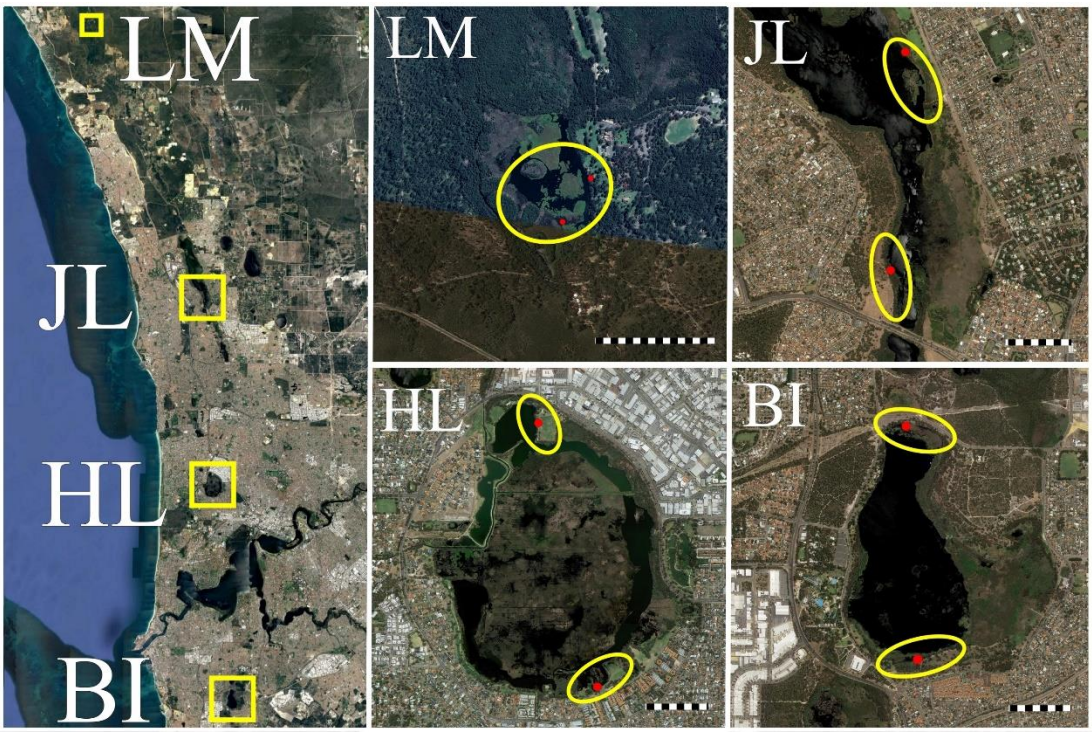

Fig. 3.1 Wetlands in the Swan Coastal Plain where sediments and tiger snakes were collected for contaminant analyse. Red dots indicate collection points of sediment samples, yellow circles indicate tiger snakes collection areas. HL = Herdsman Lake, BI = Bibra Lake, JL = Lake Joondalup, LM = Loch McNess (located in Yanchep National Park). Satellite images were obtained from Google Earth Pro in 2019. Scale bar = 500m.

Table 3.1 Brief description and history of contaminants detected within the wetland sites used for study. Area was calculated using Google Earth Pro polygon tool based on the most recent aerial photographs. Sites are listed in order of most urbanised to least.

Site	Brief description of urbanisation	References	Contaminants detected (S = sediment, W = water)	References
Herdsman Lake (HL) 31° 55'12 S, 115° 48'19 E 3.07 km^2	Heavily modified; since 1850s has been subject to agriculture, industrial dumping, dredging and storm water inflow; located among heavy urbanisation	Clarke et al. (1990); Gentilli and Bekle (1993); Kobryn (2001)	(**W**) Cr, Cu, Pb, Ni, Zn, Fe, Mn (**W**) Cd, Cu, Pb, Zn; (**W**) Dieldrin, heptachlor (**S**) Cu, Zn, As; (**S**) Aldrin, Chlordane, dieldrin, DDT (**W**) Cd, Cu, Pb, Zn (**W**,**S**) Al, Cu, Pb, Zn, As; (**S**) PCBs, PHCs	ESRI (1983) Clarke et al. (1990) Davis et al. (1993) Kobryn (2001) Department of Water (2009)
Bibra Lake (BI) 32° 5'32 S, 115° 49'27 E 1.93 km^2	Partially modified; some fringe is urbanised; located among heavy urbanisation	Sinang et al. (2015)	(**S**) Dieldrin (**W**) Cu, Pb, Zn (**W,S**) Al, Cr, Cu, Pb, Zn; (**S**) PHCs, PAHs	Davis et al. (1993) Burkett (2005) Department of Water (2009)
Lake Joondalup (JL) 31° 45'34 S, 115° 47'33 E 7.33 km^2	Partially modified; some fringe is urbanised; located on edge of recent urbanisation	Congdon (1986); Gonzalez-Pinto et al. (2017)	(**S**) As (**W**) Al, As, Hg, Zn (**W**) Al, Cd, Hg, Zn	Davis et al. (1993) Newport and Lund (2014) Gonzalez-Pinto et al. (2017)
Loch McNess (LM) 31° 32'44 S, 115° 40'50 E 0.36 km^2	Minimally modified; surrounded by Yanchep National Park; located outside of urbanisation	Department of Water (2011)	(**S**) As (**W**) As, B, Cr, Mn, Ni	Davis et al. (1993) Department of Water (2011)

3.3.2 Study species

The tiger snake (*Notechis scutatus*) is a polymorphic Australian elapid occurring in disjunct populations that vary in diet, habitat and ecology (Aubret et al. 2004; Aubret et al. 2006). However, across most of its range, including the mainland Western Australian subspecies *N. scutatus occidentalis*, tiger snakes are an abundant higher trophic reptile predator in wetlands that have a preference for frogs (Aubret et al. 2006; Shine 1987). There is no long-term monitoring data available for tiger snakes to determine life expectancy, yet the oldest captive record was up to 24 years (Fearn and Norton 2011). Tiger snakes and dugites (*Pseudonaja affinis*) are the only two large snake species to persist within urban Perth and dugites prefer woodland and heath over wetlands, thus we selected tiger snakes as our bioindicator model species based on their abundance, habitat preference, high trophic position and prey preference. Frogs are known to bioaccumulate contaminants indirectly through their food or directly through their water permeable skin when in contact with contaminated waters or sediments (Bruhl et al. 2011; Hopkins 2007; Ohlendorf et al. 1988), and therefore provide a crucial link for contaminant transfer throughout the food web.

3.3.3 Sediment collection and analysis

Sediment samples were collected 18 December 2018 during the dry season when Perth receives little rainfall. Each wetland was sampled in two locations and sediments were collected in duplicates. Each duplicate was collected three metres away from each other. Sampling locations are shown in Fig. 3.1 and corresponded to areas of the wetland with the highest capture rate of tiger snakes. The first 10 cm of sediment was collected using a metal scoop and glass jar for organic contaminant samples and a plastic scoop and jar for metal and trace element samples. Sediment samples were kept cool until submitted for chemical analysis at the end of the day of collection. All samples were analysed for 17 metals and trace elements (from hereon in collectively referred to as metals), 21 organochlorine pesticides and 14 PAHs by ChemCentre (Perth, Western Australia). These contaminants were chosen based on the limited historical data and regular screening of this suite by ChemCentre for environmental monitoring (Leif Cooper, pers. comm.). The specific contaminants, method of analysis, detection limits and quality assurance are reported in Chapter 3 Supplementary material. Metals were determined using methods iMET2SAMS and iMET2SAMS based on the US EPA method 3051A (US EPA 2007). Sediment

samples were extracted with concentrated nitric and hydrochloric acid, and then analysed for metals using a combination of inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS). Organochlorine pesticides were determined using method ORG141S and PAHs were determined using method ORG100S, both based on US EPA method 8270D (US EPA 1998). Contaminant concentrations are reported as mg/kg dry weight, and are compared to the Australian and New Zealand guidelines (ANZECC & ARMCANZ 2000) and the revised ANZECC/ARMCANZ Sediment Quality Guidelines (Simpson et al. 2013). The lower trigger value (sediment quality guideline value (SQGV)) represents the threshold for biological effects, and the higher sediment quality guideline trigger value (SQGV-High) represents the high probability of biological effects (Simpson et al. 2013). If there was no guidelines for a particular contaminant they are compared to alternative guidelines (Lemly 1996; Ontario Ministry of Environment and Energy 1993).

3.3.4 Snake liver collection and analysis

Five tiger snakes were collected from each site by hand between February and April 2019, and euthanised humanely by blunt force trauma to the head. Sex, snout-vent length (SVL), total mass and liver mass were recorded for all snakes (Table 3.2). We selected livers for analysis due to their potential to retain contaminants exposed from feeding. Whole livers were extracted using ceramic blades and frozen, at – 20 ^{o}C prior to submission to ChemCentre for chemical analysis. All liver samples were analysed for the same contaminants as were the sediments; the specific contaminants, method of analysis, detection limits and quality assurance are listed in Chapter 3 Supplementary material. Whole livers were homogenised and extracted with concentrated nitric and hydrochloric acid, and then analysed for metals were determined using methods iMETBTMS and iMETBTICP based on APHA methods 3120 and 3125 (APHA 1998) using a combination of inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS). Organochlorine pesticides and PAHs were determined using method ORG100B based on US EPA methods 8270D (US EPA 1998). Contaminant concentrations are reported as mg/kg wet weight.

Table 3.2 Morphological measurements (mean ± one SE (range)) of tiger snakes (*Notechis scutatus occidentalis*) collected at each site. SVL = snout-vent length.

Site	Sex (*n*)	SVL (mm)	Body mass (g)	Wet liver mass (g)
Herdsman Lake	Male (4)	855 ± 12.6 (825 – 877)	250 ± 15.15 (216 – 282)	5.2 ± 0.6 (4.0 – 6.3)
	Female (1)	776	277	3.9
Bibra Lake	Male (4)	807 ± 21.9 (746 - 847)	283 ± 20.2 (233 – 329)	9.1 ± 1.2 (6.6 – 11.7)
	Female (1)	704	212	4.8
Lake Joondalup	Male (4)	779 ± 15.5 (735 - 807)	221 ± 26.6 (171 – 296)	7.4 ± 0.8 (6.8 – 9.3)
	Female (1)	706	205.6	11.1
Loch McNess (Yanchep)	Male (3)	847 ± 43.6 (760 – 899)	294 ± 44.2 (235 – 381)	11.2 ± 2.0 (7.3 – 13.5)
	Female (2)	719 ± 47.5 (671 – 766)	194 ± 14.4 (180 -208)	4.2 ± 0.9 (3.2 – 5.1)

3.3.5 Data analysis

Concentration means and standard deviation were calculated for each contaminant for each site. For statistical analysis samples that were recorded below detectable limits (BDL) were entered as half the detection limit (ANZECC & ARMCANZ 2000; Zeghnoun et al. 2007). Due to the limited number of animals allowed to be sacrificed at each site, no statistical differences could be established between male and female contaminant burdens. Consequently contaminant data were pooled for male and female snakes. The Shapiro-Wilk test was used to determine all data (sediment and livers) had a non-parametric distribution. Hence a Kruskal-Wallis test was used for both sediments and tiger snake livers to determine if there were significant differences (p <0.05) in contaminant concentrations between sites. Following, a Dunn post-hoc test was performed to identify the pairs of sites that showed significant differences. *p*-values were adjusted using the Benjamini-Hochberg method. We used Spearman rank correlations to explore the relationship between SVL (age) of snakes and each metal and trace element within each site. We present and consider *rho* values > 0.5 as moderate positive correlations and allow the reader to assess the significance themselves. All statistical analysis were conducted in R Studio (R Core Team 2021).

3.4 Results and Discussion

3.4.1 Wetland sediment contaminants

All 17 metals tested for were detected in Herdsman Lake, 16 were detected in Bibra Lake and Loch McNess, and 15 were detected in Lake Joondalup (discussed data presented in Table 3). Four metals (As, Cu, Pb and Zn) were detected exceeding trigger values in at least one sample, and Zn was detected exceeding the high trigger value in one sample from Herdsman Lake. Selenium was detected exceeding the trigger value in one sample at Bibra Lake and two samples at Loch McNess. Mercury was detected exceeding the trigger value in two samples in Lake Joondalup and one sample in Loch McNess. Generally, mean concentrations of contaminants decreased with less urbanised sites except for nine metals that were high at Loch McNess.

Table 3.3 Concentrations (mg/kg, dry weight) of contaminants detected in sediments (*n* = 4/site). Given as: mean ± one SE (range). BDL = below detectable limits. Concentrations in bold indicate values higher than the ANZECC sediment quality guideline value (SQGV). Lower-case letter indicates Kruskal-Wallis significant difference (p = <0.05) between sites. # = alternative guidelines (Lemly 1996; Ontario Ministry of Environment and Energy 1993).

Contaminant	Herdsman Lake	Bibra Lake	Lake Joondalup	Loch McNess	ANZECC SQGV (mg/kg, dry weight)
Aldrin	<0.01	**0.14** ± 0.13 (BDL – **0.53**)	<0.01	<0.01	SQGV: 0.002[#] SQG-High: NA
Dieldrin	<0.01	**0.65** ± 0.65 (<0.01 – **2.60**)	<0.01	<0.01	SQGV: 0.12 SQG-High: 0.27
Phenanthrene	<0.5	**0.49** ± 0.24 (<0.5 – **1.20**)	<0.5	<0.5	SQGV: 0.24 SQG-High: 1.5
Fluoranthene	<0.5	**0.86** ± 0.61 (<0.5 – **2.70**)	<0.5	<0.5	SQGV: 0.6 SQG-High: 5.1
Pyrene	<0.5	**1.41** ± 1.17 (<0.5 – **4.90**)	<0.5	<0.5	SQGV: 0.665 SQG-High: 2.6
Benz(a)-anthracene	<0.5	**0.66** ± 0.42 (<0.5 – **1.90**)	<0.5	<0.5	SQGV: 0.261 SQG-High: 1.6
Chrysene	<0.5	**1.09** ± 0.84 (<0.5 – **3.600**)	<0.5	<0.5	SQGV: 0.384 SQG-High: 2.8
Benzo(a)-pyrene	<0.5	**0.69** ± 0.44 (<0.5 – **2.00**)	<0.5	<0.5	SQGV: 0.43 SQG-High: 1.6
Benzo(g,h,i)-perylene	<0.5	0.44 ± 0.92 (<0.5 – 1.00)	<0.5	<0.5	SQGV: NA SQG-High: NA
Antimony	0.77 ± 0.03[a,b] (0.68 – 0.83)	0.23 ± 0.5 (0.10 – 0.32)	0.18 ± 0.04[a] (<0.05 – 0.15)	0.20 ± 0.05[b] (<0.05 – 0.06)	SQGV: 2 SQG-High: 25
Arsenic	**20.78** ± 6.54[a] (9.10 – **34.00**)	1.80 ± 0.35 (1.20 – 2.80)	0.88 ± 0.17[a] (0.40 – 1.20)	7.12 ± 3.50 (0.60 – 15.00)	SQGV: 20 SQG-High: 70

Table 3 continued

Contaminant	Herdsman Lake	Bibra Lake	Lake Joondalup	Loch McNess	ANZECC SQGV (mg/kg, dry weight)
Barium	91.75 ± 30.10 (24.00 – 160.00)	85.50 ± 9.32 (65.00 – 110.00)	31.00 ± 9.19 (18.00 – 58.00)	86.75 ± 49.74 (11.00 – 230.00)	SQGV: NA SQG-High: NA
Beryllium	0.18 ± 0.12 (<0.05 – 0.28)	0.22 ± 0.07 (0.09 – 0.41)	0.04 ± 0.01 (<0.05 – 0.06)	0.08 ± 0.04 (<0.05 – 0.16)	SQGV: NA SQG-High: NA
Cadmium	0.14 ± 0.06 (<0.05 – 0.28)	0.14 ± 0.03 (0.07 – 0.22)	0.06 ± 0.02 (<0.05 – 0.10)	0.26 ± 0.19 (<0.05 – 0.80)	SQGV: 1.5 SQG-High: 10
Chromium	13.43 ± 2.99 (8.70 – 22.00)	11.23 ± 2.88 (6.10 – 19.00)	5.73 ± 1.24 (3.60 – 8.60)	20.45 ± 8.37 (2.80 – 38.00)	SQGV: 60 SQG-High: 370
Cobalt	2.23 ± 0.31[a] (1.70 - 3.10)	0.70 ± 0.14 (0.40 – 1.10)	1.20 ± 0.07[a] (0.10 – 0.40)	1.40 ± 0.64 (0.20 – 2.90)	SQGV: NA SQG-High: 50[#]
Copper	**76.50** ± 45.07[a] (20.00 – **210.00**)	7.40 ± 1.25 (3.90 – 9.80)	5.05 ± 1.54[a] (0.50 – 6.90)	10.87 ± 3.69 (3.10 – 19.00)	SQGV: 65 SQG-High: 270
Lead	43.75 ± 8.84[a,b] (31.00 – **69.00**)	20.50 ± 1.66 (18.00 – 25.00)	14.08 ± 3.57[a] (6.30 – 23.00)	9.20 ± 3.96[b] (1.50 – 17.00)	SQGV: 50 SQG-High: 220
Manganese	68.75 ± 23.26 (25.00 – 120.00)	30.08 ± 8.56 (6.30 – 47.00)	26.67 ± 17.18 (7.20 – 78.00)	20.07 ± 10.38 (2.30 – 50.00)	SQGV: 460[#] SQG-High: 1100[#]
Mercury	0.05 ± 0.01 (0.01 – 0.07)	0.05 ± 0.005 (0.04 – 0.06)	0.14 ± 0.08 (0.01 – **0.35**)	0.10 ± 0.05 (0.01 – **0.21**)	SQGV: 0.15 SQG-High: 1
Molybdenum	1.19 ± 0.27[a] (0.65 – 1.7)	0.49 ± 0.09 (0.27 – 0.71)	0.16 ± 0.07[a] (0.07 – 0.37)	0.88 ± 0.39 (0.19 – 1.7)	SQGV: NA SQG-High: NA
Nickel	7.48 ± 1.18[a] (6.00 – 11.00)	3.80 ± 0.59 (2.50 – 5.30)	0.55 ± 0.16[a] (0.30 – 1.00)	2.55 ± 0.99 0.50 – 4.60	SQGV: 21 SQG-High: 52
Selenium	0.59 ± 0.14 (0.38 – 0.97)	0.96 ± 0.36[a] (0.45 – **2.00**)	0.13 ± 0.29[a,b] (0.08 – 0.21)	**4.04** ± 2.14[b] (0.23 – **7.90**)	SQGV: 2[#] SQG-High: 4[#]
Silver	0.09 ± 0.01 (0.07 – 0.11)	<0.05	<0.05	<0.05	SQGV: 1 SQG-High: 3.7
Tin	8.10 ± 5.31[a] (2.00 – 24.00)	1.00 ± 0.14 (0.60 – 1.30)	<0.5	0.60 ± 0.21[a] (<0.5 – 1.10)	SQGV: NA SQG-High: NA
Zinc	**221.00** ± 105.15[a] (41.00 – **510.00**)	60.50 ± 14.91 (18.00 – 84.00)	12.12 ± 5.57[a,b] (<5.00 – 28.00)	31.00 ± 12.57[b] (11.00 – 67.00)	SQGV: 200 SQG-High: 410

Herdsman Lake generally had the highest mean concentration of metals including significantly higher concentrations of Pb, As, Co, Cu, Mo, Ni, Sn and Zn compared with at least one other site, as well as the only detection of Ag. Herdsman Lake is particularly susceptible to contamination from several point sources. Since urbanisation in Perth began, the wetland has suffered from considerable changes in land use including: stock grazing (1850s), market gardening (1910s), drainage for irrigation and land reclamation (1920s), sanitary landfill (1930s), compensation basin for urban drainage (1930s+), intense pesticide treatment (1950 – 1980s), reserved for public recreational space (1970s+), dredging (1980s) and periodic illegal rubbish

disposal (Clarke et al. 1990; Davis and Garland 1986; Department of Water 2009; ESRI 1983; Gentilli and Bekle 1993; Kobryn 2001). Currently, the wetland is divided into three main interconnected water bodies which receive drainage from five major and an unknown number of minor drainage systems, including the bordering industrial area. Urban and industrial development began to encroach the lake in the 1950s and currently virtually no original fringing vegetation remains. The high concentrations of As, Cu, Pb and Zn are likely to originate from industrial stormwater, urban runoff, and leaching from historical dumping. Although our sampling did not detect any pesticides they have been detected in more comprehensive studies in the past (Clarke et al. 1990; Davis and Garland 1986).

Bibra Lake generally had the second highest mean concentrations of metals, and the only detections of OCPs and PAHs (discussed later). There is limited information available on the history of Bibra Lake but it has not suffered from the same degree of urbanisation as Herdsman Lake. Although it has a much larger buffer of remnant vegetation than Herdsman Lake (Fig. 3.1) it is still in close proximity to an industrial area which began development in conjunction with suburbia in the 1970s (Department of Water 2009). The northern and southern edge of the lake are bordered closely to main roads, and the southern edge of the lake has buried sanitary landfill (Burkett 2005). Bibra Lake receives water via direct rainfall and surface runoff from the urban catchments (Sinang et al. 2015), which is might be the source of its moderate concentration of metals.

Joondalup Lake had the lowest concentration of most of the detected metals, including significantly lower concentrations of Pb, As, Co, Cu, Mo, Ni and Zn than Herdsman Lake, the latter being the most contaminated and urbanised site. Urbanisation of Joondalup only began to rapidly increase around the lake in the 1980s, which is later than the other study sites (Kinnear et al. 1997); and the wetland has a relatively large buffer of remnant vegetation surrounding the lake compared to the other urban sites, protecting the lake from urban runoff as most of its water is recharged from rainfall (Newport and Lund 2014). Interestingly, Joondalup had the highest mean and maximum concentrations of total Hg, both exceeding the guideline's low trigger value. Monitoring of Joondalup's surface water in recent years has detected Hg exceeding guideline concentrations in winter months; while the point source is still unknown the

annual spike suggests that the source is runoff from winter rains (Gonzalez-Pinto et al. 2017; Newport and Lund 2014).

Despite being outside the urban matrix, Yanchep National Park's wetland Loch McNess had elevated concentrations of As, Ba, Cd, Cr, Co, Mo and Zn. In addition, Hg and Se were detected concentrations exceeding their trigger values (Table 3). Loch McNess is defined as a 'flow-through lake', receiving water from the groundwater system to which it was connected, potentially receiving contaminants from bordering agricultural land. Yet over the past two decades it has been suffering from a severe surface water and groundwater decline resulting in its almost permeant disconnection from the groundwater (Department of Water 2011). As a result, large areas of lakebed sediment are now exposed and suffer from drying and erosion, then re-flooding with rain events. This process can release the sediments accumulated contaminants back into the wetland ecosystem (Al-Maarofi et al. 2013). Arsenic compounds are common and naturally occurring in many soils and wetlands of the Swan Coastal Plain (Appleyard et al. 2006), yet elevated concentrations in the sediment of Loch McNess could potentially be enhanced by groundwater contaminated from the historic use of pesticides on sheep (Arnold and Oldham 1997; Davis et al. 1993). Although Se is a necessary element for the normal development of organisms (Kapustka et al. 2004) it was detected at levels of concern in the sediments of Loch McNess (mean 4.04, max 7.9 mg/kg dry weight), and significantly higher concentrations than Bibra and Joondalup Lakes (Table 3). A point source cannot be determined but contamination might be from groundwater passing beneath agricultural irrigation and fertiliser use (Gardiner and Gorman 1963).

Only one sediment sample at one site (Bibra Lake) contained OCPs or PAHs, and the two detected OCPs and six out of seven PAHs exceeded sediment guidelines. The OCPs aldrin and its metabolite dieldrin have been banned from manufacture, importation, and use in Australia since the internationally legally binding agreement for OCPs (The Stockholm Convention on Persistent Organic Pollutants) was enacted in 2004 (DEH 2004; UNEP 2011). However, OCPs and their metabolites are highly persistent compounds that can be still present in the environment (Bai et al. 2015; Wu et al. 1999), and have been historically detected in the sediment of Perth's estuaries (Nice 2009) and wetlands (Davis et al. 1993). The presence of dieldrin and other OCPs detected in Perth's urban wetlands can be attributed to the state government program

to control Argentine ants (from 1950s to 1980s) and periodic termite control (Davis and Froend 1999; Davis and Garland 1986). Bibra Lake and other urban wetlands of Perth have been subject to heavy pesticide treatment in the past in an attempt to control non-biting midge and mosquito levels (City of Cockburn 2015; Davis and Froend 1999). PAHs have historically been found in the sediments of Bibra Lake (Department of Water 2009); and six out of seven detected PAHs exceeded the SQG trigger values. The hypothesized source is used vehicle oil and machinery fluids. The sampling site of our sediment containing both OCPs and PAHs is within 50 m down slope of a main road and may be frequently exposed to road pollution during rain events. Although this research only detected OCPs and PAHs in one sediment sample in one lake, the small sample sizes and large intra-site variation of contaminant concentrations between sediment samples should not rule out the possibility of OCPs and PAHs persisting in sediments at concentrations of concern in our other study sites or other wetlands of Perth.

Despite a high degree of intra-site variation in contaminant concentrations, generating knowledge on the sediment contaminant burden is important for highlighting the extent and history of wetland pollution (Förstner 2004). Generally, contaminants were in higher concentrations in the sediments of heavily urbanised compared to less-urbanised wetlands except for some metals in Loch McNess. The concentrations of Loch McNess sediments suggest that despite being outside of the urban matrix and surrounded by a protected National Park, contamination is still possible. High concentrations may originate from agriculture-contaminated groundwater that potentially contributes to wetland sediment contamination at levels comparable to highly urbanised wetlands. We recognise our number of sediment samples is both small and limited to the areas where snakes were captured, and thus might not be an accurate representation of contamination of the entire wetlands. Nevertheless, the samples do present a snapshot of contamination present in areas abundant with tiger snakes.

3.4.2 Occurrence of contaminants in snakes

Sixteen metals were detected in the livers of tiger snakes collected across Perth's wetlands in 2019 (discussed data and significant differences presented in Table 3.4). Beryllium was not analysed above detection limits and Sn was only above detection

limits in a single liver from a snake captured at Lake Joondalup. Antimony, As, Ba, Cd, Co, Hg, Mo and Se concentrations were significantly different between sites, mostly higher at Herdsman Lake compared with Lake Joondalup. Lead was detected in only four snakes: three from Loch McNess and one from Herdsman Lake. Sn was detected in only one snake from Lake Joondalup. Molybdenum concentrations in snakes from Herdsman Lake were significantly higher than in snakes at all other sites, and are as far as we can tell, the highest reported liver concentration in a reptile. These contaminants have all been documented in snakes before; however, the only one other publication reports a comprehensive suite of metals analysed in snakes (Wylie et al. 2009). Our study is the most comprehensive study to date on bioaccumulation in any terrestrial reptile in Australia. There were no significant differences between Cr, Mn, Ni, Ag and Zn liver concentrations between sites. No OCPs or PAHs were quantified above detection limits; however, both were detected from only one sediment sample at a single wetland, and PAHs are known to be metabolised quickly in vertebrates (Hylland 2006). In addition, due to small liver tissue mass (<10g) and broad-scale analysis detection limits had to be up to 2 mg/kg, thus we cannot conclude that these were not present under those concentrations. There is no quantitative toxicity thresholds available for OCPs and PAHs in reptiles; however, relative to other vertebrates toxicity results, <2 mg/kg is unlikely to induce adverse biological impact on snakes (Ball and Truskewycz 2013; Weir et al. 2013).

Table 3.4 Concentrations (mg/kg, wet weight) of metals detected in tiger snake livers (n = 5/site). Contaminants analysed at lower than detectable limits were not included. Given as: mean ± one SE (range); BDL = below detectable limits. Lower-case letter indicates Kruskal-Wallis significant difference (p = <0.05) between sites.

Contaminant	Herdsman Lake	Bibra Lake	Lake Joondalup	Loch McNess
Antimony	0.042 ± 0.005[a] (0.025 – 0.052)	0.018 ± 0.004 (0.007 – 0.03)	0.014 ± 0.002 (0.010 – 0.019)	0.006 ± 0.001[a] (0.003 – 0.008)
Arsenic	0.388 ± 0.023[a] (0.33 – 0.45)	0.098 ± 0.012[a] (0.06 – 0.13)	0.188 ± 0.082 (0.05 – 0.5)	0.276 ± 0.049 (0.18 – 0.44)
Barium	0.122 ± 0.015[a] (0.09 – 0.16)	0.039 ± 0.009[a] (<0.05 – 0.06)	0.32 ± 0.270 (<0.05 – 1.4)	0.089 ± 0.023 (0.025 – 0.140)
Cadmium	0.023 ± 0.004 (0.014 – 0.035)	0.015 ± 0.004[a] (<0.001 – 0.025)	0.02 ± 0.006 (0.006 – 0.039)	0.057 ± 0.013[a] (0.025 – 0.1)
Chromium	0.106 ± 0.016 (0.06 – 0.15)	0.123 ± 0.037 (<0.05 – 0.25)	0.137 ± 0.050 (<0.05 – 0.28)	0.068 ± 0.021 (<0.05 – 0.13)
Cobalt	0.080 ± 0.018[a,b] (0.054 – 0.15)	0.028 ± 0.004[a] (0.019 – 0.041)	0.021 ± 0.003[b,c] (0.013 – 0.031)	0.048 ± 0.006[c] (0.032 – 0.064)
Copper	7.28 ± 1.006 [a,b] (4.9 – 9.8)	3.9 ± 0.376 [a,c] (3.1 – 5.2)	3.68 ± 0.676 [b,d] (2.4 – 5.9)	9.84 ± 1.518 [c,d] (6.6 – 15.0)
Lead	0.007 ± 0.004 (<0.05 – 0.024)	<0.05	<0.05	0.047 ± 0.028 (<0.05 – 0.15)
Manganese	0.83 ± 0.048 (0.7 – 0.94)	0.75 ± 0.018 (0.70 – 0.79)	0.664 ± 0.051 (0.49 – 0.76)	0.848 ± 0.152 (0.54 – 1.4)
Mercury	0.164 ± 0.009[a,b] (0.14 – 0.19)	0.061 ± 0.02[a,c] (BDL – 0.12)	0.064 ± 0.014[b,d] (0.01 – 0.09)	0.29 ± 0.068[c,d] (0.14 – 0.39)
Molybdenum	8.32 ± 1.547[a,b,c] (4.8 – 13.0)	1.36 ± 0.214[a] (0.6 – 1.9)	1.98 ± 0.666[b] (0.7 – 4.2)	1.36 ± 0.144[c] (1.0 – 1.8)
Nickel	0.275 ± 0.121 (<0.01 – 0.64)	0.372 ± 0.100 (0.03 – 0.61)	0.42 ± 0.139 (0.05 – 0.91)	0.218 ± 0.046 (0.05 – 0.32)
Selenium	0.988 ± 0.061 (0.84 – 1.2)	1.03 ± 0.139 (0.67 – 1.4)	0.49 ± 0.058[a] (0.34 – 0.66)	1.53 ± 0.208[a] (0.95 – 2.2)
Silver	0.014 ± 0.002 (0.007 – 0.017)	0.007 ± 0.002 (0.002 – 0.012)	0.006 ± 0.002 (0.002 – 0.01)	0.008 ± 0.001 (0.004 – 0.011)
Tin	<0.05	<0.05	0.058 ± 0.033 (<0.05 – 0.19)	<0.05
Zinc	24 ± 2.168 (20.0 – 32.0)	21.4 ± 1.600 (17.0 – 27.0)	21.6 ± 1.077 (19.0 – 24.0)	24.6 ± 1.721 (21.0 – 31.0)

Currently, the information available on the bioaccumulation of contaminants and their effects on reptiles is limited but is a growing field of research. Although many studies report various contaminants in snakes (Albrecht et al. 2007; Burger et al. 2007; Burger et al. 2017; Campbell et al. 2005; Drewett et al. 2013; Heydari Sereshk and Riyahi Bakhtiari 2015; Quintela et al. 2019), we could only directly compare our data to seven metals (As, Cd, Cr, Hg, Mn, Pb, Se) commonly analysed as wet weight in the livers

of three other wetland snakes *Nerodia fasciata*, *Thamnophis gigas*, *Agkistrodon piscivorus conanti* (see summary table in Wylie et. al. 2009 and Hopkins et al. 1999; Rainwater et al. 2005; Wixon 2013). Mean and maximum concentrations of these metals in Perth's tiger snakes were similar to concentrations reported in other wetland snakes, besides As and Se that were much lower in Perth's tiger snakes compared with other snakes from contaminated sites, and Perth's tiger snakes had less than half the concentration of Mn and Hg reported in other wetland snakes regardless of site contamination. For the less frequently analysed metals, we could only compare the liver concentrations of Perth's tiger snakes to livers from *A. piscivorus* and *T. gigas* (Wixon 2013; Wylie et al. 2009). Mean liver concentrations of Sb, Co, Ag and Sn were similar in tiger snakes compared with those reported in *A. piscivorus* and *T. gigas*, whereas Ba, Cu, Ni and Zn concentrations varied between the three species. Molybdenum was similar for all wetland snakes except for tiger snakes from Herdsman Lake, where it was much higher (mean 8.32, max 13.0 mg/kg wet weight).

Very few metals were positively correlated with body size except for Sb (rho 0.6, *p* 0.35), Hg (rho 0.87, *p* 0.05) and Ni (rho 0.8, *p* 0.13) in Herdsman Lake snakes; Sb (rho 0.6, *p* 0.35), Cr (rho 0.7, *p* 0.23) and Ag (rho 1, *p* 0.02) in Bibra Lake snakes; As (rho 0.8, *p* 0.13) in Lake Joondalup snakes; and Sb (rho 0.82, *p* 0.09) in Loch McNess snakes. We consider these results to be exploratory, and low significances is reflective of small sample sizes due to financial and ethical limitations. These results, however, suggest the uptake of most metals does not appear to be related to body size, a common observation in bioaccumulation research (Albrecht et al. 2007; Fontenot et al. 2000; Quintela et al. 2019). Although our study didn't detect any metals at alarmingly high concentrations, we identified nine metals of interest based on their significant difference between sites in the sediment or liver samples, and we consider these to be the contaminants of most concern. We use Fig. 3.2 to compare the inter-site differences in metal levels between sediment and liver concentrations. Antimony, Cd, Co, Mo and Se had almost identical inter-site patterns between the mean contaminant concentrations of the sediment and livers. Copper, As and Ba had mostly similar inter-site differences although there was high variation in the snake's livers. Mercury concentrations in snakes from Loch McNess reflected the concentrations in the sediment, although both had high inter-sample variation; concentrations were lower in snakes from Joondalup than the sediment and much higher in Herdsman Lake snakes

than the sediment. Lead and Sn showed generally inverse inter-site relationships between sediment and snake liver concentrations. Despite Zn exceeding the SQG higher trigger value in sediment from Herdsman Lake, it was found at consistent concentrations in Perth's tiger snake livers as it is metabolically regulated in vertebrates (Sandstead 2014).

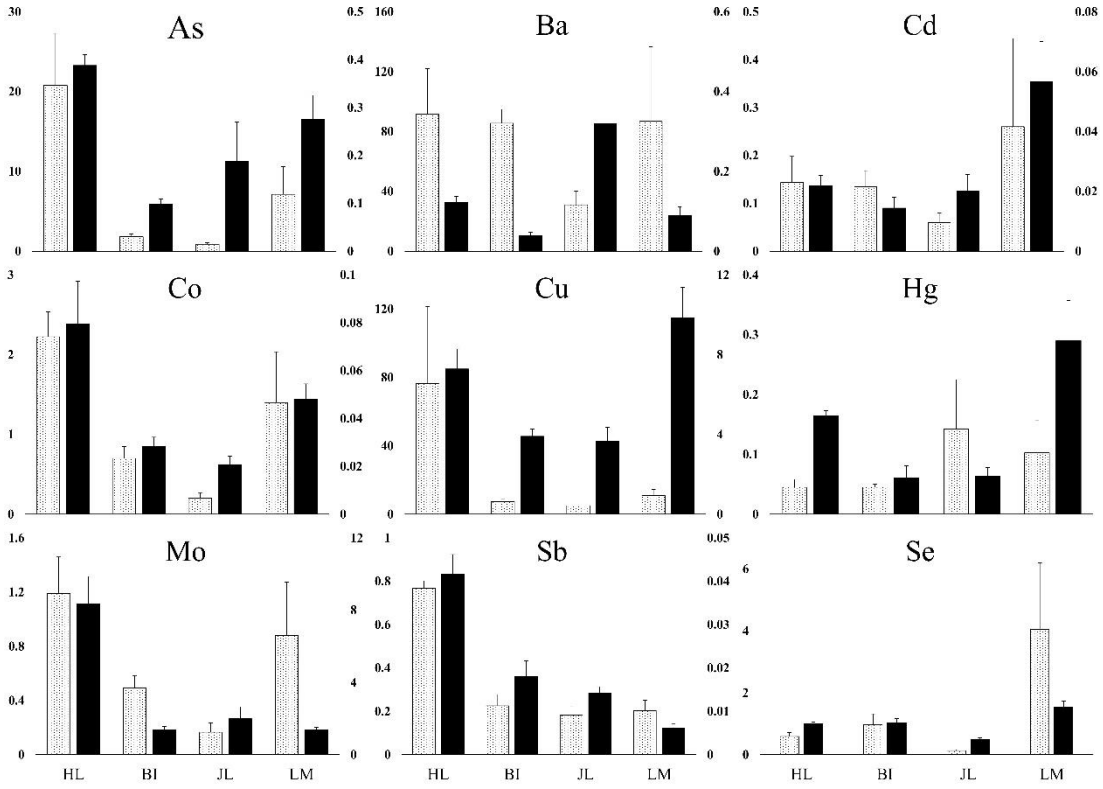

Fig. 3.2 The comparison between metals that were statistically significant in either the sediment or tiger snake livers between sites. Patterned bars = mean sediment concentration; black bars = mean snake liver concentration. Sediment concentration corresponds to the left axis and liver to the right axis, except for Hg and Se that share the same range. Error bars = SE; HL = Herdsman Lake, BI = Bibra Lake, JL = Lake Joondalup, LM = Loch McNess (located in Yanchep National Park).

The intra-site variation in contaminant concentration was generally higher in sediment than it was in snake livers (Tables 3.3 & 3.4). Variation is naturally high in sediment due to different point sources of contaminants, seasonal changes of water chemistry and soil grain-sizes. Biological tissues such as livers, are more homogenous in composition than sediments and consequently show a lesser inter-individual and inter-site variability in contaminant burdens than sediments do; thus, warrant the use of fauna as bioindicators of environmental health. The use of wetland snakes as

bioindicators has been proposed for decades (Campbell and Campbell 2001; Haskins et al. 2019; Stafford et al. 1977) based on their trophic status and site fidelity (Campbell et al. 2005). Despite limited positive correlations between body size and metals Fig. 3.2 suggests there is a strong relationship between sediment and snake liver concentrations within sites. These results are consistent with the general pattern of metal accumulation in fauna increasing if the population is exposed to a source of contamination (Nasri et al. 2017), in this case the sediment. Therefore, our results support the use of tiger snakes as a bioindicators of wetland ecosystem health based on (1) the evidence that they accumulate a range of metals, (2) their long life expectancy of up to 24 years (Fearn and Norton 2011), (3) their small home ranges (~0.05 km^2) (Butler et al. 2005) especially in wetlands isolated by urbanisation, and (4) their high trophic position in the food chain as adults (thus being exposed to all of their prey's contaminants).

Tiger snakes are directly exposed to the contaminants in their wetland through drinking the water, foraging in sediment (Orange 2007) when wetland waters annually recede, and through the consumption of their prey. Frogs, being tiger snakes' preferred prey (Aubret et al. 2006; Lettoof et al. 2020), are particularly sensitive to contaminant accumulation as they feed in sediment as tadpoles, have the ability to absorb chemicals through their skin (Bruhl et al. 2011; Smalling et al. 2015), and stay in close proximity to waterbodies (e.g. ~20 m for *Litoria raniformis*), especially in urban isolated wetlands (Hamer and Organ 2008; Hitchings and Beebee 1997). Lemaire et al. (2018) compared the Hg concentrations in scales of various populations of viperine snakes (*Natrix maura*) that predate on frogs or on aquaculture-raised fish, and found lower mean contaminant concentrations and accumulation rates in frog-eating snake populations. This difference may be due to frogs generally being at a lower trophic tier than fish and potential Hg contamination from the aquaculture farms from which snakes were sampled (Lemaire et al. 2018). We believe frogs are an important vector for contaminants in the food web, and recommend testing them to better understand the food chain transfer of contaminants in Perth's urban wetlands, a research area that is lacking in Australia (Mann et al. 2009; Sievers et al. 2019). Due to financial limitations and the wetlands being inhabited by several species of frogs we chose to investigate bioaccumulation in tiger snakes as they should reflect the contamination of that particular food web.

In addition, tiger snakes may have a shorter lifespan in urban wetlands as a result of exposure to multiple stressors and therefore may not have the opportunity to accumulate large concentrations of contaminants. Such stressors include harassment from the public, pet dogs, vegetation management and parasitism, as tiger snakes often have large burdens of gastric nematodes (Lettoof et al. 2020). Although the high frequency of parasitism suggests tolerance, the influence of contaminants and parasites on tiger snake populations may be more complex. Chronic symptoms from synergistic contaminants may result in immunosuppression and increasing nematode parasitism (Rohr et al. 2008), or a reduction in feeding behaviour (Alonso et al. 2009; Forrow and Maltby 2000) which may imperil individual snakes who cannot consume enough food to feed both themselves and the nematodes. From another point of view, a large parasite burden may have beneficial impacts on tiger snakes, because parasites are able to uptake contaminants directly and reduce the concentrations in their host (Evans et al. 2001).

3.4.3 Potential impacts to snakes

As with other high trophic predators, the presence and persistence of higher trophic snakes represents a healthy ecosystem, and yet ecotoxicological research on snakes is still relatively rare compared to research on other predators (Campbell and Campbell 2001). Snakes can provide crucial insight into the distribution and movement of contaminants through wetland food webs in comparison to most other top predators (usually mammals and birds) based on several characteristics: entirely carnivorous, comparatively small home ranges, longevity and progression up trophic tiers throughout their life history. Six metals, two OCPs and seven PAHs of the 52 screened contaminants were detected at concentrations of concern in the sediment of wetlands across Perth. Of the 16 metals detected in tiger snakes from these wetlands, nine were of significantly different concentrations between sites. Although we did not detect very high concentrations of metals that may cause acute toxicity in tiger snakes, the generally higher concentrations of most tested contaminants in snakes from the most urbanised wetland, Herdsman Lake, may have synergistic chronic effects on snake health. Only a handful of studies have been conducted on the acute or chronic impacts metal exposure and accumulation has on reptiles. For example, the presence of blood Hg concentrations have been found to negatively correlate with lymphocyte and B-cell proliferation in Loggerhead sea turtles (*Caretta caretta*), suggesting suppression

of the immune system (Day et al. 2007). Lead and Cd, both detected in snake livers at higher concentrations in Loch McNess and Herdsman Lake than other sites, have reported lethal doses. The approximate concentration of Pb and Cd required to kill western fence lizards (*Sceloporus occidentalis*) is 2000 mg/kg and 1480 mg/kg of body weight respectively (Brasfield et al. 2004; Salice et al. 2009), which is considered mid-range sensitivity when compared with other taxa. The concentrations we detected in Perth's tiger snakes were much lower than these and are unlikely to have acute clinical impacts on the populations.

Experimental exposure of several metals to reptiles has received more interest. Dietary exposure to Se, for example, can lower reproduction likelihood and potentially reduce egg and total mass of clutches in brown house snakes (*Boaedon fuliginosus*) (Hopkins et al. 2004); reduce average food intake and growth in body mass and length in leopard geckos (*Eublepharus macularius*) (Rich and Talent 2009). Banded water snakes (*N. fasciata*) from polluted wetlands with high liver concentrations of As and Se (mean As: 134 ppm; Se 140 ppm dry weight) exhibited mean standard metabolic rates 32% higher than snakes from unpolluted sites (Hopkins et al. 1999). Corn snakes (*Elaphe guttata*) feed methylmercury experienced a reduced growth rate (Bazar et al. 2002), whereas new-born northern water snakes (*N. sipedon*) contaminated with maternally-transferred Hg had less motivation to feed and had reduced strike efficiency (Chin et al. 2013). Developing embryos can also be exposed to metals through the egg shell (Marco et al. 2004). Consequently, absorption of Cd can cause malformation of eye development in the Italian wall lizard (*Podarcis sicula*) (Simoniello et al. 2014) and the running speed of hatchling Iberian rock lizard (*Iberolacerta monticola*) was negatively correlated with embryonic As absorption (Marco et al. 2004). Metal body burdens or exposure levels that cause physiological or behavioural alterations in the aforementioned studies are well above what we observed in the livers of our adult tiger snakes from the Swan Coastal Plain; however, chronically contaminated reptiles are susceptible to a variety of pernicious developmental and behavioural disorders. Snakes from the urban sites Herdsman and Bibra Lake seem to be in poor health conditions (observation by the authors), which parallels sediment and liver contaminant levels. We have not as part of the present study measured behavioural parameters or biochemical markers of health however complimentary studies are initiated to investigate potential health impacts of contaminants on tiger snakes (Chapters 4 & 7).

Interestingly, tiger snakes from Herdsman Lake had the highest mean liver concentration of Mo reported in a reptile (8.32 ± 3.458 mg/kg). Molybdenum is an essential element for vertebrate nutrition, however, excessive exposure to laboratory rats, guinea pigs and rabbits has been associated with anaemia, diarrhoea, deformities of joints and long bones, mandibular exostoses and morphological changes to the liver, kidneys and spleen (Tallkvist and Oskarsson 2015). Molybdenum exists naturally in various oxidation states but is mined as a principle ore for use as an alloy in steel and cast iron production, and also used in lubricants, chemical reagents and dyes. Stormwater drainage and urban runoff from the bordering industrial area are likely point-sources for Mo contamination in Herdsman Lake.

3.4.4 Future research and recommendations

Laboratory testing for ecotoxicology is expensive and usually charged per contaminant (for example, the cost of this study was over $11 000 AUD). This warrants a trade-off between broad-scale screening with small sample sizes, or targeted screening focussing on contaminants of concern and much larger samples. Most other studies choosing the latter had contamination spill events or access to published recent monitoring data allowing a focus on contaminants of concern. Despite the study sites being recognised as critical wetlands for the environment, major tourist and local attractions, and situated throughout a developed country's major city, contaminant monitoring of these wetlands has largely been disregarded; and as a result sample sizes were sacrificed for broad-scale screening. Periodic monitoring of contaminants should be conducted in urban wetlands to prevent researchers from investing limited resources on broad-scale screening and rather focus on contaminants of concern.

Ecotoxicological research on reptiles (especially snakes) is minimal at present, and fundamentally non-existent in Australia, despite consistent evidence of wetland snakes in the United States and other countries being good bioindicators of environmental contamination (Burger et al. 2007; Drewett et al. 2013; Lemaire et al. 2018; Quintela et al. 2019; Wixon 2013). Australia has several major cities with snakes persisting in urban wetlands or estuaries which should be susceptible to bioaccumulation of contaminants, and would be suitable systems to model the ecotoxicological impacts on snakes. Other model species include: the red-bellied blacksnake (*Pseudechis*

porphyriacus), lowlands copperhead (*Austrelaps superbus*), common tree snakes (*Dendrelaphis punctulatus*), slaty-grey snake (*Stegonotus australis*), keelback (*Tropidonophis mairii*), Australian bockadam (*Cerberus australis*), and Richardson's mangrove snake (*Myron richardsonii*).

3.5 Conclusions

This study conducted a broad-scale analysis for 52 contaminants in sediment samples of four wetlands spanning across an urban gradient of Perth, Australia. We detected six metals, two OCPs and six PAHs exceeding SQG trigger values in sediments, with generally higher mean concentrations of contaminants found in more heavily urbanised wetlands. The natural site, Loch McNess, despite being the least modified and furthest from urbanisation was an exception, as the sediments contained second highest concentrations for 8 of the 16 of the metals analysed. We hypothesize the source of contamination in Loch McNess is from inflowing groundwater contaminated by radial agricultural land, specifically the historic use of pesticides and fertilisers.

Arsenic, Cd, Co, Hg, Mo, Sb and Se measured in the livers of tiger snakes paralleled the pattern of contamination measured in the wetland sediments where snakes were collected, and should be the focus of future research. Hence we propose the use of high trophic wetland snakes, such as tiger snakes, as bioindicators of wetland contamination. Although these contaminants may not be the only ones accumulating in tiger snakes (e.g. other environments might have other persistent contaminants), we present the first data on these particular ecosystems. The potential impact of these metal mixtures is unknown; however, research is currently being conducted on the physical and biochemical markers of health in tiger snakes at these wetlands (Chapter 4 & 7). Despite wetland degradation related to urbanisation, urban wetlands often provide the last refuge for a wide diversity of wildlife species including higher trophic snakes.

Although our data are limited we chose to sacrifice sample size to allow screening of more contaminants, and quantify both environment and predator contaminants. Therefore this study presents a snapshot of the first broad-scale contaminant screening of an Australian snake, and one of few studies, to our knowledge, that complements contaminants in snakes with contaminants in wetland sediment. It also was the first detailed investigation of contaminants in four iconic wetlands of a capital city in a

developed country, reinforcing urgent government monitoring of urban wetland pollution to inform environmental management on actions required to preserve these highly productive and biodiverse habitats.

3.6 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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3.7 Chapter 3 addendum

3.4.3 – page 63

"Dietary exposure to Se, for example, can lower reproduction likelihood…".

This is a misinterpretation of the Hopkins et al. (2004) paper. The paper did not demonstrate a reduction in reproductive output (number and size of eggs) due to trace element contamination, rather found lower average reproductive likelihood (67% vs 91%) in Se exposed snakes, yet high variation made it difficult to discern from natural variability.

3.4.4 – page 64-65

"Other model species include: the red-bellied blacksnake (*Pseudechis porphyriacus*), lowlands copperhead (*Austrelaps superbus*), common tree snakes (*Dendrelaphis punctulatus*), slaty-grey snake (*Stegonotus australis*), keelback (*Tropidonophis mairii*), Australian bockadam (*Cerberus australis*), and Richardson's mangrove snake (*Myron richardsonii*)."

Water pythons (*Liasis fuscus*) and Arafura file snakes (*Acrochordus arafurae*) should also be considered as model species.

Quality Assurance Report

ChemCentre
EXPERT SOLUTIONS

Client : Curtin School of Environment & Agriculture
Client Ref No :
CoC No :
QA Report No : 18S2471-QA R 1

Analyte	Method	Unit	LoR	Blank	Sample Duplicates			Acceptable RPD	Recoveries		Acceptable Recovery
					Sample	Duplicate	RPD		LCS	Matrix Spike	
							%	%	%	%	%
Scientific Services Division											
Sample Matrix : sediment											
					18S2471/000						
Silver	iMET2SAMS	mg/kg	0.05	<0.05	-	-	-	20%	105%	-	75% - 125%
Arsenic	iMET2SAMS	mg/kg	0.2	<0.2	-	-	-	20%	106%	-	75% - 125%
Barium	iMET2SAICP	mg/kg	0.1	<0.1	-	-	-	20%	122%	-	75% - 125%
Beryllium	iMET2SAMS	mg/kg	0.05	<0.05	-	-	-	20%	100%	-	75% - 125%
Cadmium	iMET2SAMS	mg/kg	0.05	<0.05	-	-	-	20%	106%	-	75% - 125%
Cobalt	iMET2SAICP	mg/kg	0.1	<0.1	-	-	-	20%	104%	-	75% - 125%
Chromium	iMET2SAICP	mg/kg	0.05	<0.05	-	-	-	20%	115%	-	75% - 125%
Copper	iMET2SAICP	mg/kg	0.1	<0.1	-	-	-	20%	114%	-	75% - 125%
Mercury	iMET2SAMS	mg/kg	0.02	<0.02	-	-	-	20%	97%	-	75% - 125%
Manganese	iMET2SAICP	mg/kg	0.2	<0.2	-	-	-	20%	111%	-	75% - 125%
Molybdenum	iMET2SAMS	mg/kg	0.05	<0.05	-	-	-	20%	129%	-	75% - 125%
Nickel	iMET2SAMS	mg/kg	0.1	<0.1	-	-	-	20%	108%	-	75% - 125%
Lead	iMET2SAICP	mg/kg	0.5	<0.5	-	-	-	20%	103%	-	75% - 125%
Antimony	iMET2SAMS	mg/kg	0.05	<0.05	-	-	-	20%	102%	-	75% - 125%
Selenium	iMET2SAMS	mg/kg	0.05	<0.05	-	-	-	20%	106%	-	75% - 125%
Tin	iMET2SAICP	mg/kg	0.5	<0.5	-	-	-	20%	112%	-	75% - 125%

Quality Assurance Report

Analyte	Method	Unit	LoR	Blank	Sample	Duplicate	RPD	Acceptable RPD	LCS	Matrix Spike	Acceptable Recovery
Zinc	iMET2SAICP	mg/kg	5	<5	-	-	-	20%	108%	-	75% - 125%
18S2471/002											
a-BHC	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Acenaphthylene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Acenaphthene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
a-Chlordane	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
alpha-Endosulfan	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Aldrin	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Anthracene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
b-BHC	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
beta-Endosulfan	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Benz(a)anthracene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Benzo(a)pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Benzo(g,h,i)perylene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	30%	-	-	60% - 130%
Chrysene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
d-BHC	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
DDD	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
DDE	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
DDT	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Dibenzo(a,h)anthracene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Dieldrin	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endosulfan sulphate	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endrin	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endrin aldehyde	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endrin Ketone	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Fluoranthene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Fluorene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
g-Chlordane	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Moisture, loss at 105C.	ORG160	%	0.1	<0.1	81	83	2%	10%	-	-	75% - 125%
Heptachlor	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Hexachlorobenzene	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Indeno(1,2,3-cd)pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Lindane (g-BHC)	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%

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Analyte	Method	Unit	LoR	Blank	Sample	Duplicate	RPD	Acceptable RPD	LCS	Matrix Spike	Acceptable Recovery
Methoxychlor	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Naphthalene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	30%	-	-	60% - 130%
Oxychlordane	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Phenanthrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
18S2471/004											
Acenaphthene	ORG100S	mg/kg	0.5	<0.5	<0.5	-	-	20%	-	113%	60% - 140%
Pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	-	-	20%	-	110%	60% - 140%
Lindane	ORG141S	mg/kg	0.01	<0.01	<0.01	-	-	10%	-	88%	75% - 125%
Heptachlor	ORG141S	mg/kg	0.01	<0.01	<0.01	-	-	10%	-	98%	75% - 125%
Dieldrin	ORG141S	mg/kg	0.01	<0.01	<0.01	-	-	10%	-	94%	75% - 125%
Endrin	ORG141S	mg/kg	0.01	<0.01	<0.01	-	-	10%	-	81%	75% - 125%
18S2471/022											
a-BHC	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Acenaphthylene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Acenaphthene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
a-Chlordane	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
alpha-Endosulfan	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Aldrin	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Anthracene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
b-BHC	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
beta-Endosulfan	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Benz(a)anthracene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Benzo(a)pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Benzo(g,h,i)perylene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	30%	-	-	60% - 130%
Chrysene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
d-BHC	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
DDD	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
DDE	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
DDT	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Dibenzo(a,h)anthracene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Dieldrin	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endosulfan sulphate	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endrin	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%

Endrin aldehyde	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Endrin Ketone	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%

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Quality Assurance Report

Analyte	Method	Unit	LoR	Blank	Sample	Duplicate	RPD	Acceptable RPD	LCS	Matrix Spike	Acceptable Recovery
Fluoranthene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Fluorene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
g-Chlordane	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Moisture, loss at 105C.	ORG160	%	0.1	<0.1	88	89	1%	10%	-	-	75% - 125%
Heptachlor	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Hexachlorobenzene	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Indeno(1,2,3-cd)pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Lindane (g-BHC)	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Methoxychlor	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Naphthalene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	30%	-	-	60% - 130%
Oxychlordane	ORG141S	mg/kg	0.01	<0.01	<0.01	<0.01	<1%	10%	-	-	75% - 125%
Phenanthrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%
Pyrene	ORG100S	mg/kg	0.5	<0.5	<0.5	<0.5	<1%	20%	-	-	60% - 140%

Definitions:

RPD = Relative Percentage Difference
LCS = Laboratory Control Sample
LoR = Limit of Reporting

Quality Control Acceptance Criteria

Waters:

Lab Dups RPD <10% for results greater than 5 X LOR.
For results less than 5 x LOR no acceptance criteria for RPD.
Matrix spikes, LCS and Surrogate recoveries: Generally 75% - 125% for inorganics/metals, 60% - 140% for organics (+/- 50% surrogates) and 10% - 140% for labile SVOCs (including labile surrogates) unless other values are stated above.

Soils:

Soils: Lab Dups RPD<20% for results greater than 5 X LOR.
For results less than 5 x LOR no acceptance criteria for RPD.
Matrix spikes, LCS and Surrogate recoveries: Generally 75% - 125% for inorganics/metals, 60% - 140% for organics (+/- 50% surrogates) and 10% - 140% for labile SVOCs (including labile surrogates) unless other values are stated above.

Quality Assurance Report

ChemCentre
EXPERT SOLUTIONS

Client : Curtin School of Environment & Agriculture
Client Ref No :
CoC No :
QA Report No : 18S4132-QA R 0

Analyte	Method	Unit	LoR	Blank	Sample Duplicates			Acceptable RPD	Recoveries		Acceptable Recovery
					Sample	Duplicate	RPD		LCS	Matrix Spike	
							%	%	%	%	%
Scientific Services Division											
Sample Matrix : Biota											
18S4132/005											
Antimony	iMET1BTMS	mg/kg	0.001	<0.001	0.007	0.006	15%	10%	122%	-	75% - 125%
Arsenic	iMET1BTMS	mg/kg	0.01	<0.01	0.27	0.26	4%	10%	113%	-	75% - 125%
Barium	iMET1BTICP	mg/kg	0.05	<0.05	<0.05	<0.05	<1%	10%	113%	-	75% - 125%
Beryllium	iMET1BTMS	mg/kg	0.001	<0.001	<0.001	0.001	<1%	10%	105%	-	75% - 125%
Cadmium	iMET1BTMS	mg/kg	0.001	<0.001	0.037	0.037	<1%	10%	112%	-	75% - 125%
Chromium	iMET1BTMS	mg/kg	0.05	<0.05	0.06	0.06	<1%	10%	108%	-	75% - 125%
Cobalt	iMET1BTMS	mg/kg	0.001	<0.001	0.037	0.036	3%	10%	116%	-	75% - 125%
Copper	iMET1BTICP	mg/kg	0.01	<0.01	9.5	9.6	1%	10%	100%	-	75% - 125%
Lead	iMET1BTMS	mg/kg	0.005	<0.005	<0.005	<0.005	<1%	10%	94%	-	75% - 125%
Manganese	iMET1BTICP	mg/kg	0.05	<0.05	0.54	0.55	2%	10%	92%	-	75% - 125%
Mercury	iMET1BTMS	mg/kg	0.01	<0.01	0.18	0.18	<1%	10%	94%	-	75% - 125%
Molybdenum	iMET1BTICP	mg/kg	0.01	<0.01	1.1	1.2	9%	10%	103%	-	75% - 125%
Nickel	iMET1BTMS	mg/kg	0.01	<0.01	0.2	0.22	9%	10%	106%	-	75% - 125%
Selenium	iMET1BTMS	mg/kg	0.01	<0.01	1.3	1.3	<1%	10%	100%	-	75% - 125%
Silver	iMET1BTMS	mg/kg	0.001	<0.001	0.008	0.007	7%	10%	110%	-	75% - 125%
Tin	iMET1BTMS	mg/kg	0.05	<0.05	<0.05	<0.05	<1%	10%	100%	-	75% - 125%
Zinc	iMET1BTICP	mg/kg	1	<1	23	24	4%	10%	94%	-	75% - 125%
18S4132/003											
a-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%

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Acenaphthylene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Acenaphthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
a-Chlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
alpha-Endosulfan	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Aldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
b-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
beta-Endosulfan	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benz(a)anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(a)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(b)fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(e)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(k)fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(g,h,i)perylene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Chrysene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
d-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDD	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDE	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDT	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Dibenzo(a,h)anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Dieldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Endrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Endosulfan sulfate	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Fluorene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
g-Chlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Heptachlor epoxide	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Heptachlor	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Hexachlorobenzene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Indeno(1,2,3-cd)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%

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Lindane (g-BHC)	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Methoxychlor	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Naphthalene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Oxychlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
18S4132/005											
Acenaphthene	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	64%	75% - 125%
Aldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	72%	75% - 125%
Lindane (g-BHC)	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	71%	75% - 125%
18S4132/011											
a-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Acenaphthylene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Acenaphthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
a-Chlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
alpha-Endosulfan	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Aldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
b-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
beta-Endosulfan	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benz(a)anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(a)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(b)fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(e)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(k)fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(g,h,i)perylene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Chrysene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
d-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDD	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDE	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDT	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%

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Dibenzo(a,h)anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Dieldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Endrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Endosulfan sulfate	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Fluorene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
g-Chlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Heptachlor epoxide	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Heptachlor	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Hexachlorobenzene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Indeno(1,2,3-cd)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Lindane (g-BHC)	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Methoxychlor	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Naphthalene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Oxychlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
18S4132/012											
Acenaphthene	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	72%	75% - 125%
Aldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	67%	75% - 125%
Lindane (g-BHC)	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	82%	75% - 125%
18S4132/022											
a-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Acenaphthylene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Acenaphthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
a-Chlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
alpha-Endosulfan	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Aldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
b-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
beta-Endosulfan	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%

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Benz(a)anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(a)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(b)fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(e)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(k)fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Benzo(g,h,i)perylene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Chrysene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
d-BHC	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDD	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDE	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
DDT	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Dibenzo(a,h)anthracene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Dieldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Endrin	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Endosulfan sulfate	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Fluoranthene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Fluorene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
g-Chlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Heptachlor epoxide	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Heptachlor	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Hexachlorobenzene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Indeno(1,2,3-cd)pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Lindane (g-BHC)	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Methoxychlor	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Naphthalene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Oxychlordane	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
Pyrene	ORG100B	mg/kg	0.5	<0.5	<2.0	<2.0	<1%	10%	-	-	75% - 125%
18S4132/023											
Acenaphthene	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	73%	75% - 125%
Aldrin	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	74%	75% - 125%

Lindane (g-BHC)	ORG100B	mg/kg	0.5	<0.5	<2.0	-	-	10%	-	67%	75% - 125%

Definitions:
RPD = Relative Percentage Difference
LCS = Laboratory Control Sample
LoR = Limit of Reporting

Quality Control Acceptance Criteria
Waters:
Lab Dups RPD <10% for results greater than 5 X LOR.
For results less than 5 x LOR no acceptance criteria for RPD.
Matrix spikes, LCS and Surrogate recoveries: Generally 75% - 125% for inorganics/metals, 60% - 140% for organics (+/- 50% surrogates) and 10% - 140% for labile SVOCs (including labile surrogates) unless other values are stated above.
Soils:
Soils: Lab Dups RPD<20% for results greater than 5 X LOR.
For results less than 5 x LOR no acceptance criteria for RPD.
Matrix spikes, LCS and Surrogate recoveries: Generally 75% - 125% for inorganics/metals, 60% - 140% for organics (+/- 50% surrogates) and 10% - 140% for labile SVOCs (including labile surrogates) unless other values are stated above.

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12-Feb-2020

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Chapter 4. Plasma biochemistry profiles of wild western tiger snakes (*Notechis scutatus occidentalis*) before and after six months of captivity

The study presented in Chapter 4 was accepted in the peer-reviewed journal '*Journal of Wildlife Diseases*' on 14 October 2020, and is an exact reproduction of the copyright paper reformatted for this thesis.

Lettoof, D. C., Aubret, F., Spilsbury, F., Bateman, P.W., Haberfield, J., Vos, J., Gagnon, M. M. Plasma Biochemistry Profiles of Wild Western Tiger Snakes (*Notechis scutatus occidentalis*) before and after Six Months of Captivity. (2021). *J Wildl Dis*. 57(2):253-63. doi: 10.7589/JWD-D-20-00115.

4.1 Abstract

Urban wildlife often suffer poorer health than their counterparts living in more pristine environments due to exposure to anthropogenic stressors such as habitat degradation and environmental contamination. As a result, the health of urban versus non-urban snakes might be assessed by differences in their plasma biochemistries. We compared the plasma profiles of Western tiger snakes (*Notechis scutatus occidentalis*) from a heavily urbanized wetland and a natural, non-urbanized wetland. Despite the urbanized snakes having lower body mass index, we found no significant difference between the plasma profiles of the two populations. We collected snakes from each population and kept them in captivity for 6 mo providing them with stable conditions, uncontaminated (exempt from heavy metals and pesticides) food and water, and lowered parasite intensity in an attempt to promote better health through depuration. After captivity, snakes experienced a significant improvement in body mass index, and significant changes in their plasma profiles. Snakes from the natural wetland initially had more variation of DNA damage; mean concentration of DNA damage in all snakes slightly decreased, but not significantly, after captivity. We present the plasma biochemistry profiles from Western tiger snakes both before and after captivity and suggest a period of removal from natural stressors via captivity may offer a more reliable result of how plasma profiles of healthy animals might appear.

4.2 Introduction

Assessing and monitoring health and disease in reptiles is a complex and often difficult process, particularly if there are no health parameter baseline references for healthy individuals. Measurable signs of unhealthy individuals such as low body condition scores, wounds, and infections, and high parasite burdens can be obvious; however, as reptiles often do not show physiological changes until very late stages of many ailments (Selleri and Hernandez-Divers 2006) a normal-appearing reptile may not be reflecting its current health condition. As an alternative, plasma biochemistry profiles can provide an important snapshot of the physiological health of free-living individuals (Eatwell et al. 2014) as analytes and biomarker measurements can be linked to disease (Jacobson et al. 1991), tissue damage (Campbell 2006), and contaminant toxicity (Koch and Hill 2017; Komoroske et al. 2011; Villa et al. 2017) before physical signs can be detected. However, the plasma profile reference profiles for healthy individuals must first be determined for each species and, for reptiles, there are few baseline measurements available compared to other taxa (Campbell 2006). In addition, developing a reference interval is more difficult for reptiles than for other taxa as reptilian plasma analytes can be influenced by physiological traits such as age, sex, feeding and reproductive status (Coz-Rakovac et al. 2011) and environmental conditions for example, season, and toxin exposure (Arthur et al. 2008).

Many ecosystems around the world are increasingly affected by the rapid expansion of urbanization. Nevertheless, many wildlife populations persevere or thrive in urban environments (Bateman and Fleming 2012; Luniak 2004). Generalist and opportunistic species usually adjust to or benefit from disturbed environments, while species with more specific ecological requirements merely persist within suitable remnant fragmented habitats (French et al. 2018); however, urbanization is generally regarded as detrimental to wildlife health (Murray et al. 2019). Common stressors to which urban wildlife are exposed include noise, human and machine disturbance, light pollution, and toxic contaminants (French et al. 2018). Exposure to any or all these stressors can have a significant impact on the health of urban wildlife (Liker et al. 2008; Murray et al. 2019; Winchell et al. 2019). Identifying and assessing stressors and understanding their impacts on wildlife health can be a difficult task, yet this is crucial for conservation and management of urban wildlife.

The Australian tiger snake (*Notechis scutatus*) is a ~ 1 m elapid commonly found occupying wetlands and wet forests in cool climate areas with high rainfall. Populations currently persist in many urban wetlands, including those in Perth, Western Australia (Lettoof et al. 2020c) and Melbourne, Victoria (Butler et al. 2005). Western tiger snakes (*Notechis scutatus occidentalis*) have received considerable research attention focusing on evolutionary plasticity and behaviour (Aubret 2015; Aubret et al. 2011; Bonnet et al. 2002), and several studies alluding to signs of Perth urban tiger snakes suffering poor health. For example, snakes in a highly urbanized wetland (Herdsman Lake, central Perth) have a high proportion and degree of tail loss and injury (Aubret 2005; Aubret et al. 2005). Urban tiger snakes in Perth are accumulating a suite of heavy metals and anticoagulant rodenticide (Lettoof et al. 2020a; Lettoof et al. 2020b). We hypothesized that tiger snakes in urban wetlands suffer poorer health than do tiger snakes in natural wetlands and predicted that this would be reflected in their plasma biochemistry. By removing snakes from these wetlands, holding them over a period of captivity with fresh water, uncontaminated food, and worming treatment and measuring their plasma profiles before and after captivity we aimed to determine the plasma profiles of healthy tiger snakes. Our objectives were to compare the differences in plasma biochemistry profiles between tiger snakes from a highly urbanized wetland and from a natural wetland, to measure the change in plasma profiles after a period of captivity, and to explore the plasma biochemistry profile changes that may reflect healthier tiger snakes.

4.3 Methods

4.3.1 Field collection and sites

In October 2018, 20 adult (i.e., >530 mm snout-vent length; Shine (1987)), tiger snakes were hand collected from two sites in the greater Perth region of Western Australia; 10 (seven males and three females) from the heavily urbanized wetland Herdsman Lake (HL; 31°55′12″S, 115° 4819″E), and 10 (six males and four females) from Loch McNess (YC; 31°32′44″S, 115° 40′50″E), a wetland beyond the fringe of urban Perth and located within Yanchep National Park. These sites were selected based on their similarity of structure and proximity to each other, yet are characterized by very different degrees of urbanisation. The sites also differ in sediment contamination of metals. Lettoof et al. (2020b) report sediment concentrations of

arsenic, copper, lead and zinc in Herdsman Lake exceeding trigger values of the Australian and New Zealand guidelines (ANZECC & ARMCANZ 2000) and the revised Australia and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand Sediment Quality Guidelines (Simpson et al. 2013); mercury and selenium levels from Loch McNess were also found to exceed trigger values. Tiger snakes from Herdsman Lake have higher liver concentrations of antimony, arsenic, barium, chromium, cobalt, molybdenum, and silver, whereas Loch McNess snakes had higher cadmium, copper, lead, mercury and selenium (Lettoof et al. 2020a).

4.3.2 Data collection

On the day of capture, body weight, snout-vent length (SVL), and tail length were recorded for each snake. An external examination was conducted for four types of parasites (ticks, skin worms, gastric nematodes and oral trematodes), for injuries and wounds, and the stomach was palpated for detection of food. On the day of capture, a blood sample was taken from the heart using a lithium heparin coated 23G needle, placed in a heparinised tube and centrifuged within 30 min of extraction at 2000 x G for 10 min at room temperature. Plasma was separated into three vials per snake, rapidly frozen in liquid nitrogen and stored at -80 C until analysed. The first blood sample was taken within 6 h of snake capture to best represent wild plasma profiles, and the second sample was taken after 6 mo of captivity. To reduce influences of feeding on plasma analyte concentrations, snakes were fasted for 2 weeks before the second blood sampling. Body weight was recorded monthly at two weeks after feeding.

4.3.3 Snake husbandry

Snakes were housed individually in locked plastic tubs (70 x 50 x 40 cm) and provided with a water bowl, plastic hide, and shredded hemp as bedding. Water and cleaning was provided ad libitum but a maximum of once a week. Smaller snakes were offered a thawed adult mouse once every 2 weeks, and once a week for larger snakes. The majority of snakes ate every feeding day. The holding laboratory was temperature controlled, ranging from 16–20 C, and a looped strip of 32 C heat cord passed beneath one end of each tub, allowing for thermoregulation. The room was lit with natural light to allow natural periods of activity. Snakes were collected and held from October to

April reflecting their peak seasonal activity. Animal care and sample collection was approved by the Animal Ethics Committee at Curtin University (ARE2018-23) and the Department of Biodiversity, Conservation and Attractions (No. 08-002624-02).

4.3.4 Deworming treatment

We treated all snakes to a deworming regime because Western tiger snakes are frequently infected with the gastric nematode *Ophidascaris pyrrhus* (Lettoof et al. 2020c). Fenbendazole (Panacur 100, Intervet Australia, Bendigo East, Victoria, Australia) was administered orally for three monthly treatments increasing in dosage (10; 20; 40 mg/kg) and each treatment consisted of four dosages, one dose every four days. We collected faeces before and after treatment and conducted standardised faecal floats but no eggs were detected.

4.3.5 Plasma biochemistry profile, oxidative DNA damage and HSP70

The plasma concentration of aspartate aminotransferase (AST), bile acids (BA), creatine kinase (CK), uric acid (UA), glucose (GLU), total calcium (CA), phosphorus (PHOS), total protein (TP), albumin (ALB), globulin (GLOB), potassium (K) and sodium (NA) were measured using VetScan Avian/Reptilian Profile Plus reagent rotor in the VetScan Whole Blood Analyzer (Abaxis, Inc., Union City, California). The coefficients of variation for each analyte using the VetScan Avian/Reptilian Profile Plus reagent rotor are: AST (3.6-4.3%), BA (4.5-4.9%), CK (3.6-6.0), UA (3.9-4.8%), GLU (1.4-1.6%), CA (2.9-3.4%), PHOS (2.6-4.9%), TP (1.2-1.9%), ALB (3.6-4.3%), GLOB (3.5-4.4%), K (5.7-6.3%) and NA (1.6-1.8%).

Measures of DNA damage have been frequently used as a biomarker of metal-induced toxicity and carcinogenesis in organisms (Finlayson et al. 2019; Kasprzak 2002; Simonyan et al. 2018). The by-product, 8-hydroxy-20-deoxyguanosine (8-oxo-dG), is the most studied and detected indicator of oxidative DNA damage (Olsson et al. 2012) and is formed during DNA replication (Haghdoost et al. 2005). We measured the concentration of 8-oxo-dG in tiger snake plasma before and after captivity using the HT 8-oxo-dg ELISA Kit II (Trevigen, Gaithersburg, Maryland, United States). The analysis was performed as outlined by the manufacturer.

The stress protein HSP70 is a commonly used biomarker of environmentally induced stress (Tsan and Gao 2004). As there are no commercial HSP70 ELISA kits available

specifically for reptiles, we tested an avian HSP70 ELISA for measurement of HSP70 in tiger snake plasma (Chicken Heat Shock 70 KDa Protein ELISA Kit, Abbexa, Milton, Cambridge, United Kingdom). This trial was unsuccessful, however, with only non-specific binding evident.

4.3.6 Statistical analysis

We compared changes in body condition calculated as Body Mass Index (BMI=[body mass (g)/SVL^2 (cm)]x100) of snakes over the period of captivity using a repeated measures analysis of variance (ANOVA) with individual snakes as the random factor (Zipkin et al. 2020). We assigned each snake a single SVL measurement (the mean of their before and after captivity lengths) due to the margin of error when measuring snakes (Rivas et al. 2008).

A Principle Component Analysis (PCA) was undertaken to identify plasma profile differences between site, sex and captivity status (Le et al. 2008). The PCA analysis does not allow for missing data and so for the purposes of statistical analysis, analytes below the limit of detection (LOD) were assumed to be the value of half the LOD, analytes above the detection range were assumed to be the upper detection limit, and individual analytes that were missing (due to instrument error or to death of an individual before completion of the trial) were assumed to be the mean of that analyte for snakes before and after captivity, respectively.

Multiple comparisons of analytes were performed by one-way ANOVA followed by Tukey's test. Data were considered significantly different with alpha = 0.05. To account for variation due to dehydration, 8-oxo-dG concentrations were normalised by total serum protein (Gagnon and Rawson 2016). All statistical analyses were conducted using R statistical software (R Studio version 4.0.2; R Development Core Team 2019).

Detection of outliers was done with Reference Value Advisor, version 2.1 (Geffre et al. 2011). Outliers were identified visually as well as statistically using Tukey methods and the Dixon-Reed test, and removed if deemed an aberrant observation (e.g., high AST and CK values due to failed cardiac puncture attempts).

4.4 Results

One individual from each site died prior to the conclusion of the experiment. The HL snake was in the poorest condition of all snakes upon capture and rarely ate; it died within 2 mo of captivity. The post-mortem examination revealed no obvious pathologies so we assumed that this snake may have been close to its natural death. The YC snake ate every feeding day and was in good condition for 4 mo. It regurgitated a semi-digested mouse 6 d after feeding, and died 2 d after that. The post-mortem revealed no obvious abnormalities except it had a large nematode burden (n=42). It is possible that the snake's stomach may have been blocked by the dead nematodes after the deworming treatment. Plasma analytes and DNA damage concentrations from these snakes were included in the mean values for before captivity.

4.4.1 Body condition

Snakes from HL were of lower mean (± SD) body condition (BMI 3.82 ± 0.33 SD) than were YC snakes (BMI 4.19 ± 0.69 SD) upon capture. There was no significant difference between the mean body condition of both sexes from each site throughout the experiment (t-test, p = 0.975). Snakes increased in body condition significantly over the 6 mo of captivity (ANOVA, $p < 0.001$; Fig. 4.1), but the interaction between site and month was not significant (p = 0.069). At the end of captivity, the mean body condition of HL snakes was similar (BMI 5.05 ± 0.43 SD) to YC snakes (BMI 4.95 ± 1.01 SD). The YC snakes had higher variation in body condition (sample variance 0.86) than did HL snakes (sample variance 0.54) throughout the entire captive period.

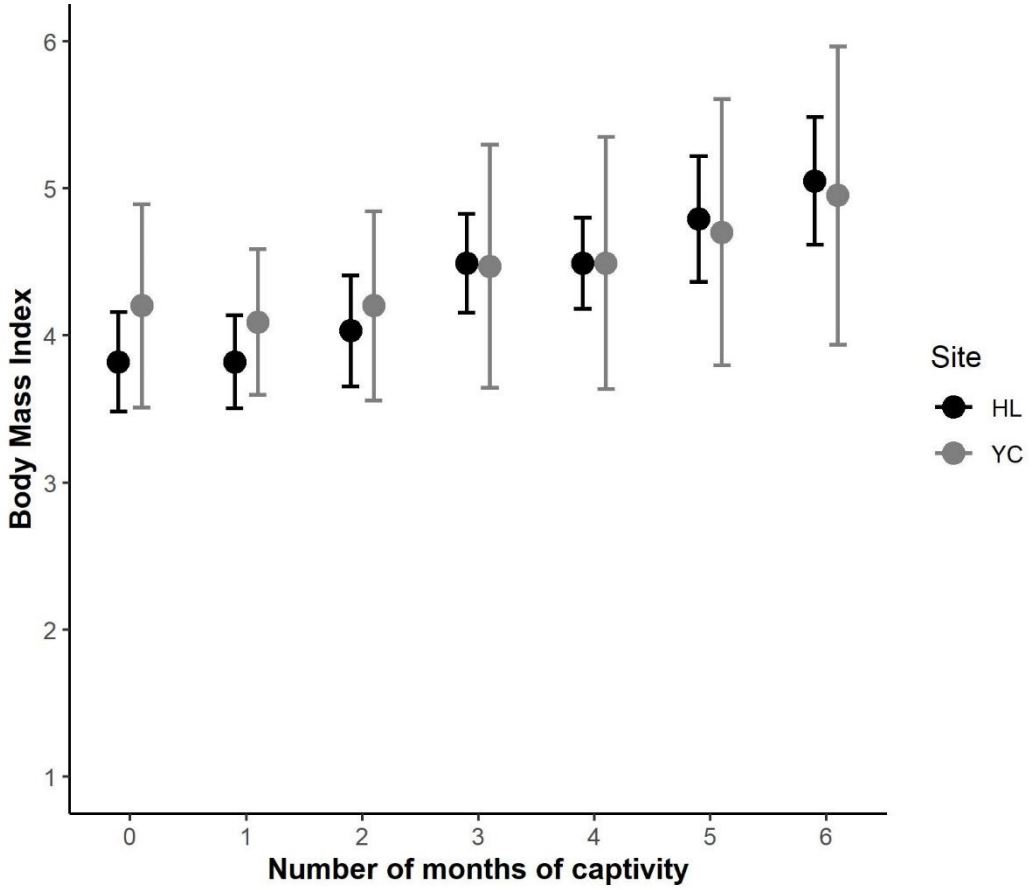

Fig. 4.1 The mean Body Mass Index (BMI) change of Western tiger snake (Notechis scutatus occidentalis) from two populations over 6 mo of captivity. Month 0 = BMI upon capture. Error bars represent standard deviation. HL = Herdsman Lake (urbanised wetland) and YC = Loch McNess, Yanchep National Park (natural wetland).

4.4.2 Plasma biochemical profiles

As the concentrations of BA were below the limit of detection for all but two snakes, we did not report them. The PCA revealed two principle component axes, Dim1 and Dim2 (dimension), which account for 53.6% of the total variability. Plasma biochemistry profiles were significantly different for snakes before and after captivity (Dim1 ANOVA, $p < 0.001$), but not between snakes from sites (Dim1 ANOVA, $p = 0.625$), nor between snakes of different sexes (Dim1 ANOVA, $p = 0.342$; Fig 4.2a, b, c). Specifically, UA, GLU, PHOS were significantly lower ($p < 0.001$) after captivity, and TP, ALB and GLOB were significantly higher ($p < 0.001$) after captivity (Fig 4.3). The DNA damage biomarker was not significantly different after captivity, (ANOVA, $p = 0.551$) with mean 8-oxo-dG concentrations decreasing from 0.83 ± 0.33 SD nmol/g

to 0.77 ± 0.35 SD nmol/g pre- and post-captivity, respectively. Plasma analyte profiles are presented for both before and after captivity in Table 4.1.

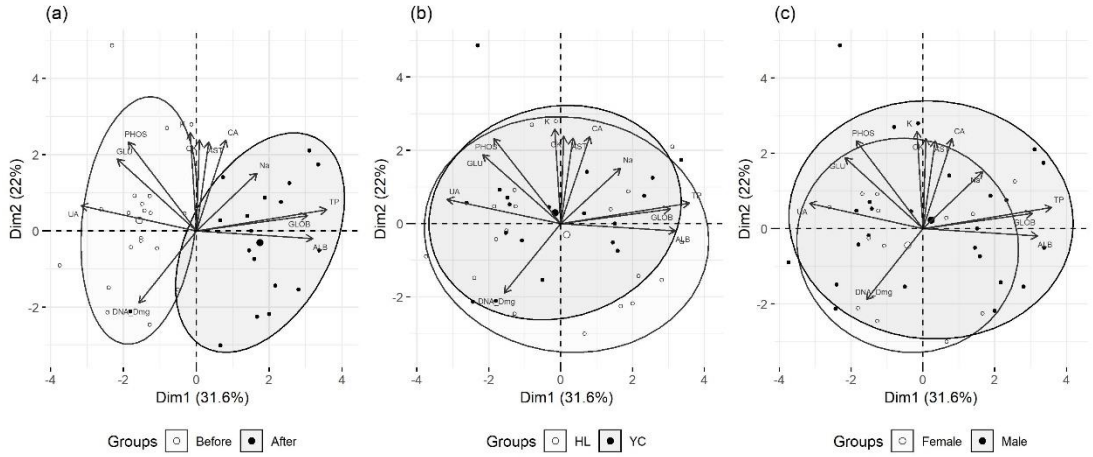

Fig. 4.2 Principle Component Analysis of Western tiger snake (*Notechis scutatus occidentalis*) plasma biochemistry profiles grouped by a) change over captivity, b) site and c) sex. Before = wild snakes upon capture, After = the same snakes after 6 mo of captivity. Snakes are from two populations around Perth, Western Australia: HL = Herdsman Lake (urbanized wetland) and YC = Yanchep National Park (natural wetland).

Table 4.1: Biochemistry profiles and descriptive statistics of Western tiger snakes (*Notechis scutatus occidentalis*) before and after 6 mo captivity in 2019 - 2020. Snakes are from Herdsman Lake (urbanized wetland) and Yanchep National Park (natural wetland) around Perth, Western Australia. Outliers were removed if $n < 20$ for before captivity and $n < 18$ after captivity.

Analyte (units)	Captivity	*n*	Mean	Standard deviation	Median	Range
Aspartate	Before	18	28.9	14.1	30.5	3 – 57
transaminase (U/L)	After	16	32.2	17.3	29.5	3 - 70
Creatine kinase	Before	19	920.7	439.2	907	249 – 1924
(U/L)	After	16	876.6	543.5	734	122 - 1682
Uric acid (μmol/L**)**	Before	18	323.3	103.7	302.5	197 – 522
	After	18	172.4	67.0	162	106 – 333
Glucose (mmol/L)	Before	20	6.7	2.5	6.9	2.1 – 11.7
	After	16	2.8	0.6	2.8	2 – 3.9
Calcium (mmol/L)	Before	20	3.8	0.9	3.5	2.2 – 6
	After	18	3.8	0.5	3.7	2.4 – 4.8
Phosphate (mmol/L)	Before	20	1.3	0.3	1.2	0.8 – 2.1
	After	18	0.9	0.2	0.8	0.5 – 1.4
Total protein (g/L)	Before	19	58.4	8.5	60	42 – 69
	After	17	79.9	10.4	81	51 – 96
Albumin (g/L)	Before	20	12.3	3.7	13	5 – 19
	After	15	18.1	3.7	18	9 – 23
Globulin (g/L)	Before	17	45.7	7.3	46	31 – 56
	After	16	60.8	10.1	61	43 – 78
Potassium (mmol/L)	Before	20	6.1	0.9	5.9	4.9 – 7.9
	After	18	5.9	0.6	5.9	4.9 – 6.7
Sodium (mmol/L)	Before	20	150.8	11.6	153.5	123 – 169
	After	18	156.9	7.1	158.5	142 – 167

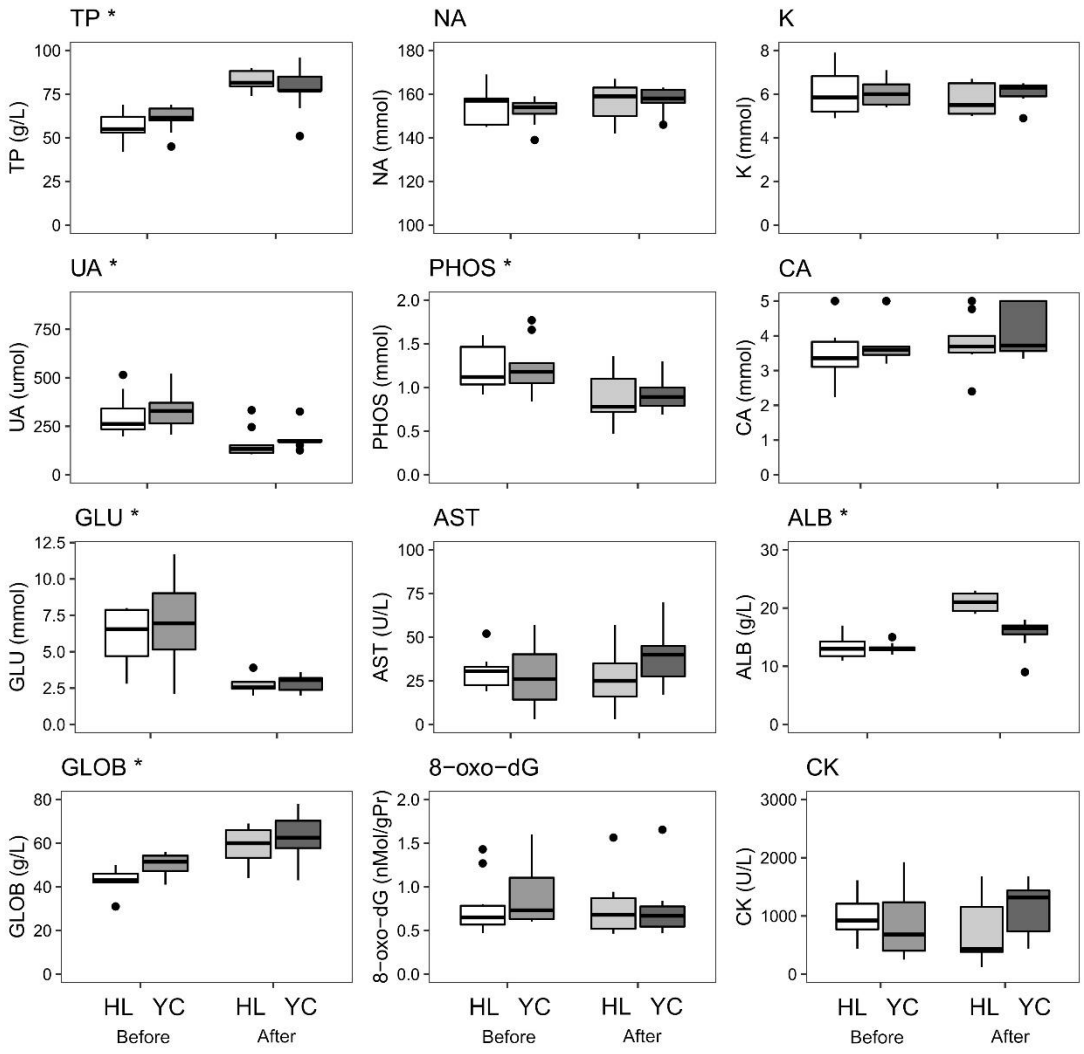

Fig. 4.3 Box-and-whisker plots of Western tiger snake (*Notechis scutatus occidentalis*) plasma analytes from HL = Herdsman Lake (urbanised wetland) and YC = Yanchep National Park (natural wetland) before and after 6 mo of captivity. Black horizontal line = median. Analytes with an asterisk indicate significant ($p < 0.05$) changes after captivity.

4.5 Discussion

We compared the plasma profiles of wild-caught (before captivity) adult tiger snakes from a heavily-urbanized wetland (HL) to those from a natural wetland (YC), with the hypothesis that snakes from the former site would be in poorer health than those from the latter and this would be reflected by differences in their plasma profiles. Surprisingly, there was no significant difference in the plasma profiles of tiger snakes between the urbanized wetland (HL) and the natural wetland (YC) or between male and female snakes (Fig. 4.2b, c), despite snakes from HL being of lower body condition and generally exposed to higher concentrations of metals and anthropogenic

disturbance compared to snakes from YC (Lettoof et al. 2020a). Other studies have found strong differences in plasma profiles of organisms exposed to different environmental stressors, for instance in green turtles (*Chelonia mydas*) exposed to the toxic cyanobacterium *Lyngbya majuscula* (Arthur et al. 2008), pigeon guillemots (*Cepphus columba*) from oil-spill areas (Seiser et al. 2000) and Libyan jirds (*Meriones libycus*) from heavy metal contaminated urban sites (Adham et al. 2011). Our results suggested either the stress or contaminants accumulated by tiger snakes at HL are not different enough to influence the plasma profiles, or tiger snakes from YC are experiencing similar stress and contaminant accumulation. However, snakes from these sites may suffer from other impacts to their health, such as immunosuppression and aberrant behaviour, which are not detected in plasma profiles but warrant further investigation.

Our results also suggested that developing baseline plasma profiles based on wild populations may not accurately represent the plasma profiles of healthy individuals, or the analytes we measured may not be diagnostically sensitive for poor health in tiger snakes, as snakes from HL were noticeably in poorer condition upon capture yet their plasma profiles were similar to YC snakes. Following 6 mo of captivity with minimal stressors, the experimental depuration period resulted in a significant increase in body condition for all snakes (Fig. 4.1), and a significant change in plasma profiles (Fig. 4.2a).

By the end of captivity snakes from both sites were of similar mean body condition. Yanchep snakes had much higher body condition variation throughout the experiment than those from HL as YC snakes took food infrequently, possibly due to what appeared to be their more defensive and nervous behaviour. The increase in body condition for all snakes was likely influenced by the captive diet. Western tiger snakes primarily eat frogs; as frogs are not commercially available as pet food in Australia, we were restricted to feeding them mice, which provide more calories compared to frogs (Wiseman et al. 2019). Also, snakes from both sites may have been of lower than normal condition and returned to a healthier condition during captivity. Shedding nematodes by worming treatment may have also contributed to an increase in body condition.

Stress can negatively impact the health of snakes. Some stressors of wild snakes include fluctuations in food availability, habitat disturbance and contamination from urbanization, predation, and conspecific competition (Van Waeyenberge et al. 2018). We removed snakes from these stressors, but captivity can present different stressors, such as unfamiliar scents, reduced freedom of movement, and different food. Snakes exhibit acute stress responses from capture and short-term (3 days – 8 weeks) captivity (Mathies et al. 2001; Sykes and Klukowski 2009), but only one study measured stress in snakes over a longer period. Sparkman et al. (2014) found wild-caught gravid garter snakes (*Thamnophis elegans*) plasma corticosterone levels and heterophil-to-lymphocyte ratios increased over 4 mo of captivity. We had no obviously pregnant females so these results may not be comparable to our study.

Acute and chronic stress responses are also highly variable between species and individuals (Fischer and Romero 2019; Sparkman et al. 2014). We attempted to reduce acute stress in snakes by minimizing interaction (3 days/week) and feeding them uncontaminated food and water over the duration of captivity. We consider any potential stress to be chronic. Measuring chronic stress in captive snakes is difficult but obvious symptoms are behaviour changes, increase in lesions and infections, and weight loss (Van Waeyenberge et al. 2018). We did not detect any increase in lesions or infections, and most tiger snakes ate almost immediately when presented with food, and increased in weight. Although we attempted to measure stress (HSP70) there was no reactivity between the avian assay and tiger snake plasma; however, we consider the lack of chronic stress symptoms to be an indication that these snakes were less stressed than in the wild.

Baseline healthy plasma profiles are difficult to establish for reptiles due to their variable physiology. The concentration of plasma analytes can be influenced by the season (Bryant et al. 2012; Machado et al. 2006), sex and reproductive status (Christopher et al. 1999; Coz-Rakovac et al. 2011), diet, and time between feeding (Moon et al. 1999; Smeller et al. 1978; Stinner and Ely 1993). We kept tiger snakes in stable conditions for 6 mo to minimize these variables and consider the plasma analyte concentrations after captivity to better reflect those of healthier Western tiger snakes freed of multiple environmental stressors.

By the end of this period of captivity concentrations of TP, ALB and GLOB in tiger snake plasma significantly increased and UA, GLU and PHOS concentrations significantly decreased. Total protein, UA, and GLU, can be influenced by the diet, hydration, and nutritional status in reptiles (Campbell 2006; Hamilton et al. 2016). We could not determine when the snakes had last eaten when they were sampled before capture, but they were fasted for 2 weeks before they were sampled after being placed into captivity so after captivity values were not influenced by recent feeding. We doubt that an increase in TP was caused from dehydration as snakes had a constant supply of fresh water, and there was neither a corresponding increase in UA nor electrolytes (NA, K and PHOS). Rather, TP concentrations may have increased in captivity from a more calorie-rich diet of mice, as well as from a lowered parasite intensity. As ALB concentrations generated by the dye-binding methods are inaccurate compared to protein electrophoresis (Muller and Brunnberg 2010), caution must be taken when interpreting reptile ALB for health assessments. Furthermore, as GLOB concentration is calculated from the TP and ALB values, our globulin results were likely influenced by the change in diet and hydration during captivity.

Glucose and UA of tiger snakes before captivity were significantly higher and highly variable compared to those after captivity. As both of these analytes will increase from recent feeding (Lam and Halán 2017; Moon et al. 1999; Smeller et al. 1978) and GLU can increase due to stress (Laderberg 2015; Skoczylas and Sidorkiewicz 1974; Stinner and Ely 1993), it is possible that the high concentrations and variations of these analytes can be attributed to recent feeding and stress of capture. Concentrations of GLU also increase and UA concentration can decrease during the season of peak activity (Coz-Rakovac et al. 2011; Silva et al. 2011). However, a decrease in GLU concentration can also be influenced by high protein diets (Campbell 2006) and lower body temperatures (Laderberg 2015). Plasma PHOS concentrations are often used to test for renal disease and nutritional deficiency in reptiles (Campbell 2006; Knotek et al. 2003); however, the concentrations and the PHOS:CA ratio were not high enough to suggest that either of these conditions occurred in our snakes. The concentration of PHOS can also increase during periods of sexual activity (Coz-Rakovac et al. 2011); the lower PHOS levels after captivity probably reflect both the end of the breeding season and the fasting period.

We could not determine what parameters influenced the snake plasma profiles, but they were likely a combination of fasting, diet, and season. However, with the provision of an uncontaminated diet, deworming treatment, and reduction of stressors throughout captivity we consider the plasma profiles of snakes after captivity to be a better representation of healthier tiger snakes. We recommend plasma profiles be measured in wild Western tiger snakes at our sites during similar seasons (October and April) to disentangle the influence of season on analytes. We also recommend measuring plasma profiles of snakes in October from sites with notably fewer stressors to see if plasma profiles are closer to our after-captivity results, to establish if these profiles reflect healthier snakes. Our results can be used as baselines for future comparative studies to assess the health of other Western tiger snake populations and for veterinary diagnosis of captive snakes.

Surprisingly, snakes from HL expressed lower mean 8-oxo-dG concentrations than did YC snakes upon capture. After captivity, snakes from HL had slightly higher variation in 8-oxo-dG and YC snakes less variation (Fig. 4.3). A slight, not statistically significant, reduction in mean 8-oxo-dG concentrations for all snakes after captivity suggested that their removal from contaminated environments for 6 mo may have instigated depuration, but the elapsed time was insufficient to see statistically significant changes due to the slow metabolism of reptiles. Measuring oxidative DNA damage is often used as a biomarker for metal toxicity (Collins et al. 1996; Kasprzak 2002), and high variation in biomarkers is often an indicator of contaminant accumulation (Gagnon and Rawson 2016; van der Oost et al. 2003). Despite being exposed to generally lower concentrations of contaminants, snakes from YC were found to have higher liver concentrations of cadmium, copper, lead, mercury, and selenium (Lettoof et al. 2020a), and perhaps these metals are more genotoxic than the metals (antimony, arsenic, barium, chromium, cobalt, molybdenum and silver) that HL snakes are exposed to. The limited number of studies make it difficult to conclude whether changes in reptilian DNA are influenced by genotoxic agents or are a result of natural phenomena (Novillo et al. 2006), and more research is needed to determine if 8-oxo-dG is a reliable biomarker of contaminant toxicity in reptiles.

4.6 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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4.7 Chapter 4 addendum

4.3.4 – page 96

Further details relating to the lack of nematode egg detection, that were removed from the published manuscript due to word limit.

We collected each scat and conducted standardised faecal floats in attempt to quantify presence of nematode eggs and thereby monitor loss of infection; however, no eggs were detected. This could be due to the rapid loss of available eggs (through desiccation or hatching larvae) if the scats are not inspected soon after deposition (Crawley et al. 2016). The lack of eggs could also be due to O. pyrrhus not laying in the snakes' digestive tract; although this is the presumed life history of this nematode species it has never been confirmed (Lettoof et al. 2020a). All but one snake did, however, pass dead nematodes in the scats post-treatments.

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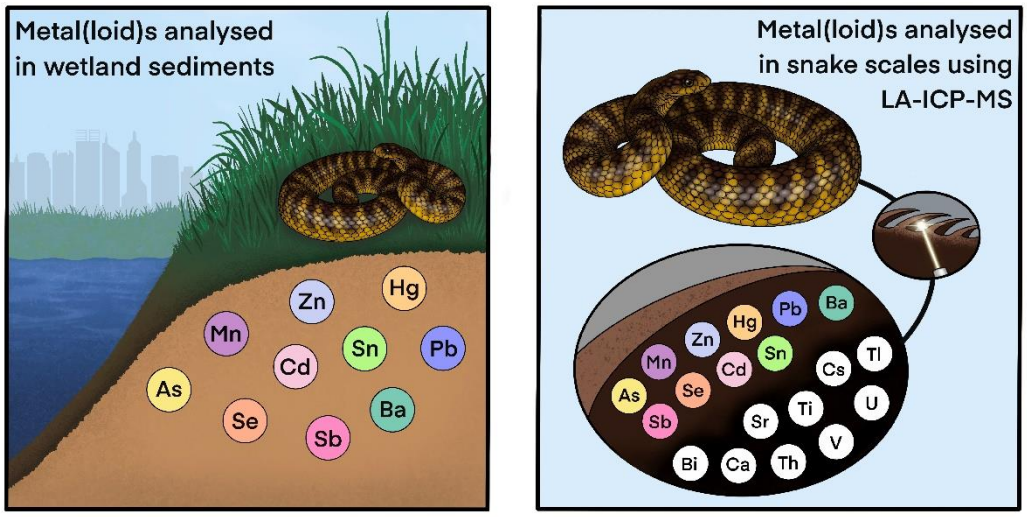
Chapter 5. Snake scales record environmental metal(loid) contamination

The study presented in Chapter 5 was accepted in the peer-reviewed journal '*Environmental Pollution*' on 16 January 2021, and is an exact reproduction of the copyright paper reformatted for this thesis.

Lettoof, D. C., Rankenburg, K., McDonald, B. J., Evans, N. J., Bateman, P. W., Aubret, F., Gagnon, M. M. (2021). Snake scales record environmental metal(loid) contamination. *Environ Pollut*. 274:116547 doi: 10.1016/j.envpol.2021.116547.



Credit: Alana de Laive

5.1 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.

5.2 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.

5.3 Methods

5.3.1 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 5.1 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).

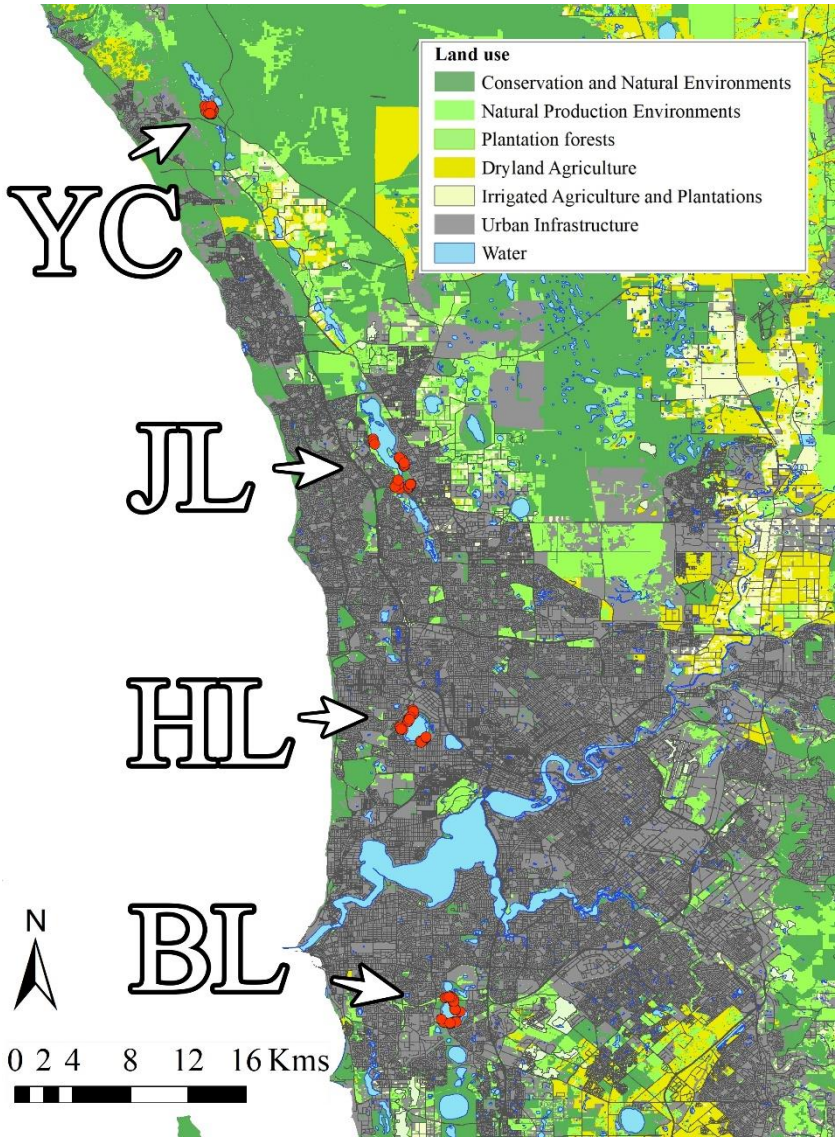


Fig. 5.1 Map of the land uses of the study area, Perth, Western Australia, based on the 2016 Australian Land Use and Management Classification (Version 8). Red dots indicate Western tiger snakes (*Notechis scutatus occidentalis*) sampled for scale analysis. YC = Loch McNess in Yanchep National Park, JL = Lake Joondalup, HL = Herdsman Lake and BL = Bibra Lake.

5.3.2 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°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.

5.3.3 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.

5.3.4 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.

5.4 Results and Discussion

5.4.1 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 5.1). 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.

Table 5.1 The analytical precision and accuracy of elements between testing runs. LOD = Limit of detection, all values are ppm, n.m. = not measured, n.r. = not reported, 2s = two standard error either side of the mean.

Metal	Typical LOD	1st run	2nd run	3rd run	4th run	5th run	6th run	Average	2s	Cert. value	2s	Potential isobaric interferences	Accuracy
^{25}Mg	0.06	95	94	71	97	65	108	**88**	33	**248**	14	C_2H	Rejected
^{27}Al	0.04	26	21	35	110	46	62	**50**	66	**23.2**	2	CN, HCN	Rejected
^{43}Ca	7	1267	1400	1122	1291	1109	1322	**1252**	230	**1537**	68		High
^{47}Ti	0.03	1.23	1.45	1.39	2.79	0.83	2.08	**1.63**	1.4	**2.1**	n.r.	SNH, SN, NO_2, NOH, CCl, PO	High
^{51}V	0.005	0.08	0.09	0.08	0.14	0.07	0.13	**0.099**	0.06	**0.089**	n.r.	SOH, ClO, ArC, ArN, ArNH, ClN, SN, SO	High
^{52}Cr	0.005	0.49	0.71	0.59	0.67	0.53	0.50	**0.58**	0.2	**8.74**	0.97	ClOH, ArC, ArO, ArN, ArNH, ClN, ClO, SO	Rejected
^{55}Mn	0.06	1.96	2.78	2.13	3.02	1.66	2.19	**2.29**	1.0	**3.83**	0.39	ArNH, ClO, ArN, ArO, ArOH, ClOH	Low
^{59}Co	0.001	0.046	0.094	0.027	0.029	0.034	0.036	**0.044**	0.05	**0.153**	0.015	ArOH	Rejected
^{60}Ni	0.001	0.449	0.081	0.059	0.194	0.104	0.36	**0.208**	0.32	**5.77**	n.r.		Rejected
^{63}Cu	0.002	16	21.6	13	22.9	11.6	17.1	**17.0**	9.1	**33.6**	2.3	PO_2, ArCNH, NCCl, OCCl	Rejected
^{66}Zn	0.03	282	477	315	374	318	349	**352.6**	137	**191**	16		Low
^{75}As	0.02	0.59	0.71	0.47	0.46	0.4	0.64	**0.55**	0.24	**0.198**	0.023	ArCl, Ar_2H, CPO_2	Low
^{77}Se	0.03	0.54	0.43	0.52	0.55	0.55	0.38	**0.49**	0.14	**0.59**	0.04	ArCl, Ar_2H	High
^{88}Sr	0.001	5.37	5.79	4.97	5.71	4.79	5.46	**5.35**	0.8	**8.17**	0.69		Low
^{95}Mo	0.001	0.2	0.36	0.21	0.19	0.21	0.24	**0.23**	0.13	**1.06**	0.12		Rejected
^{111}Cd	0.01	0.13	0.23	0.14	0.14	0.08	0.17	**0.15**	0.1	**0.072**	0.01		Low
^{118}Sn	0.001	0.25	0.22	0.13	0.5	0.13	0.23	**0.25**	0.27	**n.m.**	n.m.		Indicative
^{121}Sb	0.004	0.15	0.22	0.12	0.17	0.12	0.19	**0.16**	0.08	**0.12**	0.02		High
^{133}Cs	0.001	0.002	0.002	0.002	0.004	0.001	0.003	**0.002**	0.002	**n.m.**	n.m.		Indicative
^{137}Ba	0.001	7.06	7.1	6.32	8.37	5.81	8.00	**7.11**	1.94	**11.1**	1.3		Low
^{201}Hg	0.02	0.59	0.54	0.63	0.46	0.73	0.71	**0.61**	0.21	**1.06**	0.28		Low
^{205}Tl	0.003	0.012	0.014	0.01	0.011	0.01	0.01	**0.011**	0.003	**n.m.**	n.m.		Indicative
^{208}Pb	0.001	8.49	8.60	3.63	4.95	4.15	4.78	**5.77**	4.41	**3.83**	0.18		Low
^{209}Bi	0.001	0.030	0.055	0.02	0.045	0.01	0.03	**0.031**	0.033	**n.m.**	n.m.		Indicative
^{232}Th	0.0001	0.009	0.006	0.011	0.026	0.007	0.02	**0.014**	0.017	**n.m.**	n.m.		Indicative
^{238}U	0.0001	0.005	0.007	0.005	0.007	0.006	0.01	**0.007**	0.004	**n.m.**	n.m.		Indicative

Comparing the accuracy of measured concentrations in GBW09101b to the certified values led us to divide the data set into three categories of elements (Table 5.1): 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 5.1). 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.

5.4.2 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).

5.4.3 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 5.1), 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.

5.4.4 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.

5.4.5 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.

5.4.6 Comparison of metal content in wild snakes 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 5.2). 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. 5.2).

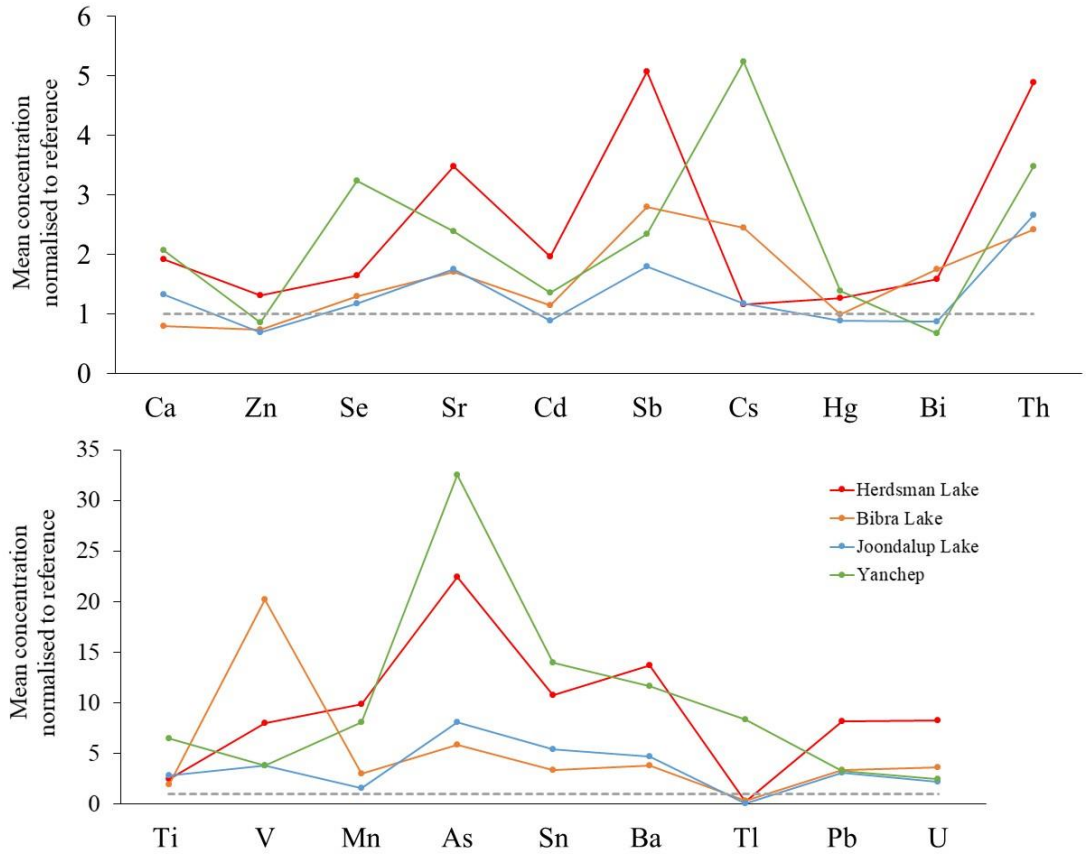


Fig. 5.2 A comparison of mean inter-site metal concentrations (ppm) in Western tiger snake (*Notechis scutatus occidentalis*) scales normalised to mean concentrations in reference tiger snake scales. Grey dotted line = normalised reference concentration.

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.

Table 5.2 Concentrations (mean ± 1 SE (range), ppm) of elements analysed in Western tiger snake (*Notechis scutatus occidentalis*) ventral scales. Lower case letters indicate Kruskal-Wallis significant difference ($p < 0.05$) between sites, # = all sites significantly different from each other.

	Reference (n = 9)	Herdsman Lake (n = 30)	Bibra Lake (n = 28)	Lake Joondalup (n = 29)	Yanchep (n = 26)
High accuracy					
Ca	379.61 ± 165.16[#]	729.07 ± 143.09[#]	301.59 ± 127.91[#]	506.83 ± 58.15[#]	785.18 ± 121.34[#]
	(24.35 – 1651.87)	(102.87 – 3694.93)	(48.59 – 3715.08)	(99.75 – 1305.41)	(217.42 – 3129.63)
Ti	0.153 ± 0.081[a]	0.382 ± 0.116	0.304 ± 0.052	0.433 ± 0.157	0.990 ± 0.580[a]
	(0.017 – 0.791)	(0.027 – 3.348)	(0.021 – 1.315)	(0.014 – 4.395)	(0.029 – 15.368)
V	0.057 ± 0.019[#]	0.458 ± 0.117[#]	1.156 ± 0.185[#]	0.220 ± 0.058[#]	0.219 ± 0.043[#]
	(0.006 – 0.138)	(0.011 – 2.827)	(0.027 – 3.433)	(0.006 – 1.654)	(0.007 – 0.763)
Se	0.413 ± 0.072[#]	0.681 ± 0.080[#]	0.538 ± 0.061[#]	0.489 ± 0.049[#]	1.334 ± 0.179[#]
	(0.195 – 0.949)	(0.210 – 1.912)	(0.264 – 1.956)	(0.204 – 1.402)	(0.385 – 4.029)
Sb	0.007 ± 0.002[a,b]	0.035 ± 0.007[a,c]	0.019 ± 0.004[b,e]	0.012 ± 0.003[e]	0.016 ± 0.003[c]
	(0.002 -0.017)	(0.003 – 0.210)	(0.003 – 0.097)	(0.001 – 0.087)	(0.003 – 0.077)
Low accuracy					
Mn	0.484 ± 0.120[a,b]	4.789 ± 2.078[a,c]	1.450 ± 0.444[d]	0.787 ± 0.128[c,e]	3.927 ± 1.580[b,d,e]
	(0.036 – 1.044)	(0.177 – 60.894)	(0.109 – 12.676)	(0.063 – 3.264)	(0.297 – 41.956)
Zn	49.379 ± 30.780	64.629 ± 23.104	36.667 ± 7.982	34.434 ± 3.809	42.395 ± 5.075
	(7.066 – 294.226)	(11.693 – 699.618)	(8.586 – 213.453)	(7.503 – 82.134)	(6.532 – 92.358)
As	0.047 ± 0.013[#]	1.052 ± 0.144[#]	0.273 ± 0.050[#]	0.381 ± 0.059[#]	1.525 ± 0.157[#]
	(0.008 – 0.135)	(0.159 – 3.304)	(0.050 – 1.140)	(0.078 – 1.860)	(0.534 – 3.353)
Sr	0.591 ± 0.274[a,b,c]	2.059 ± 0.569[a,d]	1.009 ± 0.472[d,e,f]	1.032 ± 0.150[b,e]	1.410 ± 0.262[c,f]
	(0.013 – 2.623)	(0.298 – 16.838)	(0.142 – 13.675)	(0.212 – 3.293)	(0.265 – 6.661)
Cd	0.005 ± 0.001	0.010 ± 0.004	0.006 ± 0.001	0.004 ± 0.001	0.007 ± 0.001
	(0.001 – 0.013)	(0.002 – 0.012)	(0.001 – 0.017)	(0.001 – 0.021)	(0.0004 – 0.017)
Ba	0.216 ± 0.064[#]	2.974 ± 1.002[#]	0.831 ± 0.411[#]	1.012 ± 0.238[#]	2.525 ± 0.634[#]
	(0.007 – 0.451)	(0.213 – 28.963)	(0.066 – 11.838)	(0.113 – 6.217)	(0.193 – 16.373)
Hg	0.228 ± 0.058[#]	0.290 ± 0.049[#]	0.225 ± 0.048[#]	0.204 ± 0.015[#]	0.317 ± 0.028[#]
	(0.092 – 0.637)	(0.103 – 1.221)	(0.109 – 1.457)	(0.096 – 0.412)	(0.150 – 0.672)
Pb	0.022 ± 0.009[a,b,c]	0.183 ± 0.073[a]	0.075 ± 0.025	0.070 ± 0.013[b]	0.074 ± 0.010[c]
	(0.004 – 0.085)	(0.012 – 2.164)	(0.011 – 0.693)	(0.004 – 0.269)	(0.013 – 0.247)
Indicative accuracy					
Sn	0.005 ± 0.012[#]	0.054 ± 0.015[#]	0.017 ± 0.012[#]	0.027 ± 0.008[#]	0.070 ± 0.018[#]
	(0.001 – 0.010)	(0.001 – 0.425)	(0.001 – 0.331)	(0.001 – 0.186)	(0.010 – 0.439)
Cs	0.005 ± 0.064[#]	0.005 ± 0.001[#]	0.012 ± 0.002[#]	0.005 ± 0.001[#]	0.025 ± 0.006[#]
	(0.008 – 0.020)	(0.001 – 0.031)	(0.001 – 0.036)	(0.0002 – 0.012)	(0.003 – 0.142)
Tl	0.017 ± 0.005[#]	0.004 ± 0.001[#]	0.005 ± 0.001[#]	0.002 ± 0.0004[#]	0.143 ± 0.032[#]
	(0.006 – 0.055)	(0.001 – 0.014)	(0.001 – 0.027)	(0.0004 – 0.009)	(0.014 – 0.783)
Bi	0.0004 ± 0.0001	0.0006 ± 0.0002	0.001 ± 0.0001[a,b]	0.0003 ± 0.00003[a]	0.0002 ± 0.00002[b]
	(0.0001 – 0.0010)	(0.0001 – 0.0048)	(0.0001 – 0.003)	(0.0001 – 0.0006)	(0.0001 – 0.0005)
Th	0.002 ± 0.001	0.012 ± 0.003	0.006 ± 0.002	0.007 ± 0.001	0.009 ± 0.002
	(0.0001 – 0.0063)	(0.001 – 0.074)	(0.0001 – 0.051)	(0.0001 – 0.027)	(0.001 – 0.031)
U	0.0016 ± 0.0004[a,b]	0.0132 ± 0.0045[a,c,d]	0.0058 ± 0.0013[b]	0.0035 ± 0.0006[c]	0.0040 ± 0.0009[d]
	(0.0001 – 0.0049)	(0.0016 – 0.1342)	(0.0002 – 0.0352)	(0.0002 – 0.0148)	(0.0001 – 0.0193)

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 near-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.

5.4.7 Metals in wild snake scales vs. sediment

The inter-site differences of metal concentrations in sediment and snake scales were visually compared using Fig. 5.3, 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. 5.3).

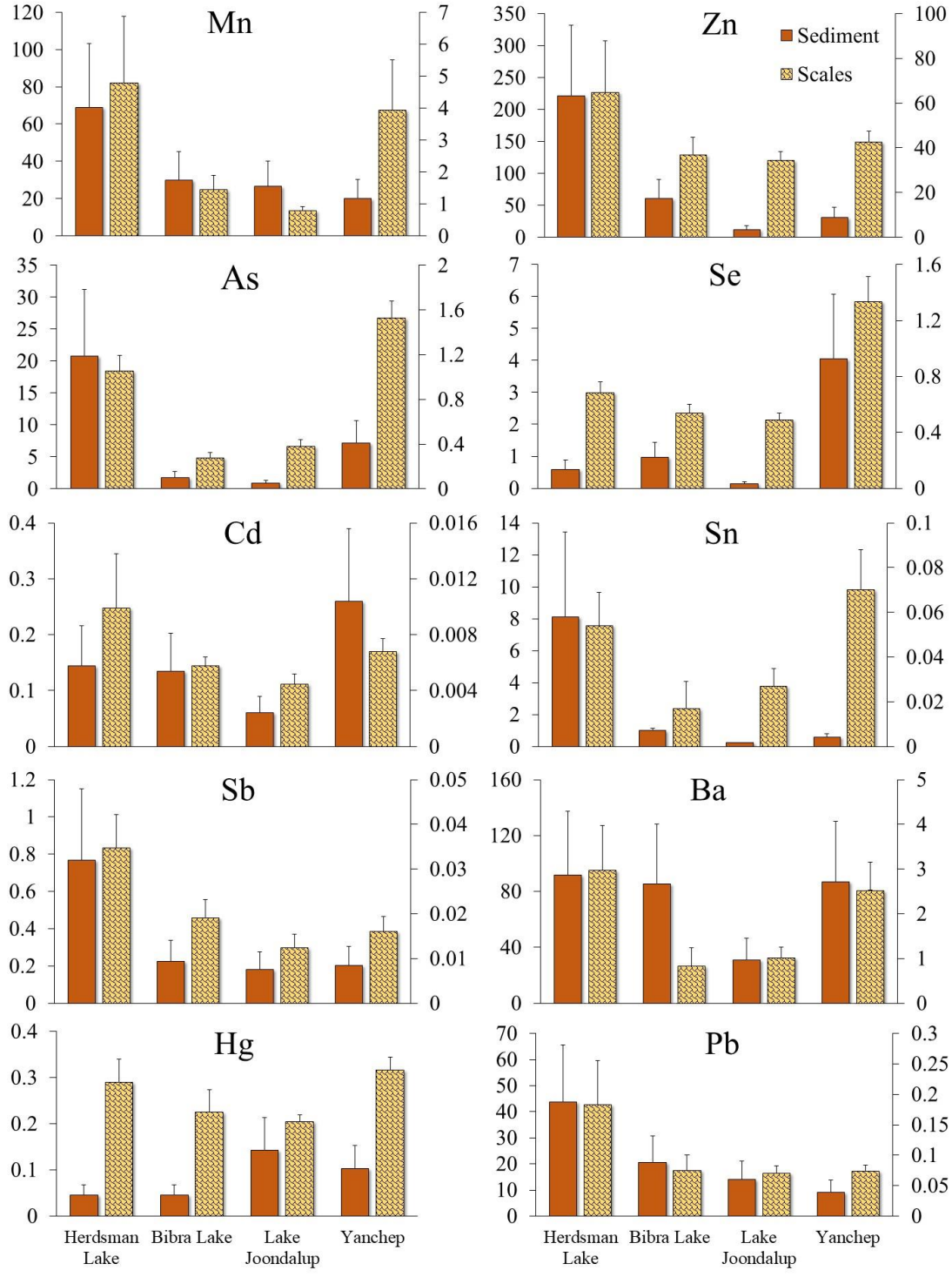


Fig. 5.3 A comparison of inter-site metal concentrations (ppm) in Western tiger snake (*Notechis scutatus occidentalis*) scales determined by LA-ICP-MS (this study) and sediments determined by acid digestion ICP-MS (Lettoof et al. 2020a). Left *y* axis represents mean sediment metal concentration and 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.

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. 5.3). 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. 2021; 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).

5.4.8 Scales as an indicator of internal accumulation

Scale concentrations could be compared to liver concentrations (from Lettoof et al. 2020a) for Mn, Zn, As, 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 ($F_{1, 19} = 3.96$, $p = 0.049$). 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).

5.4.9 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. 5.2). 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.

5.4.10 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.

5.5 Conclusions

This research successfully demonstrated and progressed the use of LA-ICP-MS for quantifying a suite of metals in 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.

5.6 References

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5.7 Chapter 5 addendum

5.4.6 – page 127

"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 5.2). 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."

This statement is confusing and seems to have a contradictory statement with Bi and Th. It should read as: The concentrations of Ca, Ti, V, Se, Sb, Mn, As, Sr, Ba, Hg, Pb, Sn, Cs, Tl and U in reference snake scales were significantly lower ($p < 0.05$) than in wild-caught snake scales from at least one site (Table 5.2). The concentrations of Zn, Bi, Cd and Th in reference snake scales were not statistically different ($p > 0.05$) from wild-caught snakes.

Chapter 6. Bioindicator snake shows genomic signatures of natural and anthropogenic barriers to gene flow

The study presented in Chapter 6 was accepted in the peer-reviewed journal '*PLoS One*' on 16 October 2021, and is an exact reproduction of the copyright paper reformatted for this thesis.

Lettoof, D. C., Thomson, V. A., Cornelis, J., Aubret, F., Bateman, P. W., Gagnon, M. M., von Takach, B. Bioindicator snake shows genomic signatures of natural and anthropogenic barriers to gene flow. (2021). *PLoS One.* 16: e0259124. doi: 10.1371/journal.pone.0259124.

6.1 Abstract

Urbanisation alters landscapes, introduces wildlife to novel stressors, and fragments habitats into remnant 'islands'. Within these islands, isolated wildlife populations can experience genetic drift and subsequently suffer from inbreeding depression and reduced adaptive potential. The Western tiger snake (*Notechis scutatus occidentalis*) is a predator in wetlands in the Swan Coastal Plain, a unique bioregion that has suffered substantial degradation through the development of the city of Perth, Western Australia. Within the urban matrix, tiger snakes now only persist in a handful of wetlands where they are known to bioaccumulate a suite of contaminants, and have recently been suggested as a relevant bioindicator of ecosystem health. Here, we used genome-wide single nucleotide polymorphism (SNP) data to explore the contemporary population genomics of seven tiger snake populations across the urban matrix. Specifically, we used population genomic structure and diversity, effective population sizes (N_e), and heterozygosity-fitness correlations to assess fitness of each population with respect to urbanisation. We found that population genomic structure was strongest across the northern and southern sides of a major river system, with the northern cluster of populations exhibiting lower heterozygosities than the southern cluster, likely due to a lack of historical gene flow. We also observed an increasing signal of inbreeding and genetic drift with increasing geographic isolation due to urbanisation. Effective population sizes (N_e) at most sites were small (< 100), with N_e

appearing to reflect the area of available habitat rather than the degree of adjacent urbanisation. This suggests that ecosystem management and restoration may be the best method to buffer the further loss of genetic diversity in urban wetlands. If tiger snake populations continue to decline in urban areas, our results provide a baseline measure of genomic diversity, as well as highlighting which 'islands' of habitat are most in need of management and protection.

6.2 Introduction

Urbanisation, the anthropogenic transformation of natural ecosystems via the growth of cities (Vlahov and Galea 2002), introduces wildlife to a myriad of stressors such as dynamic availability of resources (Kristan et al. 2004), pollution (Luo et al. 2019; Rodriguez Martin et al. 2015), novel environments (Bower et al. 2014; Newbery and Jones 2007) and human disturbance (Doherty et al. 2021). These novel stressors affect the behaviour, physiology and health of wildlife (Murray et al. 2019), and consequently create strong selection pressures driving evolution (McDonnell and Hahs 2015; Miranda 2017; Rivkin et al. 2019). Additionally, urban development fragments habitats resulting in remnant patches becoming isolated islands in a matrix of urbanisation (Bryant et al. 2017; Fusco et al. 2021; Wodkiewicz and Gruszczynska 2014). The less-mobile, philopatric or more habitat-specialist species may persist only in these islands and not the surrounding matrix, and thus face the random genetic pressures inherent to isolated populations of such species with reduced or non-existent gene flow between sub-populations (Macdonald et al. 2020; Miles et al. 2019).

Isolated populations are expected to experience increased levels of genetic drift—stochastic loss of alleles through time—and differentiation, in conjunction with reduced genetic diversity within populations (Miles et al. 2019). In instances where the remnant population is small, a reduction in genetic diversity can potentially lead to signs of inbreeding depression (Keyghobadi 2007; Madsen et al. 1996). This inbreeding depression, the overexpression of deleterious recessive alleles in homozygotes, can also lead to a reduction in individual fitness (Hedrick and Garcia-Dorado 2016; Johansson et al. 2007), while genetic drift can lead to reduced adaptive potential (Miles et al. 2019; Reed and Frankham 2003). Consequently, urban 'island' populations are in a fitness and adaptation arms race against the constant stressors of urbanisation.

The Australian city of Perth is built on the Swan Coastal Plain (SCP); a bioregion characterised by banksia woodland on sand dunes, intersected by north-south connected chains of ephemeral wetlands. Since 1850, urban development and agriculture in the SCP led to the loss of 70% of the original wetland area (Davis and Froend 1999), with most of the remaining wetlands suffering from severe degradation. Tiger snakes (*Notechis scutatus*) are a ~ 1 m elapid snake restricted to the cooler, wetter climates of Australia (Wilson and Swan 2017); they prefer wetland habitats on the mainland, yet numerous populations exist on very dry off-shore islands (Aubret 2015). Tiger snakes were once considered under threat of extinction for a number of reasons, but primarily the destruction and degradation of wetland habitats due to urbanisation (Softly 1971). Although tiger snakes are still regionally common, there is anecdotal evidence of their decline in some cities and on some islands. For example, Eastern tiger snakes (*N. scutatus scutatus*) were once locally abundant in greater Sydney but now only persist on the outskirts of the city (Shea 2010).

The loss of tiger snakes across some of their distribution is not enough to label the species with conservation concern; however, snakes can be useful indicators of ecosystem health (Bauerle et al. 1975; Beaupre and Douglas 2009; Haskins et al. 2021). Perturbation of their populations can thus inform land managers of the integrity of the environment. Prior to urbanisation, Western tiger snakes (*N. scutatus occidentalis*) in the SCP likely moved among ephemeral wetlands as these environments dried throughout the warmer months, but now populations only persist around, and appear restricted to, several large lakes and river edges with sufficient fringing vegetation (DL pers. obs.). Despite persisting in these fragmented wetland habitats thus far, they are exposed to and bioaccumulate a suite of contaminants that likely contribute to poorer health and decreased survival of individuals (Lettoof et al. 2020a; Lettoof et al. 2020b; Lettoof et al. 2021b). However, the degree to which small population sizes, geographic isolation and inbreeding effects contribute to population health in these urban and peri-urban populations has not yet been investigated.

To address these knowledge gaps, we assessed the population structure and patterns of genomic diversity in tiger snake populations persisting in and around the city of Perth in Western Australia. We included a ‘recently-introduced’ off-shore island population to allow for a comparison with the genomic structure of a true island population. Analyses included calculating and comparing the effective population

sizes across populations to explore the impacts of genetic drift and isolation, and compare individual heterozygosity to a body condition index (Gibbs and Chiucchi 2012; Moss et al. 2019) to investigate the potential relationship between fitness and heterozygosity. We predicted that the major river systems that divide Perth (Fig 6.1) would be barriers to gene flow between the northern and southern localities, as observed in other species (Ottewell et al. 2019), and that those populations more isolated by urbanisation would show lower levels of genomic diversity, higher pairwise genomic differentiation and stronger signals of inbreeding, which would correlate with lower fitness. This study explores the contemporary population genomics of a large elapid persisting in wetlands threatened by ever-increasing urbanisation, and highlights which populations are at risk of extirpation in the future.

6.3 Methods

6.3.1 Study sites and sample collection

We sampled 150 tiger snakes from six wetlands around Perth: Loch McNess (*n* = 22; within Yanchep National Park, 31°32'45" S, 115°40'50" E), Lake Joondalup (*n* = 23; 31°45'37" S 115°47'36" E) and Herdsman Lake (*n* = 57; 31°55'14" S 115°48'18" E), located north of the Swan/Canning River system, and Bibra Lake (*n* = 29; 32°05'33" S 115°49'31" E), Kogolup Lake (*n* = 10; 32°07'40" S 115°50'05" E) and Black Swan Lake (*n* = 9; 32°28'32" S 115°46'22" E) located south of the Swan/Canning River system. These study sites represent the northern extremity of the Western tiger snake distribution (Fig 6.1). We also collected nine samples from Carnac Island, approximately 7 km off-shore (Fig 6.1). Carnac Island is a small freshwater-devoid island (19 ha) with the tiger snake population thought to originate from human introduction approximately 90 years ago, with the suspected source population coming from the nearby mainland (Aubret 2015; Ladyman et al. 2020). Kogolup Lake, Black Swan Lake and Carnac Island were surveyed less than the other sites (a few days compared with several weeks), which resulted in lower sample sizes for these sites.

We took ventral scale clips from each snake collected from the six mainland sites during September–October 2020. We stored individual scales in 95% ethanol at -20°C until extraction. The Carnac Island tiger snakes also had scale clips collected from April 2018–January 2020 that were stored in 95% ethanol at 4°C until extraction. We also included two additional snakes from eastern Australia, > 2000 km from the Perth

populations, as outgroup samples. Tiger snakes from the mainland sites were sampled under Curtin University's Animal Research Ethics approval: ARE2018-23 and ARE2020-6; and Western Australia's Department of Biodiversity, Conservation and Attractions permits: FO25000149 and FO25000294-2. The nine tiger snakes from Carnac Island were sampled under the University of Adelaide's Animal Research Ethics permits: S-2016-111; and Western Australia's Department of Biodiversity, Conservation and Attractions permits: 01-000069-3, FO25000008, and FO25000008-2.

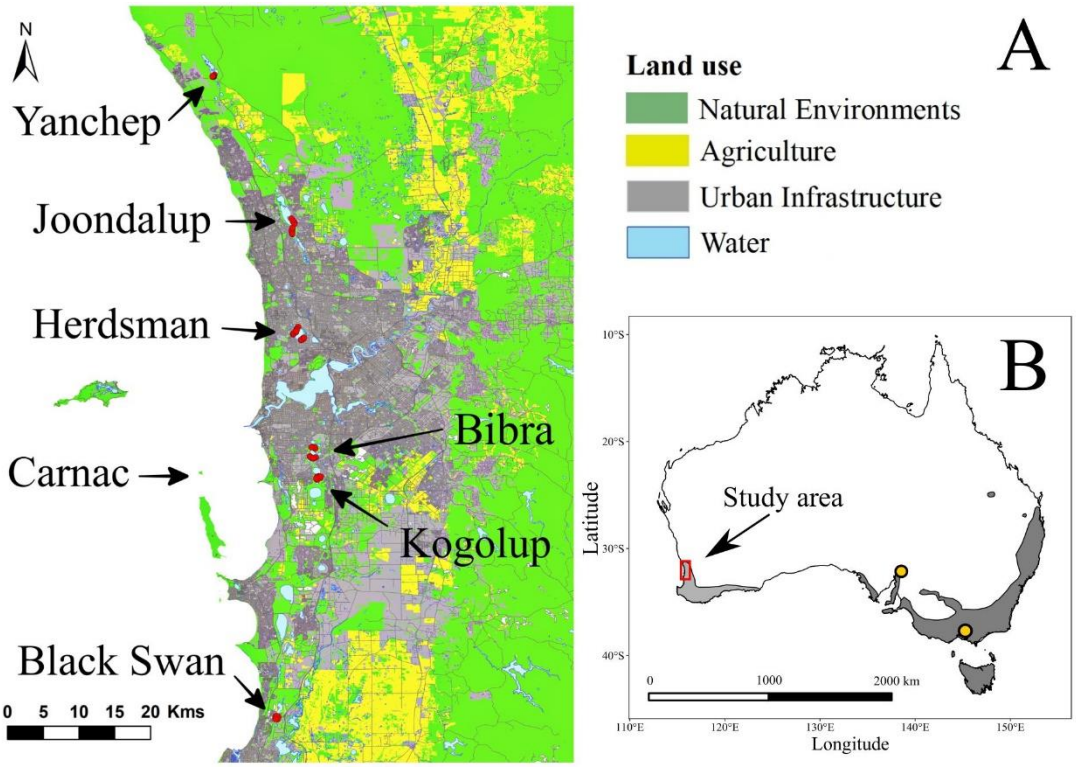

Fig. 6.1. Map the studied populations of *Notechis scutatus occidentalis* and land-use of Perth, Western Australia. Red points represent individual Western tiger snakes, and yellow points represent Eastern tiger snakes (*Notechis scutatus scutatus*). Grey shading represents the current distribution extent of the species (light = Western, dark = Eastern, modified from the IUCN Red List of Threatened Species; https://www.iucnredlist.org/resources/spatial-data-download). Land-use was classified by the 2016 Australian Land Use and Management Classification (Version 8; http://www.agriculture.gov.au/abares/publications).

6.3.2 DNA extraction, sequencing and bioinformatics pipeline

Tissue samples were sent to the DArTseq laboratory in Canberra, ACT for DNA extraction, library preparation and double-digest restriction-site associated DNA next-

generation sequencing. Briefly, the library preparation consisted of DNA digestion using the restriction enzymes PtsI and HpaII, as these enzymes had previously been used for tiger snake RAD-seq library preparation. Following digestion, adapter ligation and PCR amplification, DNA libraries were sequenced on a single lane of an Illumina Hiseq 2500 platform. The DArTseq proprietary bioinformatic pipeline (Kilian et al. 2012) was used to demultiplex, clean, and filter reads, then map reads to the Notechis scutatus reference genome (NCBI PRJ: PRJEB27871) and call single-nucleotide polymorphisms (SNPs). Detailed methods covering DArTseq library preparation, sequencing, read filtering and SNP calling have been provided in previous publications (Melville et al. 2017; Sansaloni et al. 2011). We received a SNP-by-sample matrix consisting of 161 samples and 22542 SNPs, which was read into R v4.0.3 for subsequent SNP filtering and analysis.

6.3.3 SNP filtering

We used a custom R script to prune unwanted SNPS and retain SNPs of interest. To remove potential bias due to sequencing or genotyping errors, we retained SNPs with total read depth > 10 and < 100, as well as those with high DArTseq reproducibility scores (no SNP < 100% reproducible in technical replicates). Reads that did not map to the reference genome in the DArTseq pipeline were also discarded. To account for bias due to linkage disequilibrium (Price et al. 2008), we filtered out SNPs in close proximity to one another, by retaining just one SNP from each RAD locus. We also retained only SNPs that were genotyped in a high proportion of samples (callrate > 0.95), had a minor allele count ≥ 3, and observed heterozygosity < 0.6. Finally, we removed any sample that had > 20% missing data. This produced a large, high-quality SNP-by-sample matrix, with a low overall level of missing data (1%). Once filters had been applied we retained 4688 SNPs from 159 Western and two Eastern tiger snake individuals.

As a preliminary measure to investigate whether our filtered dataset was appropriate for inferring relationships between individuals and populations, we created and visually inspected a hierarchical clustering dendrogram based on Nei's genetic distance (Fig S6.1). All individuals fell within their sampled localities, and populations clustered in line with expected geographic distances and landscape barriers, including the two individuals from eastern Australia, suggesting that the retained SNP dataset

was adequate for further analysis. The eastern individuals were then removed from subsequent analyses. As further confirmation of data integrity, we investigated pairwise kinship coefficients within each population. To ensure relatedness between individuals was not high enough to impact our data analysis or interpretation, we calculated pairwise kinships via the 'beta.dosage' function of the *hierfstat* package (version 0.7) (Goudet 2005; Goudet et al. 2018). Four pairwise values from a total of 2604 comparisons had kinships > 0.25 but < 0.3 (commonly values of 0.25 indicate full siblings), therefore no individuals were removed.

6.3.4 Regional population structure

To investigate genomic distance between individuals we calculated the individual pairwise genetic distances using the 'prevosti.dist' function in the *poppr* package (version 2.9.1) (Kamvar et al. 2014). We visualised these distances via the 'cmdscale' function, plotting the first two dimensions of the genetic distance matrix in a multidimensional scaling plot – where the distances between points are approximately equal to the dissimilarities. We then calculated pairwise population genomic differentiation values (G''_{ST}) among all sampling localities using the 'pairwise_Gst_Hedrick' function of the *mmod* package (version 1.3.3) (Winter 2012), and obtained *p* values for all population pairs using the *StAMPP* package (version 1.6.2) (Pembleton et al. 2013). The G''_{ST} metric is an F_{ST} analogue representing a standardised measure of allelic isolation that corrects for the number of subpopulations being considered.

To explore genomic structure and assess potential gene flow among populations, we searched for genomic groups using the TESS3 algorithm – a spatially explicit ancestry estimation model from the *tess3r* package (version 1.1) (Caye et al. 2016; Caye et al. 2018). The 'tess3' function incorporates the latitudes and longitudes of each sampled individual to account for the influence of isolation-by-distance on ancestry coefficients, with large drops or plateaus in the scree plot identifying useful values of *K* for inference of population genetic structure (Fig S2). We considered *K* values of 2, 3 and 4 most useful for describing the high-level genetic structure across the region, as these values showed the largest reductions in cross-entropy, with a plateau starting to form at $K > 4$. For finer-scale investigation of population structuring we also plotted ancestry coefficients of $K = 5$, 6 and 7 (Fig S6.3). To confirm whether there SNPs

under putative selection were driving patterns of population structure, we conducted a genome scan for outlier loci using the 'pvalue' function. This outlier test uses overall differentiation to discern if a portion of SNPs have greater allele frequency differences than expect from a neutral distribution (Narum and Hess 2011). We used K = 4 as we considered this the most useful for inference, and a Benjamini-Hochberg correction to achieve a false discovery rate of one in 10 000.

To investigate isolation-by-distance, we calculated the multilocus spatial autocorrelation for the mainland, and subset of northern and southern populations of snakes, respectively. The spatial autocorrelation analysis was conducted in GENALEX (version 6.5) add-on in Excel (Peakall and Smouse 2006, 2012).

6.3.5 Population genomic diversity

To investigate patterns of within population genomic diversity, we calculated standard genetic diversity metrics for all *a-priori* populations using the GENALEX in Excel. The diversity metrics included mean values for the number of alleles (N_A), effective number of alleles (A_E), information index (I), observed heterozygosity (H_O), expected heterozygosity (H_E), and the fixation index (Wright's inbreeding coefficient, F_{IS}). We also calculated the number of private alleles in each population as an additional measure of genetic distinctiveness. To standardise the number of private alleles for different sample sizes among populations we bootstrapped private allele calculations by resampling nine individuals per population 100 times, and taking the mean and SE of all bootstraps.

6.3.6 Heterozygosity-fitness correlations

As individual heterozygosity is often related to individual fitness (Gkafas et al. 2020; Reed and Frankham 2003; Sovic et al. 2019), we modelled the relationship between individual heterozygosity and body condition of all genotyped individuals. Previous work has shown that approximately 50% of the variation in snake body condition estimates results from stored body fat, while organ mass such as muscle and liver account for the remainder (Madsen and Shine 2002; Weatherhead and Brown 1996). To determine body condition we calculated a scaled mass index (SMI) for each snake using the formula: $SMI = Wi \left(\frac{L0}{Li}\right)^{b\mathrm{SMA}}$ where W_i and L_i are the weight and snout-vent length (SVL) of individuals, L_0 is the arithmetic mean length of all sampled individuals, and b_{SMA} is the scaling exponent estimated by the standardised major axis

regression of mass on length of all sampled individuals (Peig and Green 2009, 2010). We consider the SMI to be an estimate of 'fitness' with higher values corresponding to fitter individuals (Gibbs and Chiucchi 2012). To increase accuracy of body condition calculations, we excluded snakes with obvious gastric food items or pregnancy. We also excluded Carnac Island snakes as this population was sampled in summer, a time when these individuals potentially have low body condition (from low prey and water availability). Based on 91 snakes, L_0 used in the equation was 757.1 mm, and the b_{SMA} was 2.98. To explore evidence of heterozygosity-fitness correlations we ran a general linear mixed model (GLMM) using the 'glmer' function of the *lme4* package (version 1.25) (Doran et al. 2007); with SMI as the response variable, individual heterozygosity and site as fixed predictor variables and to account for sex-biased differences, sex as a random factor. We used a histogram of the model residuals to confirm the assumptions of linearity.

In addition, we compared body condition with g^2 – a proxy for identity disequilibrium (inbreeding) (David et al. 2007; Gkafas et al. 2020). A $g^2 = 0$ means no variance of inbreeding in the sample. With the *inbreedR* package (version 0.3.2) (Stoffel et al. 2016), we used the 'r2_wf' function to calculate the expected correlation between inbreeding level (*f*) and body condition, and the 'r2_hf' to calculate the correlation between inbreeding level (*f*) and individual heterozygosity.

6.3.7 Effective population size

The effective population size (N_e) of a site represents the estimated number of breeding adults in a single generation of an ideal population that shows the same degree of genetic diversity as the measured population (Luikart et al. 2010; Wright 1931). Theoretically, small estimates of N_e reflect small, isolated populations suffering increased drift and lower fitness (e.g. through inbreeding), whereas large values of N_e reflect large and genetically diverse populations (Frankham 2012; Frankham et al. 2014). An effective population size can also be used to estimate adult census size (Wood et al. 2020). To estimate N_e of each population we used the widely-used linkage disequilibrium method, as it is considered one of the most suitable for single-sample datasets (Luikart et al. 2010). N_e estimates were calculated using the NEESTIMATOR v2.1 (Do et al. 2014).

6.4 Results

6.4.1 Regional population structure

We found expected levels of genomic differentiation between populations, based on geographic distance and landscape barriers between sites (Table 6.1). Carnac Island was the most differentiated from other populations (pairwise G''_{ST} = 0.29–0.40). The most geographically distant pair of sites, Yanchep and Black Swan, exhibited a moderate level of differentiation (G''_{ST} = 0.26). Black Swan Lake was less differentiated from Kogolup and Bibra Lakes (G''_{ST} = 0.13–14) than Yanchep was to Herdsman Lake (G''_{ST} = 0.23), despite these pairs of locations being a similar geographic distance from one another (~45 km). Analysis of spatial autocorrelation identified significant values of the autocorrelation coefficient *r* – persisting for distances up to 38 km (Fig S6.4). We found a difference in the decay of spatial autocorrelation between the northern and southern clusters, with the northern cluster intercepting *r* = 0 at about 30 km and the southern cluster intercepting *r* = 0 at about 12 km.

The outlier test identified 131 (2.8% of 4688) SNPs as being under putative selection. These loci demonstrate significantly higher or lower among-population genetic differentiation than expected under neutrality, many of which are possibly driven by the differentiation between Carnac Island and the mainland populations. While this small number of SNPs is unlikely to have substantially influenced our observed population structure, these loci could be responsible for influencing fitness under differing environmental conditions, and thus provide a basis for further study.

Table 6.1. Pairwise values of population genomic differentiation (G''_{ST}) of *Notechis scutatus occidentalis* around Perth, Western Australia. All non-zero values are highly significant ($p < 0.001$).

Site	Yanchep	Lake Joondalup	Herdsman Lake	Bibra Lake	Kogolup Lake	Black Swan Lake
Yanchep (*n* = 22)	0					
Lake Joondalup (*n* = 23)	0.14	0				
Herdsman Lake (*n* = 57)	0.24	0.18	0			
Bibra Lake (*n* = 29)	0.24	0.20	0.21	0		
Kogolup Lake (*n* = 10)	0.22	0.19	0.20	0.07	0	
Black Swan Lake (*n* = 9)	0.26	0.22	0.23	0.14	0.13	0
Carnac Island (*n* = 9)	0.40	0.38	0.38	0.31	0.29	0.29

The multidimensional scaling plot (Fig 6.2) showed four main population clusters of tiger snakes in the Perth region, representing (1) Carnac Island, (2) Herdsman Lake, (3) Yanchep and Joondalup lakes, and (4) all three lakes (Bibra, Kogolup and Black Swan Lakes) on the southern side of the Swan River. 24.5% of the total variation was explained by the first two axes. The Carnac Island cluster separated strongly from the other three clusters on both the first and second coordinate.

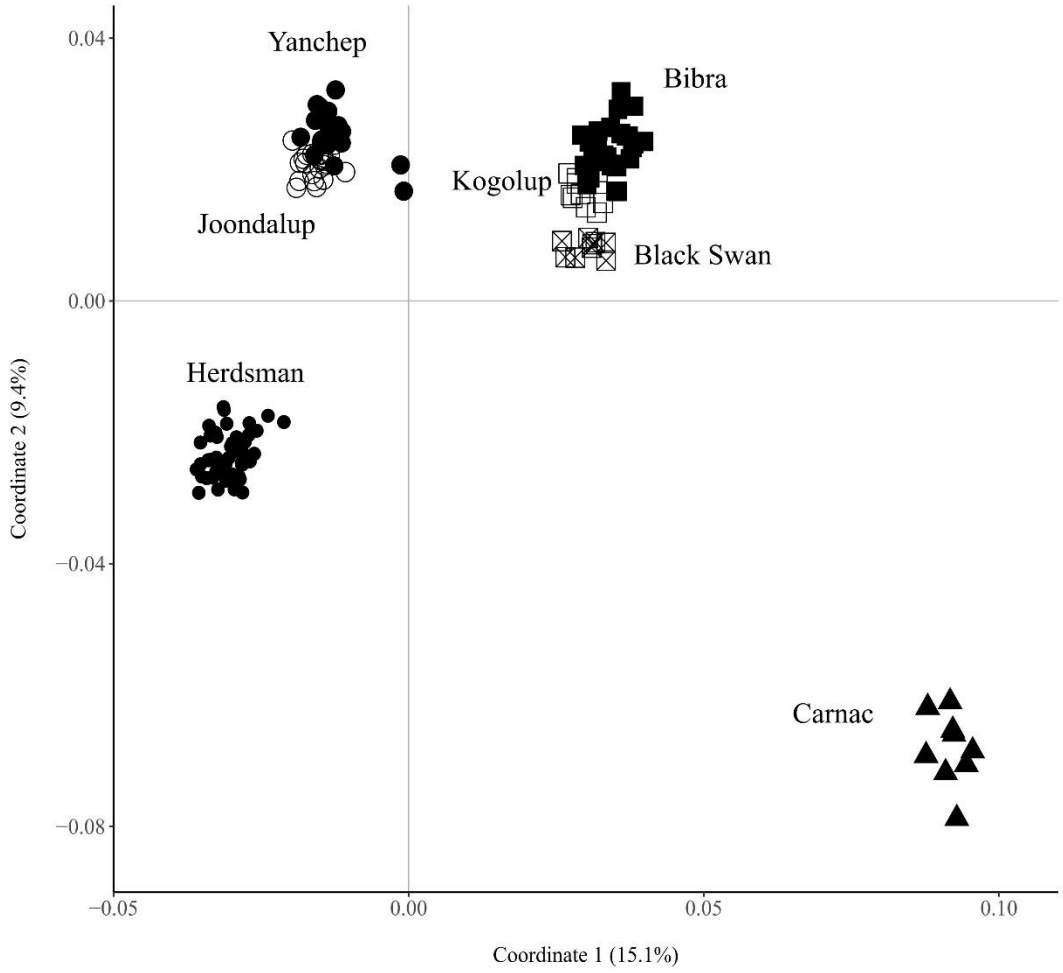

Fig 6.2 Multidimensional scaling plot of genetic distance between *Notechis scutatus occidentalis* individuals from Perth, Western Australia. Each population has been given a unique icon. Coordinate one explained 15.1% and coordinate two explained 9.4% of the total variance.

Investigation of the cross-entropy scree plot produced using the *tess3r* package indicated a likely number of ancestral linages at $2 \leq K \leq 4$ (Fig S6.2). Plotting ancestry coefficients for all individuals at these *K* values highlights the genomic separation of the northern lakes from the southern lakes ($K = 2$), with Herdsman Lake separating

from the two other northern lakes at K = 3, and Carnac Island separating from the southern lakes at K = 4 (Fig 6.3). With finer-scale population splitting, Lake Joondalup separates from the northern lakes (K = 5) while Kogolup Lake and Black Swan Lake share ancestry with Carnac Island (Fig S6.4). At K = 6, Black Swan Lake separates from the southern lakes, and Kogolup Lake is recognised as a unique different population at K = 7 (at which all *a-priori* populations cluster separately). Fig S6.5 visualises the hierarchical population genomic structure for K values between 2 and 6.

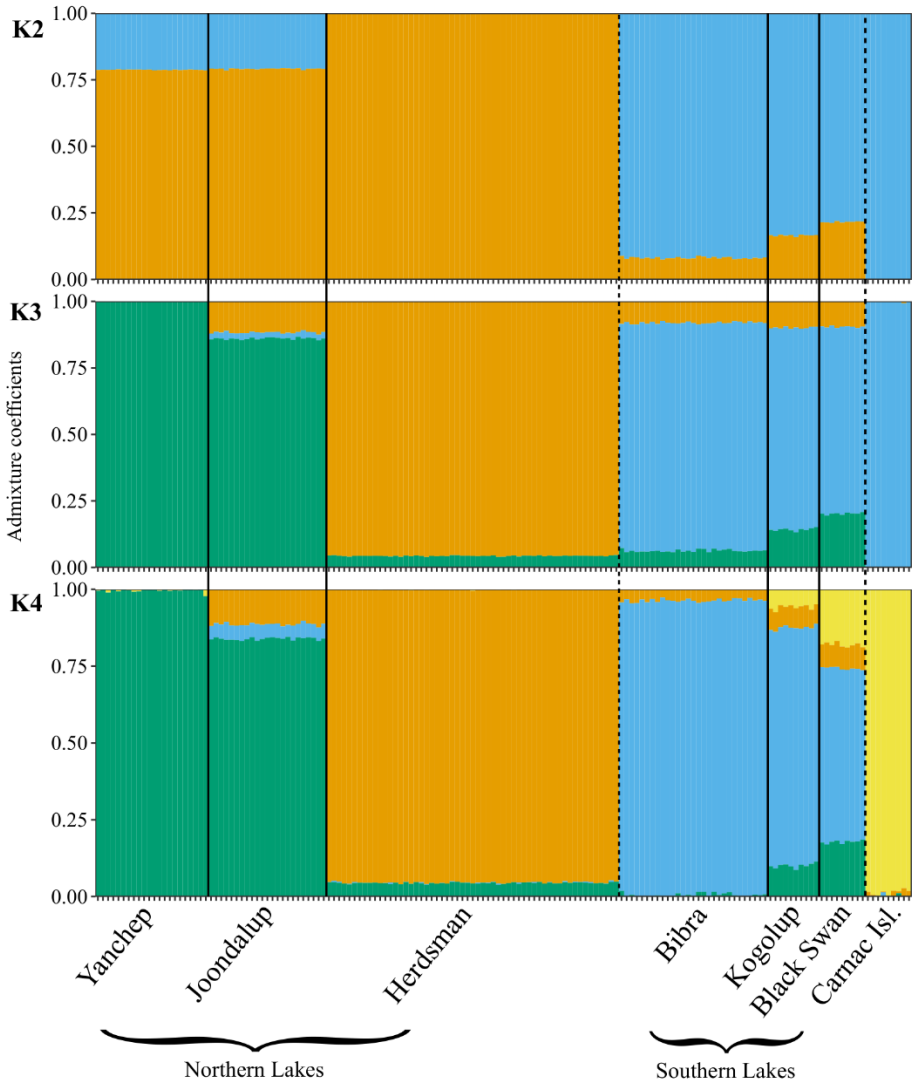

Fig 6.3 Admixture bar plot comparing population structure in *Notechis scutatus occidentalis* from Perth, Western Australia. Each tick mark on the x-axis represents an individual snake, which are grouped by sampling locations. The dashed line represents the biogeographic barrier of the Swan/Canning Rivers separating the northern and southern sampling localities. The y-axis represents the fraction of individuals' genome that originates from a particular ancestral population, each of which has been given a unique colour.

6.4.2 Genomic diversity and health

Genomic diversity was generally lower in populations north of the river than in populations south of the river, with the Carnac Island population having the highest heterozygosity of all studied populations (Table 6.2). Observed heterozygosity, specifically, was 25 – 33% lower in the northern populations. Observed heterozygosity did not differ from expected heterozygosity in most populations, although Carnac Island showed a slightly lower *H*o (0.13) than *H*e (0.14). Carnac Island had the highest relative number of private alleles (328). The signal of inbreeding (i.e. F_{IS} values) increased with the level of geographic isolation in *a-priori* populations; the true island population (Carnac Island) had the highest F_{IS} value (0.05), with the mainland populations most impacted by urbanisation having higher F_{IS} values (0.04-0.02) than less disturbed populations (≤ 0). While these values are close to zero, they likely reflect genome-wide patterns and differences between populations, with low values not unexpected when using a low minor allele frequency threshold.

Table 6.2 Genetic diversity estimates of seven populations of *Notechis scutatus occidentalis* around Perth, Western Australia. Presented as mean values across all SNPs; (<), standard error < 0.01; *N*a, no. of alleles; A_E, effective number of alleles; *I*, information index; *H*o, observed heterozygosity; *H*e, expected heterozygosity; F_{IS}, fixation index (Wright's inbreeding coefficient).

Site	N_A	A_E	*I*	*H*o	*H*e	F_{IS}	Private alleles
Yanchep (*n* = 22)	1.32 (0.01)	1.14 (<)	0.13 (<)	0.08 (<)	0.08 (<)	0.00 (<)	55.79 (0.89)
Lake Joondalup (*n* = 23)	1.33 (0.01)	1.15 (<)	0.14 (<)	0.09 (<)	0.09 (<)	0.02 (<)	56.27 (0.53)
Herdsman Lake (*n* = 57)	1.39 (0.01)	1.15 (<)	0.14 (<)	0.09 (<)	0.09 (<)	0.04 (<)	69.49 (0.73)
Bibra Lake (*n* = 29)	1.45 (0.01)	1.20 (<)	0.19 (<)	0.12 (<)	0.12 (<)	0.03 (<)	81.73 (0.86)
Kogolup Lake (*n* = 10)	1.39 (0.01)	1.19 (<)	0.18 (<)	0.12 (<)	0.12 (<)	-0.02 (<)	68.17 (0.59)
Black Swan Lake (*n* = 9)	1.38 (0.01)	1.19 (<)	0.18 (<)	0.12 (<)	0.12 (<)	-0.02 (<)	111.30 (0.46)
Carnac Island (*n* = 9)	1.41 (0.01)	1.25 (0.01)	0.21 (<)	0.13 (<)	0.14 (<)	0.05 (0.01)	328.03 (0.97)

The three northern populations, Yanchep, Lake Joondalup and Herdsman Lake, had lower mean body conditions compared to the southern populations (Fig 6.4). The GLMM (r^2 = 0.43) showed no evidence for a significant relationship between individual heterozygosity and body condition (F: 0.51, df; 1, p = 0.48); however, there was a strong effect of site (F: 3.22, df: 5, p = 0.01). A Tukey HSD test found the greatest, and only significant, difference in body condition was between Herdsman Lake and Black Swan Lake snakes (p = 0.03; Table S6.1). Of the snakes with body condition data, the inbreeding among loci was significantly different from zero (g^2 = 0.025 ± 0.003 S. E., p = 0.01). We found a high correlation between inbreeding level (f) and heterozygosity (r^2 = 0.94), strongly suggesting that heterozygosity is a good proxy for inbreeding. Furthermore, we found no correlation between inbreeding level (f) and body condition (r^2 = 0.07).

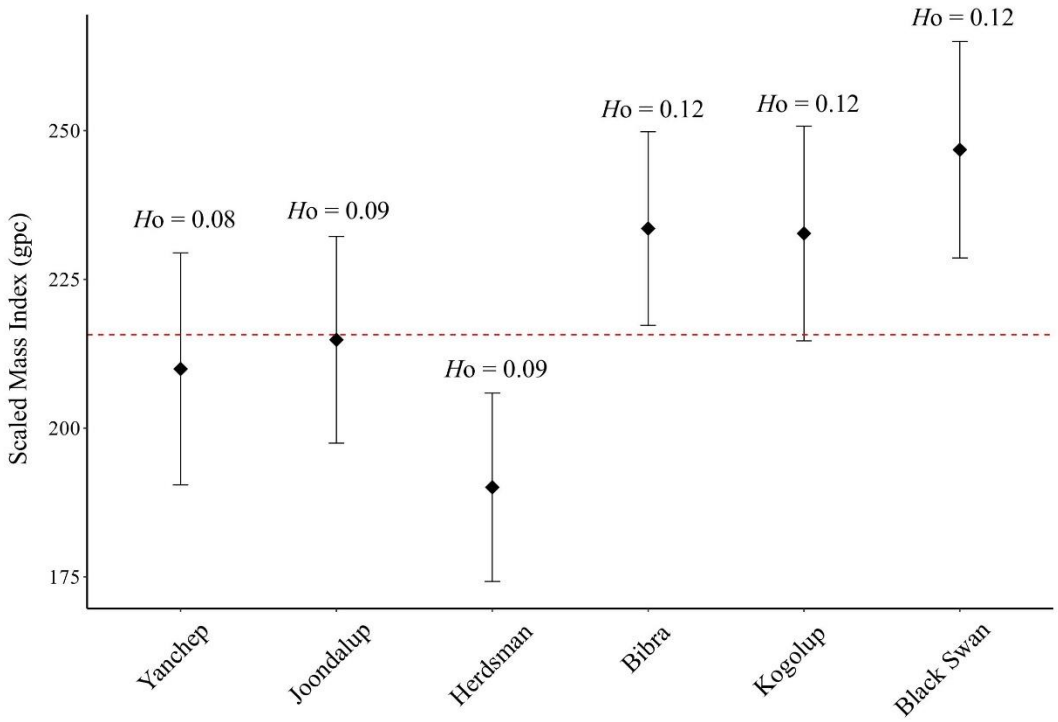

Fig 6.4 Mean body condition of mainland *Notechis scutatus occidentalis* populations around Perth, Western Australia. Body condition is presented as scaled mass index (SMI). gpc, grams per cm; filled diamonds are the population mean; error bars represent 95% confidence intervals; dashed line is mean SMI of 215.7 gpc at the mean population SVL of 757.1 mm; *H*o is the mean observed heterozygosity for each population.

6.4.3 Effective population size

We found the largest N_e at Herdsman Lake (95% CI N_e = 152 – 162), with the smallest effective population size at Black Swan Lake (95% CI N_e = 24 – 26; Table 6.3). The effective population size at Kogolup Lake was 'infinite', likely due to small sample size preventing calculation of N_e. The r^2 – a sample-size bias corrected value of linkage-disequilibrium across all loci pairs – was lower in the northern populations than in the southern populations.

Table 6.3 Effective population size (N_e) of seven populations of *Notechis scutatus occidentalis* around Perth, Western Australia. 95% CI represent both upper and lower confidence intervals; INF, 'infinite', usually a result of not enough information to obtain a reliable estimate. Overall r^2 represents a composite measure of average linkage-disequilibrium across all pairs of loci.

Site	N_e	Overall r^2	Parametric 95% CI
Yanchep	92	0.056	86–99
Lake Joondalup	110	0.053	104–118
Herdsman Lake	156	0.021	152–162
Bibra Lake	84	0.042	82–87
Kogolup Lake	INF	0.138	3581–INF
Black Swan Lake	25	0.170	24–26
Carnac Island	90	0.168	82–101

6.5 Discussion

6.5.1 Regional population structure

Population genomic analysis generally supported our *a-priori* predictions. At the highest hierarchical level, we identified population clusters that aligned with geographic regions north and south of the Swan/Canning River systems (hereafter the northern and southern cluster). Broadly, our findings reflect the results of Ottewell et al. (2019), who found that the quenda (*Isoodon fusciventer*), a small marsupial persisting in Perth bushland patches, showed a similar pattern of genomic differentiation on either side of the major rivers. Although tiger snakes are capable swimmers (Aubret et al. 2005; Cornelis and Lettoof 2020) they are unlikely to swim across the width of the Swan/Canning rivers, and these rivers have likely been historic

natural landscape barriers reducing gene flow among tiger snake populations in this region.

Finer-scale patterns identified populations at Herdsman Lake and Carnac Island as being more genetically distinct within these broader clusters, likely due to a combination of genetic drift and isolation. At $K = 3$, Herdsman Lake is recovered as a distinct cluster, potentially due to isolation from a sea of urban infrastructure; at $K = 4$, Carnac Island is distinct, likely due to its isolation by 7 km of ocean. We also observed increased genomic distinction in populations reflecting the history and level of surrounding urbanisation. Remnant patches of habitat provide connectivity and allow for persistence of wildlife in urban areas (Angold et al. 2006; Ottewell et al. 2019); however, as Western tiger snakes prefer wetland habitats, they are unlikely to disperse through the urban matrix or use remnant vegetation patches without waterways. Consequently, populations persisting in wetland habitats now surrounded by urban infrastructure are essentially fragmented islands.

Within the northern cluster, Joondalup and Yanchep populations were less differentiated than were Joondalup and Herdsman. The Herdsman Lake population is closest to the city centre and has been within the urban footprint for the longest of all our study sites (Kelobonye et al. 2019), suggesting that urban development has led to reduced gene flow between this population and the remaining northern populations, with resultant isolation and/or genetic drift. Herdsman Lake was naturally an ephemeral swamp, but since the 1850s, it has been subjected to stock grazing, market cropping, and attempted draining for land reclamation until it was finally dredged and modified to be a compensation basin for urban drainage (Clarke et al. 1990; Gentilli and Bekle 1993). As Perth's urban footprint has grown over the last two centuries, tiger snakes may have contracted from the surrounding inter-linking wetlands into the Herdsman Lake reserve. Yanchep and Joondalup wetlands are the northern and southern extremities of the Spearwood Dune System chain of lakes (CALM and Dooley 2003), and tiger snakes would have had historic population connectivity along this dune system. Urban development began around Joondalup Lake in the 1970s (Kelobonye et al. 2019), and based on the current land-use (Fig 6.1) and our results, the Yanchep and Joondalup wetland populations may still be connected. However, continuing urban development around Joondalup Lake may result in this population

developing similar genomic characteristics to the Herdsman Lake population in the near future.

As expected, Bibra and Kogolup lake populations were very closely related. These lakes are part of the Beeliar Regional Park, a chain of wetlands and woodlands currently managed as conservation land (Dooley et al. 2006). The Beeliar Regional Park is the only remaining connected wetland and woodland ecosystem in the Perth metropolitan area and should provide population connectivity for tiger snakes as long as it remains undeveloped. While there is likely to be some level of mortality due to the presence of arterial roads through the region, it appears there may still be some connectivity between these populations based on the close relationship shared by the Beeliar wetland populations and the Black Swan population.

Interestingly, the southern cluster exhibited a lack of spatial autocorrelation at 12 km, compared to 30 km in the northern cluster. While there appears to be a clear difference in the patterns between subregions, interpreting these differences is difficult without more detailed sampling at intervals of equal distance between populations. This is potentially very difficult to achieve as tiger snakes may not be present at many locations other than those already sampled in this study. The differences may also be partly the result of lower levels of genomic variation present in the northern cluster, with less variation leading to increased spatial autocorrelation of genotypes.

6.5.2 Genomic diversity and health

As predicted, the northern cluster of tiger snakes had lower genomic diversity than the southern cluster, reflecting a similar pattern seen in quenda (Ottewell et al. 2019). Yanchep is near the northern extent of the Western tiger snakes species range (Fig 6.1). As edge populations often harbour lower diversity than core populations (De Kort et al. 2021), we suspect the relatively low diversity in the northern cluster is probably caused by the Swan/Canning river system isolating populations at the northern edge of the species range from gene flow and increasing genetic drift. Interestingly, genomic diversity was not lowest in the two sites with the highest genomic differentiation and geographic isolation, Herdsman Lake and Carnac Island, suggesting that isolation of ~90–150 years has not increased genetic drift in these populations.

Although none of the studied populations appeared to be strongly inbred, we found that F_{IS} values closely reflected contemporary isolation of populations. For example, Carnac Island is insular and Herdsman Lake is isolated due to urbanisation and showed the highest signal of inbreeding, whereas the study sites with the most habitat connectivity (Yanchep, Kogolup and Black Swan) showed no signal of inbreeding. Inbreeding depression reduces individual fitness, survival and reproduction and can lead to rapid decline and extirpation of populations (Hedrick and Garcia-Dorado 2016; Hedrick et al. 2014; Madsen et al. 1996). Despite small F_{IS} values, population-level changes may not be seen for many generations in longer-lived vertebrates (Keyghobadi 2007; Kuussaari et al. 2009) – such as tiger snakes, estimated at average 10 years (Ludington and Sanders 2021). Thus, we expect to see inbreeding increase through time, especially in sites that become completely isolated from urbanisation.

Phenotypic signatures of inbreeding depression can be measured in wild populations using heterozygosity-fitness correlations. We found a strong correlation between inbreeding (*f*) and heterozygosity, justifying our use of heterozygosity as a proxy for inbreeding. Our model found no effect of individual genomic heterozygosity on snake body condition, despite the broad pattern of the northern cluster sharing both lower heterozygosity and lower body condition (Fig 6.4). Body condition is a single measurement of fitness and low heterozygosity can translate to many other measures of fitness such as reduced body size in neonates (Moss et al. 2019), higher parasitism (Shaner et al. 2013) and reduced survival probability (Johansson et al. 2007). Populations with low heterozygosity may be experiencing changes in other life history traits that could directly or indirectly affect fitness. Similar to Sovic et al. (Sovic et al. 2019), our results show that body condition was strongly influenced by site. This suggests site-specific environmental stressors such as differences in prey availability (Zipkin et al. 2020), anthropogenic disturbance (Lomas et al. 2015) or physiological changes from bioaccumulation of contaminants (Lettoof et al. 2021a; Lettoof et al. 2021b; Takekawa et al. 2002) are probably more important factors than heterozygosity for reducing body condition in tiger snakes from our study sites.

In addition, our use of genome-wide loci possibly includes many loci in non-coding regions of the genome (von Takach et al. 2021), which would conceal the signal from SNPs under selection. The outlier test identified 131 loci that are potentially influencing fitness in different environments in the Perth region, and these candidate

loci can be investigated in future analyses. However, as many traits that contribute to local adaptation are polygenic and may not exhibit high F_{ST} values, we suggest that a genotype-environment association analysis would be a better method for investigating adaptive genomic architecture (Berg and Coop 2014; Kemper et al. 2014). Further investigation sampling tiger snake populations across their entire distribution – covering a range of native and urban wetland areas – would help identify SNPs that play a role in urban adaptation.

The above results demonstrate that contemporary genomic diversity in Western tiger snakes is more affected by population edge effects in conjunction with historical landscape isolation (Swan/Canning river playing the major role in population isolation and Yanchep at the northern edge experiencing lowered diversity) as opposed to fragmentation and isolation from urbanisation. The health of the northern SCP wetlands are continuously being threatened by anthropogenic water abstraction and climate change (Semeniuk and Semeniuk 2013) in conjunction with ever-encroaching urbanisation (MacLachlan et al. 2017). Eventually, the larger urban wetlands (such as Herdsman Lake) will be the only islands of refuge for the northern cluster of tiger snakes. The northern SCP population already shows the lowest genomic diversity – hence adaptive potential – and poorest body condition, and thus is most likely at risk of future extirpation as urbanisation amplifies isolation as well as introducing novel environmental stressors.

6.5.3 Effective population sizes

The largest estimates of N_e came from the largest wetlands: Herdsman and Joondalup Lakes, despite these populations having relatively lower heterozygosity values and positive inbreeding coefficients. In contrast, the Black Swan population (the smallest lake) showed the lowest N_e value despite high genomic diversity and no evidence of inbreeding. Rather than indicating isolation and genetic drift, our N_e estimates appear to reflect the area and quality of available habitat at each locality. Similarly, Wood et al. (2020) found that despite high levels of isolation due to urbanisation, a population of the wetland snake *Thamnophis sirtalis tetrataenia* had the largest N_e compared to other studied populations, suggesting that habitat restoration and enhancements may have facilitated high adult abundance at this locality. While Fraser et al. (2019) suspect that large available habitat is responsible for maintaining large population sizes in deer

populations isolated by urbanisation. Together, these results suggest that the quality and area of suitable habitat at our sampling sites is driving the effective population sizes of tiger snakes.

The N_e estimate for Kogolup Lake population was infinite, and the lower parametric confidence interval was 3581 (Table 6.3). As our sample size for that population was n = 10, the infinite estimate is likely due to a small sample size; however, an infinite estimate can also mean there is no evidence for drift in that population, or the N_e is actually large (>1000). Consequently, our dataset is unable to distinguish whether or not the population is 'very large'. The regional population structure, negative inbreeding coefficients, and current landscape connectivity between Black Swan, Bibra and Kogolup Lakes suggest that together these populations actually represents a broader Beeliar Regional Park meta-population, and we speculate that the infinite N_e estimates could be a result of a large population and therefore not likely to suffer from genetic drift in the near future. Without increasing the sample size and recalculating the effective population size, we cannot confirm this population size. However, if the Kogolup Lake N_e is actually large then the lower bound may provide useful information about plausible N_e estimates (Do et al. 2014; Waples and Do 2010).

In conservation genetics, small population sizes limit adaptive potential (Hoffmann et al. 2017). Specifically, $N_e \geq 100$ is recommended to avoid inbreeding depression over the next five generations, while $N_e \geq 1000$ is recommended to maintain evolutionary potential; populations below this N_e will suffer a reduced ability to evolve to cope with environmental change over time (Frankham et al. 2014). Four of our seven study populations are at an N_e <100 (Table 6.3), and Joondalup and Herdsman lakes were not substantially higher than N_e = 100. Since most of our study populations are already showing signals of inbreeding, are completely isolated, or are in the process of becoming isolated due to urbanisation, ultimately all these populations are at risk of genetic degradation. If N_e is strongly influenced by area and quality of available habitat, then habitat conservation, management and restoration may be the best method to buffer the further loss of genetic diversity in urban island tiger snake populations.

6.5.4 On the origin of Carnac Island snakes

The Carnac Island tiger snakes showed a high level of genetic distinctiveness; this is not surprising given this population lives on an off-shore island. The Carnac Island population unexpectedly also had the highest level of genomic diversity, and the highest frequency of private alleles (328 compared to 56–111 in mainland populations). For a population that was suspected to be introduced ~ 90 year ago, and shares ancestry with the geographically-closest populations of the southern cluster (Black Swan Lake), this is surprising. Considering the small size of Carnac Island (19 ha), we expected the tiger snake population to show low genomic diversity as island populations are renowned for having lower genetic diversity compared to adjacent mainland populations (Clegg et al. 2002; Frankham 1997; Suezawa et al. 2021), even when the island introduction is less than 100 years (Hedrick et al. 2014). A large founding population could have resulted in high heterozygosity (Clegg et al. 2002; Kaňuch et al. 2020), however just ~ 40 adult snakes (Ladyman et al. 2020) were speculated to have been released on Carnac Island. Maintaining a large population size over time would also be necessary, as extended bottlenecks in the population size would have led to reductions in genetic diversity (Kaňuch et al. 2020; Nelson et al. 2021). It is possible that the founding population was sourced from many genetically diverse populations (e.g. including the east coast subspecies), if that was the case however, we would expect the Carnac Island snakes to separate from our sampled populations at lower *K* values, and the geographically-closest sampled populations to show little-to-no shared ancestry with Carnac Island in our admixture plots.

Surprisingly, we found the Carnac Island population had more than three to five times the private alleles compared to the mainland populations, much more than we would expect from *de novo* mutations over 90 years of isolation. We propose three hypotheses: (1) the snakes originated from other unsampled populations and the mutations are ancestral; (2) the mutation rates have increased as a response to novel selection pressures (Sniegowski et al. 2000), since the ecosystem on Carnac Island is very different to the habitat tiger snakes usually prefer on the mainland. This hypothesis could be supported by the phenotypic plasticity shown in the population (Aubret 2015), if epigenetically-driven plasticity has increased genome evolution (Ashe et al. 2021); or (3) the snakes are a naturally-occurring remnant population that is much older than 90 years, and the mutations are *de novo*. This hypothesis could be

supported by Black Swan Lake's – the geographically-closest sampled population – shared ancestry, and tiger snakes naturally occurring on the nearby Garden Island (Pearson et al. 2002), historically part of the land-bridge that connected these islands to the mainland roughly 6000 years ago (Playford 1983).

6.6 Conclusions

Urbanisation modifies ecosystems around the world, creating a range of stressors for wildlife living in cities. By investigating population genomic structure of species persisting in urban environments, we can gain useful information for conservation management of urban wildlife. Here, we genotyped urban and peri-urban populations of tiger snakes with the aim of understanding natural and anthropogenic influences on genomic diversity and population connectivity. We found that the major river system that runs through our urban study area has been a strong historical barrier to gene flow, resulting in the partial isolation of populations to the north of the river from the remainder of the species distribution. These northern populations also exhibited lower genomic diversity and lower body condition than southern populations, suggesting that they are most at risk of extirpation as urbanisation further encroaches upon their sensitive wetland habitats. As we expected, the populations most exposed to isolation—both geographic and urban—showed the strongest signal of inbreeding, although the maintenance of large effective population sizes appears to be driven primarily by the amount of available habitat. Together, these findings suggest that that increasing population connectivity and maximising the area of habitat in urban areas will help improve the adaptive capacity of urban wildlife. We also recommend further investigation into the genomic architecture of adaptation to urbanisation in this species, which will improve our understanding of the genetic and physiological pathways by which species adapt to urban environments.

6.7 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Fig S6.1 Hierarchical clustering dendrogram representing genetic distance relationships between *Notechis scutatus occidentalis* populations around Perth, Western Australia. Calculated using 4688 single-nucleotide polymorphisms from across the genome. Colours reflect unique sampled sites. Note that these relationships do not necessarily reflect a true phylogeny.

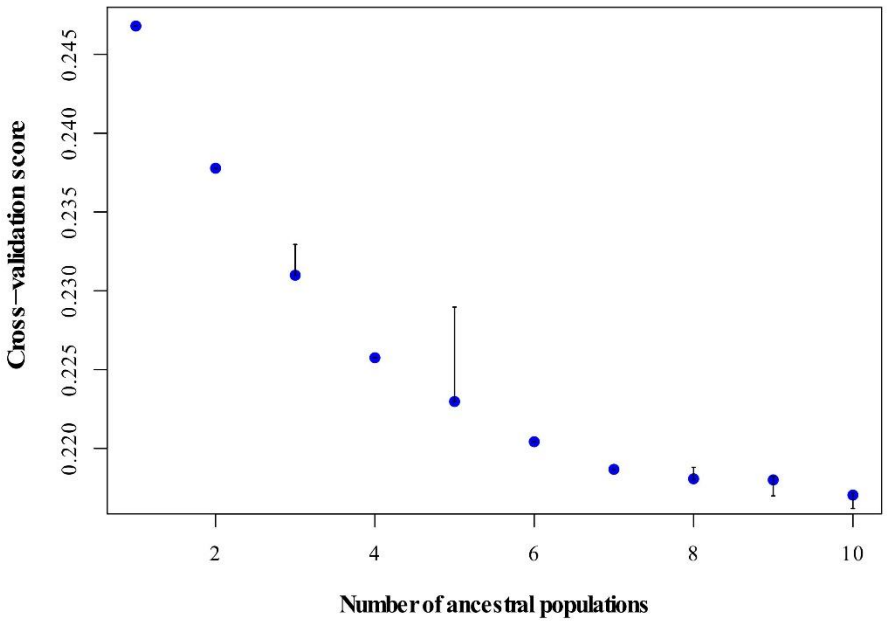

Fig. S6.2 Cross-entropy scree plot used to identify hierarchical population structuring in the genomic dataset for *Notechis scutatus occidentalis*. Lower values of the cross-entropy criterion indicate a better fit to the data.

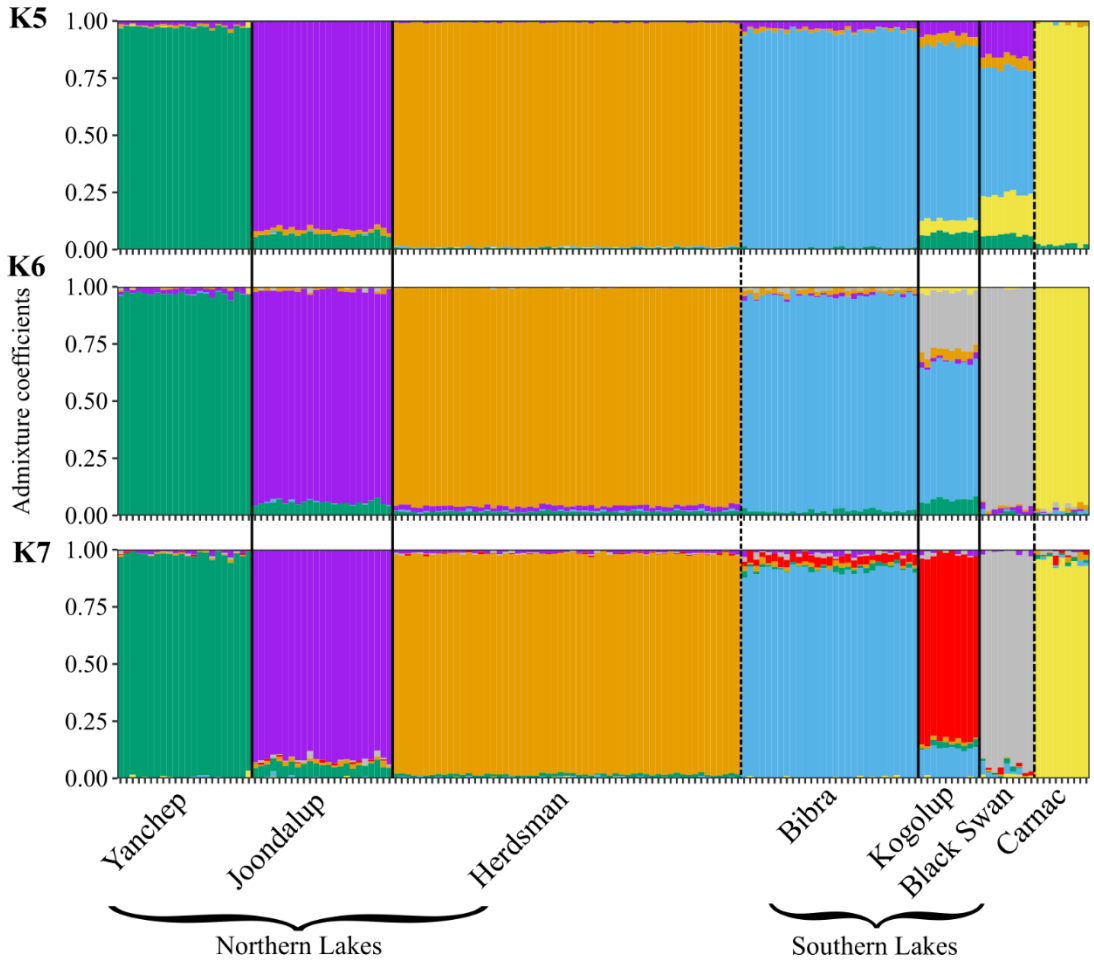

Fig. S6.3 Admixture bar plot comparing population structure in *Notechis scutatus occidentalis* from Perth, Western Australia. Each tick mark on the x-axis represents an individual snake, which are grouped by sampling locations. The dashed line represents the biogeographic barrier of the Swan/Canning Rivers separating the northern and southern sampling localities. The y-axis represents the fraction of individuals' genome that originates from a particular ancestral population, each of which has been given a unique colour.

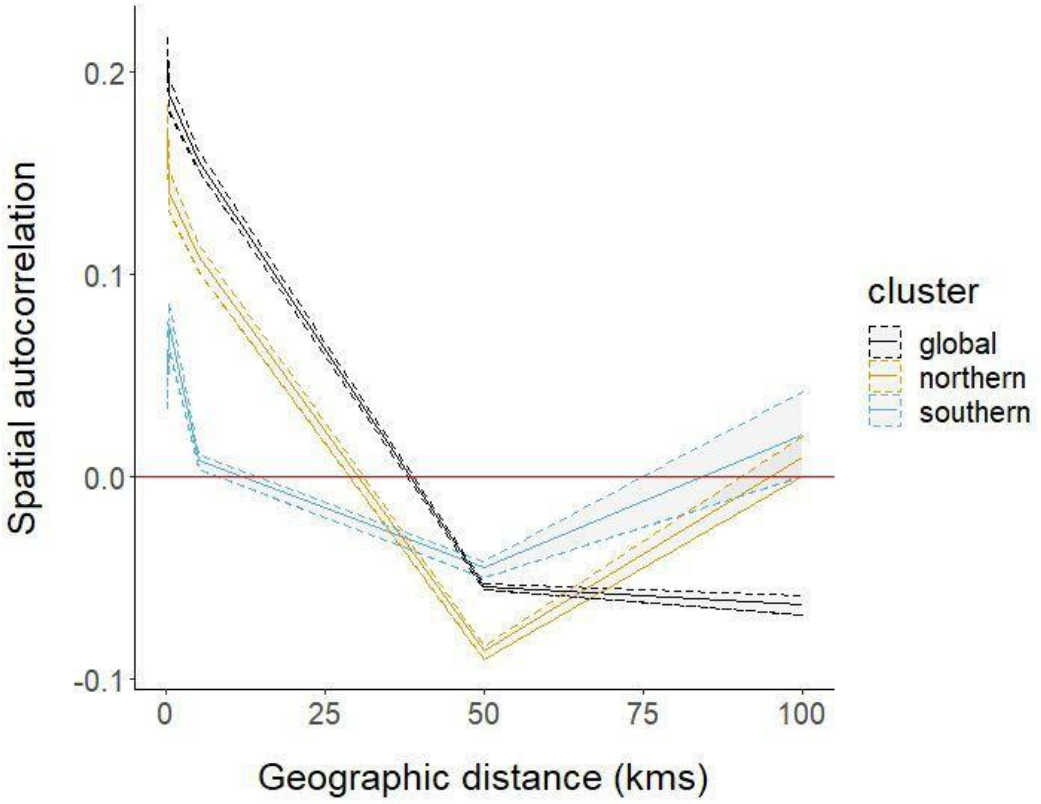

Fig. S6.4 Spatial autocorrelation of multilocus genotypes for *Notechis scutatus occidentalis* from Perth, Western Australia at five distance classes. Cluster indicates the population used for analysis. Global is all mainland snakes, Northern and Southern are the populations either side of the Swan/Canning River system. The probability value at each distance class shows the proportion of permuted *r* values greater than the observed value in that distance class, based on 999 permutations of the SNP by sample matrix.

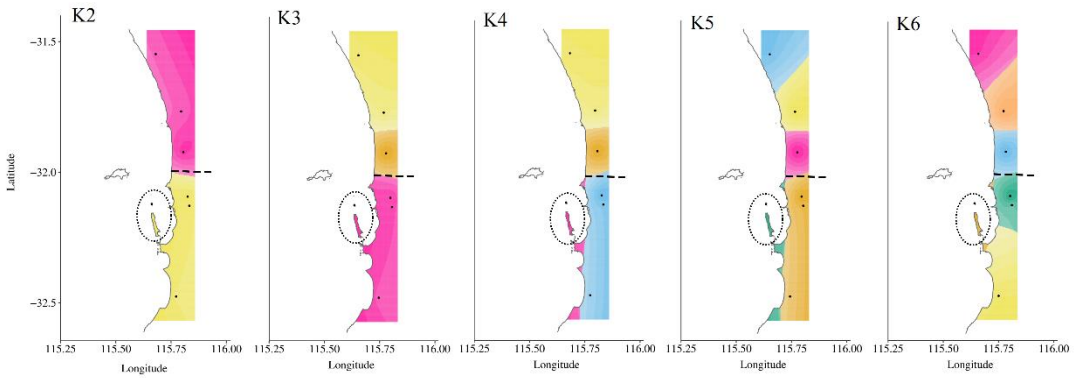

Fig. S6.5 Hierarchical population genomic structure of *Notechis scutatus occidentalis* around Perth, Western Australia. Unique colours in each panel represent an ancestral cluster. Black points indicate sampling sites. The dashed line represents the Swan/Canning River systems, while the dashed ring outlines Carnac and Garden Islands.

Table S6.1 Tukey HSD pairwise post-hoc test comparing body condition among six populations of *Notechis scutatus occidentalis* around Perth, Western Australia.

Sites	Estimate	SE	Df	t-ratio	*p*
Bibra Lake – Black Swan Lake	-13.24	11.9	76.3	-1.12	0.87
Bibra Lake – Herdsman Lake	43.49	15.4	76.6	2.82	0.06
Bibra Lake – Lake Joondalup	18.71	16.6	76.5	1.13	0.87
Bibra Lake – Kogolup Lake	0.83	11.3	76.1	0.07	1.00
Bibra Lake – Yanchep	23.60	20.7	76.9	1.14	0.86
Black Swan Lake – Herdsman Lake	56.73	18.1	76.9	3.14	**0.03**
Black Swan Lake – Lake Joondalup	31.95	19.1	76.8	1.68	0.55
Black Swan Lake – Kogolup Lake	14.07	13.8	76.5	1.02	0.91
Black Swan Lake – Yanchep	36.84	23.2	77.0	1.59	0.61
Herdsman Lake – Lake Joondalup	-24.79	11.2	76.0	-2.21	0.25
Herdsman Lake – Kogolup Lake	-42.67	17.5	76.3	-2.44	0.16
Herdsman Lake – Yanchep	-19.89	11.9	76.8	-1.67	0.55
Lake Joondalup – Kogolup Lake	-17.88	18.4	76.3	-0.97	0.93
Lake Joondalup- Yanchep	4.89	14.5	76.5	0.34	0.99
Kogolup Lake – Yanchep	22.77	22.3	76.7	1.02	0.91

Chapter 7. Metal(loid) pollution, not urbanisation nor parasites predicts low body condition in a wetland bioindicator snake

The study presented in Chapter 7 was accepted in the peer-reviewed journal '*Environmental Pollution*' on 10 December 2021, and is an exact reproduction of the copyright paper reformatted for this thesis.

Lettoof, D. C., Cornelis, J., Jolly, C., Aubret, F., Gagnon, M. M., Hyndman, T., Barton, D., Bateman, P. W. Metal(loid) pollution, not urbanisation nor parasites predicts low body condition in a wetland bioindicator snake. (2021). *Environ Pollut*. 118674. doi: 10.1016/j.envpol.2021.118674.

7.1 Abstract

Urban ecosystems and remnant habitat 'islands' therein, provide important strongholds for many wildlife species including those of conservation significance. However, the persistence of these habitats can be undermined if their structure and function are too severely disrupted. Urban wetlands, specifically, are usually degraded by a monoculture of invasive vegetation, disrupted hydrology, and chronic contamination from a suite of anthropogenic pollutants. Top predators—as bioindicators—can be used to assess and monitor the health of these ecosystems. We measured eight health parameters (e.g., parasites, wounds and scars, tail loss and body condition) in a wetland top predator, the western tiger snake, *Notechis scutatus occidentalis*. For three years, snakes were sampled across four wetlands along an urban gradient. For each site, we used GIS software to measure the area of different landscapes and calculate an urbanisation–landscape score. Previously published research on snake contamination informed our calculations of a metal-pollution index for each site. We then used generalised linear mixed models to assess the relationship between all health parameters and site variables. We found the metal-pollution index to have the most significant association with poor body condition. Although parasitism, tail loss and wounds differed among sites, none of these parameters influenced body condition. Additionally, the suite of health parameters suggested differing health status among sites; however, our measure of contemporary landscape urbanisation was never a

significant predictor variable. Our results suggest that the health of wetland predators surrounding a rapidly growing city may be offset by higher levels of environmental pollution.

7.2 Introduction

Conservation biologists are struggling against the tide of a rapidly progressing global extinction crisis (Ceballos and Ehrlich 2002; Gibbons et al. 2000; Szabo et al. 2012). Habitat modification and destruction, to accommodate the demands of an ever-expanding human population, is the leading cause of species extinction (Davies et al. 2006; Pimm and Raven 2000). The growth of urban areas is responsible for the highest degree of landscape change by destroying, displacing and degrading natural habitats while establishing entirely novel ecosystems (Hobbs et al. 2009). Consequently, urbanisation can impact wildlife populations via isolation (Miles et al. 2019), fluctuating resources (Kristan et al. 2004), constant disturbance (Doherty et al. 2021), and pollution (Müller et al. 2020). Yet, within our urban ecosystems, many remnant habitats persist and provide important strongholds for a surprising array of threatened species (Ives et al. 2016; Soanes and Lentini 2019).

The persistence of these valuable habitat remnants is adversely affected when the structure and function of these systems are disrupted (Knapp et al. 2021). For example, there is an increasing awareness that anthropogenic pollutants and parasites can simultaneously impact the health of resident fauna, and in turn impact population persistence and urban ecosystem function (Grimm et al. 2008; Koprivnikar et al. 2007; Koprivnikar and Redfern 2012; Martin et al. 2010; Rhind 2009). Although monitoring of populations via abundance counts is commonplace, only recently has the health of urban wildlife individuals been considered when assessing the resilience of populations to anthropogenic impacts (Kophamel et al. 2021; Murray et al. 2019). A focus purely on population size and dynamics without a measure of population health may miss subtle signs of perturbation and, without wildlife health assessments, could easily miss indications of a population on the verge of collapse.

The monitoring of ecosystem health and function requires complex, multifaceted data and substantial resources. A pragmatic alternative is to use bioindicator species; an organism that is easily detectable and exhibits a response to an environmental stressor, but is not so sensitive that minor or biologically unimportant alterations stimulate a

change (O'Connor and Dewling 1986; Paoletti and Sommaggio 1996; Sharma and Rawat 2009; Siddig et al. 2016). Top predators are useful bioindicators because their high trophic position often means their health reflects the dynamics of bottom-up processes of their ecosystem (Brown et al. 2013; Sergio et al. 2008). Large reptilian predators, such as snakes and crocodilians, are becoming increasingly recognised as excellent bioindicators of ecosystem health (Haskins et al. 2021; Manolis et al. 2002). Over the course of their life, large reptilian predators function as both predators and prey, and have shown resistance to the toxic effects of contaminant concentrations that would be lethal to other taxa (Beaupre and Douglas 2009; Haskins et al. 2019; Weir et al. 2015).

To assess how environmental factors associated with urban ecosystems impact the health of resident fauna, we compared a suite of health parameters in populations of a native top predator—tiger snakes (*Notechis scutatus*)—from sites differing in urbanisation intensity and contamination. To do this, we leveraged a substantial population-level dataset across multiple landscape-scale sites. Because tiger snakes bioaccumulate environmental contaminants, we anticipated that population health would be negatively associated with pollution, and potentially by parasites and by urban landscape modification. Here, we present a suite of tiger snake health parameters compared among sites, and use a series of analyses to explore which biotic and abiotic variables best explain poor health.

7.3 Methods

7.3.1 Study sites and species

Western tiger snakes (*Notechis scutatus occidentalis*) are a large (~1 m), terrestrial elapid, which are typically associated with wetland and wet forest habitats on the Australian mainland. Although a dietary generalist, they have a strong preference for frog prey (Aubret et al. 2006; Lettoof et al. 2020c). Because western tiger snakes are an abundant wetland top predator, they are a good model to examine the effects of urbanisation and contamination in wetlands (Lettoof et al. 2020a; Lettoof et al. 2021b).

We studied populations of western tiger snakes located in wetlands in and surrounding Perth, Western Australia. Perth is built on the Swan Coastal Plain (SCP), a bioregion characterised by sandy dunes, *Banksia* woodlands and a chain of wetlands interconnected by the groundwater table (Thackway and Cresswell 1995). Over the

past 200 years, approximately 70% of SCP wetlands have been lost, while remaining wetlands have been subject to degradation through anthropogenic disturbance and urbanisation (Davis and Froend 1999; Gentilli and Bekle 1993). In Perth, tiger snakes persist in a handful of wetlands. Based on abundance, we chose to assess the health of four snake populations from wetlands differing in degree of anthropogenic disturbance and pollution (Fig 7.1; in order of most to least urbanised): Herdsman Lake (HL), Bibra Lake (BL), Lake Joondalup (JL) and Loch McNess (YC), the latter located within Yanchep National Park. These sites are located within a 60 km north-to-south range and experience similar annual rainfall and temperatures (Bureau of Meteorology 2021). Recent studies have found tiger snakes at these sites are exposed to and accumulate a suite of contaminants (Lettoof et al. 2020a; Lettoof et al. 2020b; Lettoof et al. 2021b), and populations north of the Swan/Canning river systems have lower genomic diversity than the southern populations (Chapter 6).

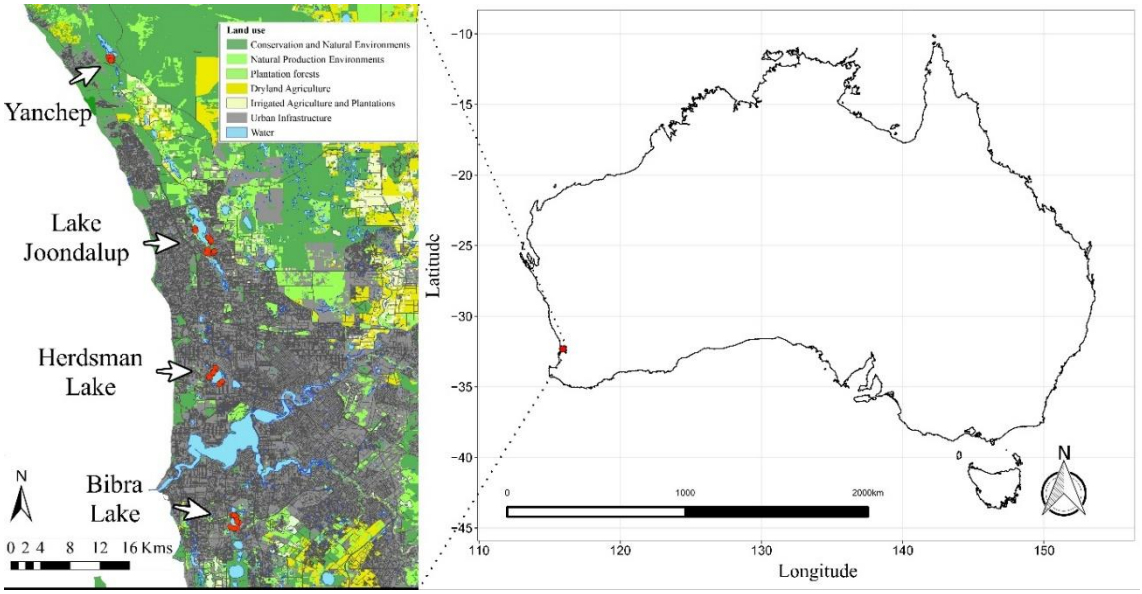

Fig. 7.1 Study sites and land use in Perth, Western Australia. Red dots represent a sample of individual western tiger snakes (*Notechis scutatus occidentalis*) used in this study.

7.3.2 Data collection

Between 2018 and 2020, DCL and JC hand-caught tiger snakes during a six-week period of peak activity (September–October), when tiger snakes finish overwintering and begin breeding activity (Shine 1979, 1987). For all snakes, we measured the snout-vent length (SVL; mm), tail length and body mass (g), and identified sex by probing

the hemi-penal pocket. Prey were detected by palpation of the stomach. To identify prey species, we would attempt to palpate prey to the mouth of each snake. We then returned prey to the stomach to prevent interfering with the long-term health of individuals.

We also used palpation to determine the presence of gastric nematodes (*Ophidascaris pyrrhus*), where we could positively detect a nematode infection of > 12 individuals (D. Lettoof, unpubl. data). As the mean nematode infection intensity was 31 (Lettoof et al. 2020c), we considered an infection of < 12 nematodes to not be pathogenic and count as an absence. We counted intensity of three types of parasites: ticks (*Amblyomma albolimbatum*), skin worms (plerocercoid larval *Spirometra* spp., most likely *S. erinacei*) and oral trematodes (*Dolichoperoides macalpini*). See Chapter 7 supplementary material for identification methods. We also counted visible dorsal wounds or scars, which often reflect bird or rodent attacks (Fearn 2011) and other injuries, and the number of ventral scales with dermatitis and scarring. Ventral scales affected by dermatitis were swabbed and examined for known pathogenic bacteria and fungal species (including *Ophidiomyces ophidiicola*), and one biopsy of an affected scale was histologically assessed (see Chapter 7 supplementary material). Figure 7.2 depicts these health parameters.

All snakes were marked by clipping their ventral scales in a sequential pattern (Plummer and Ferner 2012). Health parameters were only measured the first time each individual was recaptured within a season i.e. not the second time it was caught within a season. Across all three years of the study we recaptured 19 (of 171) at HL, 16 (of 93) at BL, 3 (of 83) at JL and 11 (of 59) at YC snakes, and we used these recaptures to estimate daily growth rates by calculating the change in SVL since the last capture (Brown et al. 2013). Data were collected under Western Australia's Department of Biodiversity, Conservation and Attractions Permit No. 08-002624-1 and Curtin University's Animal Research Ethics Committee Approval No. ARE2018-23.

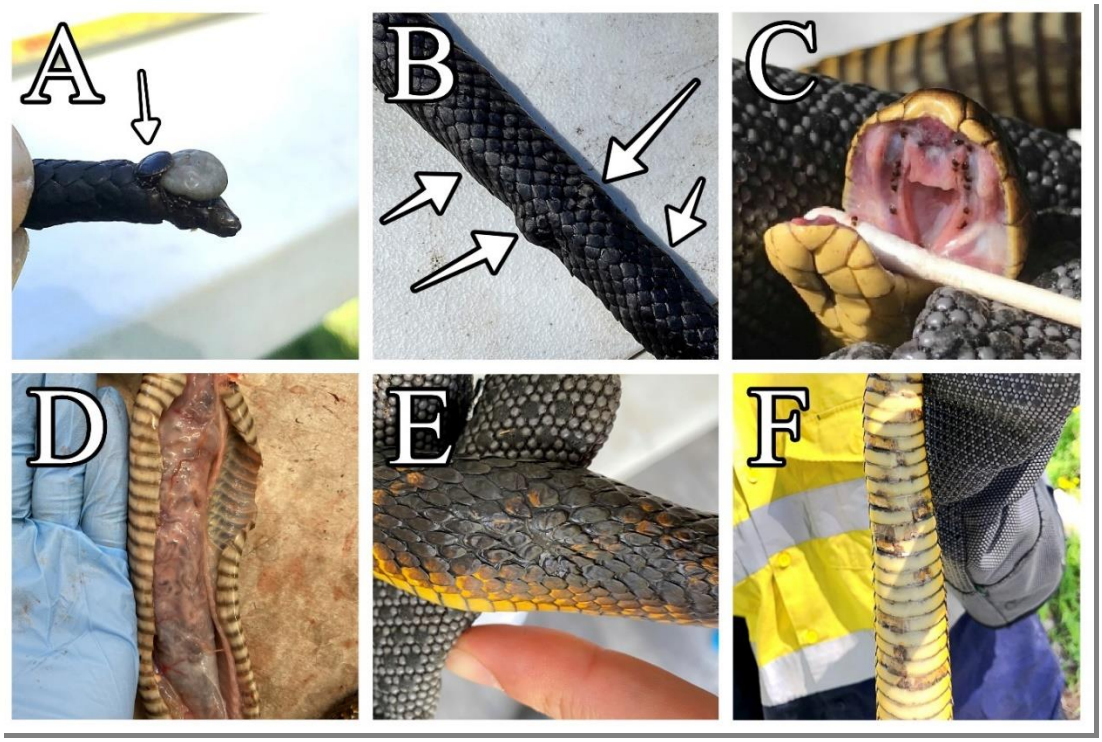

Fig 7.2 Examples of health parameters measured in western tiger snakes (*Notechis scutatus occidentalis*) around Perth, Western Australia. A) male (left; indicated by arrow) and engorged female (right) *Amblyomma* ticks on a damaged tail; B) sparganosis caused by plerocercoid larval *Spirometra* sp. (indicated by arrows); C) *Dolichoperoides macalpini* trematodes parasitising the mouth; D) a large (n > 70) stomach burden of *Ophidascaris pyrrhus* nematodes; E) a dorsal wound/scar likely caused by a bird or rodent bite; and F) ventral scales with dermal necrosis.

From these data we calculated two extra variables to include in our models: body condition and degree of tail loss. Body condition was calculated using a scaled mass index (SMI; reported as grams per cm [gpc]), from the Peig and Green (2009) equation: $\boldsymbol{SMI} = M_i \left(\frac{L_0}{L_i}\right)^{\boldsymbol{b}\mathbf{SMA}}$ where M_i = individual mass, L_0 = arithmetic mean length for the study population, L_i = individual length, and *b*SMA = standardised major axis regression of mass on length for the study population. Of the total 409 snakes, those with prey items, obvious pregnancy or that were recaptured were excluded from body condition calculations. This left 265 individuals, from these the arithmetic mean SVL used was 757.5 mm and the *b*SMA was 3.0.

Tail loss can be frequent (> 70% of individuals) and often severe (total loss of tail) in some tiger snake populations (Aubret et al. 2005). We categorised any snake missing

the terminal conical scale as having tail loss, and we calculated degree of tail loss (%) for these snakes by dividing tail length by expected normal tail length for each sex. Expected normal tail length was determined using the linear regression between SVL and intact tail length on 99 adult males ($r^2 = 0.72$, $F_{1, 97} = 244.4$, $p < 0.01$) and 54 adult females ($r^2 = 0.24$, $F_{1, 52} = 17$, $p < 0.01$). Snakes with tail loss (no conical scale), but whose tail length exceeded the expected normal tail length (based on the linear regression), were assigned a value of 1% tail loss because a more definitive value could not be assigned.

7.3.3 Site characteristics

For each site we measured the area (ha) of four land cover variables using QGIS v. 3.10.14 and ESRI satellite imaging. These variables were: urban (impervious surfaces of asphalt, concrete, buildings and roads), snake habitat (vegetation that contains mid-to-understory layers that tiger snakes could shelter in), modified vegetation (green space that contains no mid-to-understory layers, such as mowed grass) and water. Snakes were only caught around the southern half of Lake Joondalup, so we only measured this area of the wetland. We used a principal component analysis (PCA) on the land cover variables to give each site an urbanisation score. Only the first PC had an eigenvalue > 1 and explained 85.21% of the variation. It represented a major gradient of urbanisation with a positive load for urban (0.97), water (0.92), modified vegetation (0.86) and a negative load for snake habitat (-0.94). Thus, the urbanisation score for each site was its location on PC1.

We also calculated a pollution bioaccumulation score for each site using the metal pollution index (MPI; reported as mg/kg), calculated with the equation (Rzymski et al. 2013; Usero et al. 1996): **MPI** = $(Cf1 \times Cf2 \ldots Cfn)^{1/n}$ where Cfi = concentration of the metal in the sample. To calculate the MPI for each site, we first calculated the MPI for each snake (n = 5/site) using the liver concentrations of eight heavy metals: Sb, Ba, Cd, Pb, Hg, Ni, Ag and Sn; and eight trace elements: As, Co, Cu, Cr, Cu, Mo, Se and Zn (collectively referred to as metal(loid)s throughout) reported in Lettoof et al. (2020a). Metal(loid)s were analysed using a combination of inductively coupled plasma-mass spectrometry (ICP-MS) and ICP-atomic emission spectroscopy (see Lettoof et al. (2020a) for details on detection limits, quality control and raw data). Concentrations of As, Ba, Cd, Co, Cu, Hg Mo, Sb and Se showed site-specific

differences in the livers of tiger snakes (see Lettoof et al. (2020a) for more details), and 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. Sampled tiger snakes were male-biased at each site, yet we pooled sexes based on a lack of statistical difference between sex and concentrations of the metal(loid)s analysed (Lettoof et al. 2020a; Lettoof et al. 2021b). We then used the mean liver MPI for each site. The MPI does not compare contaminant concentrations with any guidelines (Caeiro et al. 2005), and since there are no known toxicity guideline values for these metals in snakes, we deemed it the most appropriate index to use.

7.3.4 Statistical analysis

All analyses were performed using R version 4.0.3 (R Core Team 2021) with the *lme4* and *glmmTMB* packages (Bates et al. 2014; Magnusson et al. 2017).

7.3.4.1 Growth rates

To compare snake growth rates between sites we generated the residual scores from a linear regression of log+1 transformed daily growth vs. SVL (Madsen and Shine 2000). We used a generalised linear mixed model (GLMM) with Gaussian error structure to test the effects of site (levels: HL, BL, JL, and YC), sex (levels: male and female) and year (levels: 2018–2019 and 2019–2020), with snake ID included as a random factor, on residual growth score.

7.3.4.2 Proportion with prey

We compared the number of snakes with prey items among sites as a proxy for prey abundance (Brown et al. 2013). We used a GLMM with a binomial error structure and logit link to test the effect of site, with year (levels: 2018, 2019 and 2020) as a random factor, on the proportion of snakes that had food in their stomach. We modelled our response variable as the number of snakes with (1s) and without (0s) food in their stomachs during a fixed number of Bernoulli trials (total number of snakes palpated).

7.3.4.3 Health profile

We used a principal component analysis (PCA) on all health parameters to create an overall health ‘profile’ for each snake. The PCA included SMI, tail loss (%), number of ventral and dorsal scars, number of ticks, skin worms and trematodes, and nematode

presence or absence (as 0 or 1). No variables showed collinearity ($r^2 < 0.3$) and all data were scaled using the 'scale.unit' argument. The PC scores were then extracted and only components with eigenvalues > 1, were considered further. We then used separate GLMMs on the PCs, to test for site and sex differences in health profiles. PCs were the response variable, site and sex were the fixed predictors, and year was included as a random effect. PCAs were calculated using the 'PCA' function of the FactoMineR package (Le et al. 2008).

7.3.4.4 Health parameters

To identify the health parameters of importance and the covariates that predict them, we used a two-step process. We first ran separate GLMMs to test whether each parameter differed in snakes as a function of site, sex and year to identify which parameters warranted further exploration, and to select random effects (Table S7.1). No parameters were correlated with SVL ($r^2 < 0.1$), so we used their normal values. Ticks were only present on 12% of snakes and were never detected on a Herdsman Lake snake, so were not explored as a response variable but included as predictor variables. Ventral dermatitis/scars and nematode presence did not differ among sites ($p = 0.45$ and $p = 0.94$, respectively) so were not explored further. For this step we used the entire dataset of 391 snakes (not including recaptures or juveniles) to assess all parameters except body condition, where we used the subset of data of 265 snakes without prey, pregnancy or recaptures.

Of all our measured parameters, we consider body condition to be our best indication of individual health. We therefore fitted a global GLMM with body condition as the response, site and all other health parameters as predictors, and to account for temporal differences—years as the random factor (Fig S7.1). We then reran this model refitting site with MPI and urbanisation PC1, and used AIC ranking as an indication of which variable best explains the site contribution. If site is a better predictor over MPI and PC1 (i.e. lowest AIC rank), this suggests an unmeasured variable offers a better explanation. The best fitting global model was dredged using the 'dredge' function from MuMIn package (Barton and Barton 2015) and all sub-models were ranked according to AICc. Only models with ΔAICc < 2 were considered further. All variables were scaled and centred to improve model fit, and data from both male and females were pooled. For exploratory purposes, we repeated this method for each

health parameter against all other ecologically relevant health parameters. These results are presented in the Tables S7.2–S7.5. The error structures used for GLMMs were: negative binomial for dorsal and ventral scars, skin worms and trematodes; binomial error for nematode presence; Tweedie for tail loss; and Gaussian for body condition. Error structures were selected based on the response variables distribution, and assessment of residuals for best model fit.

7.4 Results

7.4.1 Site characteristics

Across all sites, Herdsman Lake and Lake Joondalup had the most urban landscape and modified vegetation; however, Herdsman Lake had the least relative area of snake habitat (Table 7.1). Yanchep had the most snake habitat, and the least urban landscape and modified vegetation, as well as the smallest waterbody. Despite contrasting landscape variables, Herdsman Lake and Yanchep snakes had the highest mean liver MPI (Table 7.1).

Table 7.1 Area of landscape characteristics for the four study wetlands around Perth, Western Australia. MPI = mean metal pollution index of snake livers (n = 5/site).

Site	Total area (ha)	Urban (%)	Snake habitat (%)	Modified vegetation (%)	Water (%)	MPI (mg/kg)
Herdsman Lake	1 134.4	60.4	5.3	12.1	22.2	0.21
Bibra Lake	946.6	41.8	28.3	16.0	13.9	0.12
Lake Joondalup	1 475.3	50.1	17.5	9.7	22.7	0.13
Yanchep	1 004.5	2.1	88.6	8.2	1.0	0.18

7.4.2 Health profiles

While controlling for year, PC1 and PC4 health profiles differed significantly among sites ($F_{3,\ 264}$ = 32.70, p < 0.01 and $F_{3,\ 264}$ = 8.77, p < 0.01, respectively) and between sexes ($F_{1,\ 264}$ = 4.99, p = 0.03 and $F_{1,\ 264}$ = 7.54, p < 0.01, respectively). PC2 health profiles only differed significantly among sites ($F_{3,\ 264}$ = 22.08, p < 0.01). PC3 health profiles did not differ significantly among sites ($F_{3,\ 264}$ = 2.52, p = 0.06) or sexes ($F_{1,\ 264}$ = 0.83, p = 0.36). PC1 and PC2 explained the most variation and only these components are considered further (Table 7.2). For PC1, health profiles of snakes were characterised by higher counts of skin worms and ticks, more dorsal scars and less tail

loss (Table 7.2). For PC2, health profiles of snakes were characterised by a higher degree of tail loss, more dorsal scars and fewer trematodes (Table 7.2). A Tukey HSD test found PC1 differed significantly between Herdsman Lake and all other sites ($p < 0.01$). PC2 differed significantly between Yanchep and all other sites ($p < 0.01$), and Bibra Lake was significantly different to Lake Joondalup ($p = 0.01$). The health profiles of snakes from Herdsman Lake were the most homogenous, snakes from Yanchep showed the most heterogeneous health profiles, and the health profiles of snakes from Bibra Lake and Lake Joondalup were almost identical (Fig. 7.3).

Table 7.2 Loading values of variables used in the health profile PCA with eigenvalues > 1.

Eigenvalues and loading values	PC1	PC2	PC3	PC4
Eigenvalues	1.62	1.23	1.09	1.03
Variation explained (%)	20.22	15.38	13.67	12.81
Loading values				
SMI (gpc)	0.17	-0.18	-0.58	-0.63
Tail loss (%)	-0.33	0.37	0.21	0.19
Dorsal scar (#)	0.51	0.62	-0.04	0.07
Ventral scar (#)	0.44	0.14	-0.07	0.62
Skin worms (#)	0.67	-0.33	0.15	0.01
Trematodes (#)	0.37	-0.66	-0.14	-0.08
Ticks (#)	0.64	-0.31	-0.27	-0.28
Nematodes (pres/abs)	0.19	-0.14	0.77	0.36

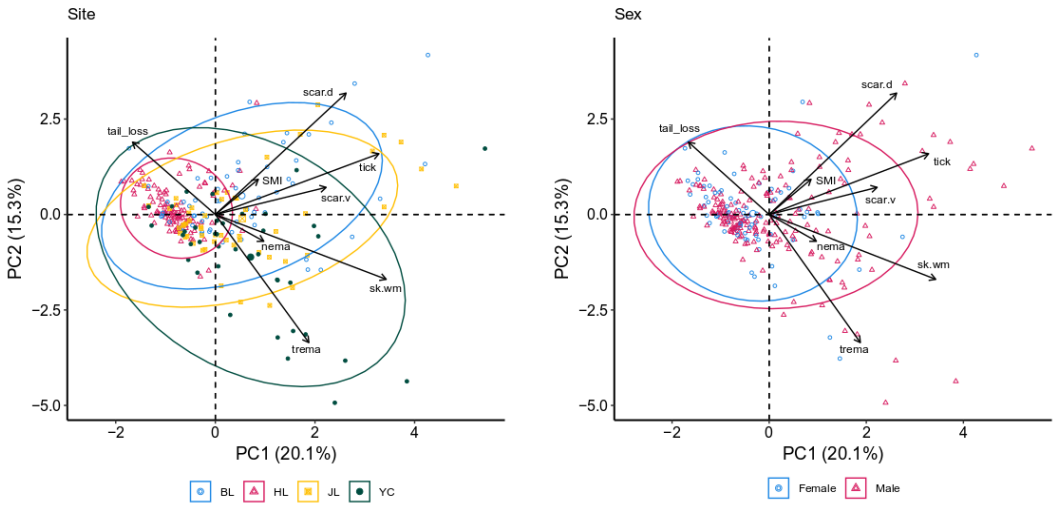

Fig. 7.3 Principal component analysis of western tiger snake (*Notechis scutatus occidentalis*) health profiles grouped by site and sex. BL = Bibra Lake, HL = Herdsman Lake, JL = Lake Joondalup and YC = Yanchep National Park; ellipses represent 90% spread of the data.

7.4.3 Growth rates

Growth rates did not differ significantly among sites ($F_{3, 48}$ = 2.38, p = 0.08) or years ($F_{1, 48}$ = 0.79, p = 0.47), nor between sexes ($F_{1, 48}$ = 2.55, p = 0.12).

7.4.4 Diet and prey proportions

Sixty of the 187 (32%) snakes from Herdsman Lake had detectable prey items in their stomach: 30 frogs, four juvenile birds, one house mouse (*Mus musculus*) and 25 could not be manipulated to their mouth (unknown). Using the same method, 38 of the 115 (33%) snakes from Bibra Lake had consumed prey items: 15 were frogs, two mice, one juvenile bird and 20 unknown. From Lake Joondalup, 30 of the 84 (36%) snakes had prey items: 17 frogs, 2 mice and 11 unknown. From Loch McNess in Yanchep National Park, 27 of the 74 (36%) snakes had prey items: 16 frogs and 58 unknown. Eight snakes were missing prey data. There was no association between site and prey proportions (χ^2_3 = 0.72, p = 0.87).

7.4.5 Ventral swabs

In total, 17 swabs and seven freshly frozen skin scrapings were tested by PCR (Table S7.1). All samples were PCR-negative for *Nannizziopsis* spp. and *O. ophidiicola*. Histological findings and interpretation are presented in the supplementary material (Fig S7.2).

7.4.6 Health parameters

The metal-pollution index was a better fit than site and urbanisation (PC1) for the body condition global model and only two top models were well-supported (ΔAICc < 2; Table 7.3). These models both identified site MPI as a strong negative predictor of body condition (Figure 7.4), and the second model found a weak positive effect of nematode presence on body condition.

Table 7.3. Site variation selection table, and top models identifying the strongest predictors of tiger snake body condition based on ΔAICc and weight. MPI = metal pollution index of tiger snake livers (site mean); PC1 = urbanisation landscape variable; R. E. = random effect; d.scar = dorsal scar; v.scar = ventral scar; nema = nematode presence; trema = oral trematode; sk.wrm = skin worm; tail = degree of tail loss. Covariates reported as estimate (standard error).

Site variation global models + (1|year) + (1|sex)	**AIC**	$R^2_{GLMM(c)}$	**Variation of R.E.**
MPI + d.scar + v.scar + nema + trema + sk.wrm + tick + tail	749.51	0.21	0.05
Site + d.scar + v.scar + nema + trema + sk.wrm + tick + tail	760.86	0.21	0.05
PC1 + d.scar + v.scar + nema + trema + sk.wrm + tick + tail	796.06	0.09	0.06

Top models (<2 ΔAICc)

	MPI	**nema**	**Intercept**	**logLik**	**AICc**	**Δ**	**weight**
Model 1	-9.75 (1.42)	-	1.70 (0.30)	-353.03	716.3	0.00	0.48
Model 2	-9.78 (1.42)	0.21 (0.14)	1.53 (0.33)	-352.94	718.2	1.93	0.18
Null	-	-	0.05	-376.00	760.16	43.88	0.00

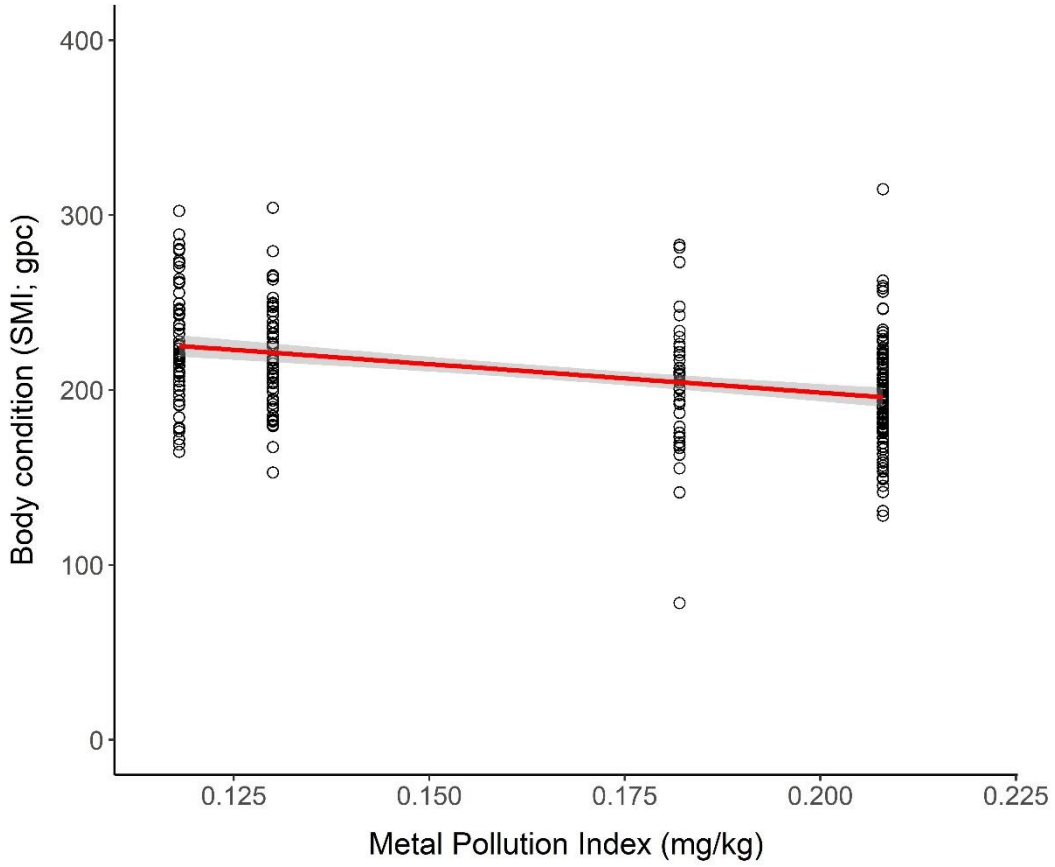

Fig. 7.4 Linear relationship between western tiger snake (*Notechis scutatus occidentalis*) body condition and site-averaged metal-pollution index. SMI = scaled mass index, gpc = grams per cm. The line of best fit in this figure is based on the linear relationship between MPI and SMI (n = 265, r^2 = 0.14, p = <0.01, y = 263.7 – 325.6x).

Ventral scars were significantly more frequent in males and different between years (Table S7.1). MPI and ticks were frequent predictors of dorsal scars for all six top models. MPI had a strong negative effect, and ticks and ventral scars had a weak positive effect on dorsal scars (Table S7.2). Site, nematodes and ticks were frequent predictors of skin worms for all seven top models (Table S7.3). Herdsman Lake snakes had the lowest intensity of skin worms, and nematode presence and tick intensity had a positive effect on skin worms. Trematode intensity was significantly higher in females; site, nematode presence, ticks and skin worms were frequent predictors of trematode intensity for all ten of the top models (Table S7.4); Bibra Lake had the lowest intensity, and nematodes, ticks and skin worms had weak positive effects on trematodes. Site was the only predictor of tail loss for all five top models, and snakes had the highest degree of tail loss at Herdsman Lake and Bibra Lake (Table S7.5). The urbanisation variable was not a significant predictor for any health parameter.

7.5 Discussion

By comparing a suite of health parameters and landscape variables among sites, we found that body condition in western tiger snakes differed among sites and that the mean metal pollution index from snake livers appears to be the most significant predictor of body condition. Nematode presence showed a weak positive association with body condition, which could be a reflection of healthier snakes having the capacity to carry more parasites (Mayer et al. 2015; Sanchez et al. 2018). Alternatively, there is the possibility that heavy nematode burdens could increase snake mass and positively bias body condition weight (Poulin and George-Nascimento 2007). Although parasitism, tail loss and wounds differed among sites, none of these parameters had a negative effect on body condition. Unsurprisingly, the snake population living in the most isolated, urbanised and polluted wetland—Herdsman Lake—had the lowest body condition, fewest parasites and most homogenous health profile of all sites. Interestingly, despite being near-free from urbanisation, the Yanchep population of tiger snakes had the second-highest liver MPI, likely caused by sediment erosion and an indirect anthropogenic source of agriculture-contaminated groundwater (Lettoof et al. 2020a; Lettoof et al. 2021b). Even though these snakes live

in habitat superficially free of anthropogenic disturbance, this cryptic contamination was reflected in their poor body condition relative to snakes from less polluted sites.

Although health parameters differed among sites, our measure of contemporary landscape urbanisation was never a significant predictor variable in any of our models. Despite studies showing linear relationships between landscape urbanisation and wildlife health (Giraudeau et al. 2014; Li et al. 2016; Saito and Sonoda 2017), it is also common for urban wildlife populations to exhibit site-specific differences in health (Winchell et al. 2019; Zhang et al. 2011). As urbanisation introduces complex stressors, our measure may not have been able to capture a pattern as we were limited to only four comparable sites with remnant populations of tiger snakes. This study provides novel evidence suggesting that environmental metal(loid) pollution, and seemingly not urbanisation nor parasites, had a significant negative association with body condition in tiger snakes surrounding a rapidly growing city.

7.5.1 Body condition

The perils of poor body condition include reduced reproductive success (Milenkaya et al. 2015), increased predation (Mattisson et al. 2016), and ultimately individual mortality (Shine et al. 2001). Therefore, despite the current abundance of tiger snakes in these sites, a population-wide reduction in body condition could be a precursor to population decline (Aubry et al. 2013; Reading 2007). Common causes of poor body condition in squamate populations are low prey availability (Gregory 2006), habitat deterioration and stress-inducing constant anthropogenic disturbance (Amo et al. 2006, 2007; Carrasco-Harris et al. 2020), and, in other taxa, high densities of conspecifics (Bucher and Entling 2011; Simard et al. 2014; Tella et al. 2001). We could not determine population density from our survey methods; however, snake population size is usually relative to prey abundance (McCauley et al. 2006; Zipkin et al. 2020) and we found the proportion of snakes with consumed prey (an index of prey availability) did not differ among sites. In addition, the snakes from the most undisturbed wetland, Loch McNess in Yanchep National Park, had relatively poor body condition. Although rarely documented, metal(loid) pollution is known to cause reduced body condition (Li et al. 2021; Newth et al. 2016). Our data suggest that no factor other than pollution explains the poor body condition of tiger snakes at Herdsman Lake and Yanchep National Park with similar parsimony.

7.5.2 Influence of pollution

Herdsman Lake and Yanchep snakes had the highest mean liver MPI (0.21 and 0.18 mg/kg, respectively), while Bibra Lake (0.12 mg/kg) and Lake Joondalup (0.13 mg/kg) were considerably less polluted. The variability in liver MPI was low (± 0.02 – 0.03 mg/kg) for all sites besides Lake Joondalup (± 0.05 mg/kg), such variability which could conceivably be driven by spatial heterogeneity in pollutant distribution across a large wetland. As the relationship between site MPI and body condition was based on a small sample size of four sites and five snakes per site, future research in this system would benefit from increasing the number of comparable study sites included, as well as increasing the number of snakes sampled for contaminants per site. Despite this study simply presenting a snap-shot of metal(loid) pollution in snake livers, the same pattern of inter-site metal(loid) pollution has been detected in a study of snake scales in these populations using a much larger sample of 25 – 30 snakes per site (Lettoof et al. 2021b).

The toxicodynamics of metal(loid) pollution has seldom been reported in reptiles (Gardner and Oberdorster 2006; Hopkins 2000). Of the few published studies, arsenic, lead, selenium and cadmium have been associated with changes in health and behaviour of reptiles. Arsenic was associated with a decrease in the running speed of hatchling *Lacerta* lizards (Marco et al. 2004). The food consumption and body weight of *Sceloporus occidentalis* lizards have shown a decrease following exposure to food consumption and body weight from lead (Salice et al. 2009), and selenium exposure has shown a decrease in male body condition in the same species (Hopkins et al. 2005). Finally, in *Nerodia fasciata* that accumulated arsenic, cadmium and selenium, the standard metabolic rate increased (Hopkins et al. 1999). Tiger snakes are exposed to, and bioaccumulate, these three metal(loid)s—and at least six others—in the wetlands we studied (Lettoof et al. 2020a). As we found no significant difference in snake prey proportion among sites, and a negative relationship between site MPI and body condition, we suspect lower body condition may be caused by the bioaccumulation of multiple metal(loid)s acting synergistically on snake physiology and metabolism. We cannot propose a toxicological mechanism, due to the dearth of reptile toxicological knowledge; however, despite having access to an abundance of resources, snakes carrying a significant metal(loid) burden may suffer from a disrupted energy budget (Jager et al. 2014) resulting in reduced muscle mass and fat storage. Further

investigation, through field and controlled toxicological experiments, is warranted to ascertain (a) whether the site-specific contaminant cocktails are the primary cause of ill-health, (b) which contaminants are inducing toxicity in snakes, and (c) what the toxicodynamics of these contaminants are in snakes.

If metal(loid) pollution induces poor body condition in tiger snakes, and if reptiles are more tolerant of toxicants—relative to other taxa (Grillitsch and Schiesari 2010)—what do these results imply for the ecosystem? Exposure to, and bioaccumulation of, a cocktail of metal(loid)s may compromise the physiology (Bernanke and Köhler 2008), biochemistry (Gavric et al. 2015; Lettoof et al. 2021a) and behaviour (Saaristo et al. 2018) of wildlife. Populations completely restricted to remnant urban 'islands' are particularly susceptible because urban runoff perpetually introduces pollution (Müller et al. 2020) and toxicants can be transmitted inter-generationally, from mother to developing offspring (Aulsebrook et al. 2020; Lettoof et al. 2021c). Moreover, urban-induced eutrophication, pH changes and heat-island effects can increase the bioavailability of contaminants (Cabral et al. 2019). Ultimately, while urban wetlands may offer vital habitat in a matrix of non-viable habitats, chronically polluted urban wetlands may come with serious associated costs for isolated, resident fauna.

7.5.3 Parasites

Host–parasite relationships are complex, and infection is often presumed to negatively affect hosts (Rynkiewicz et al. 2015). For example, ticks can harbour and transmit disease-causing pathogens to their hosts (Irwin et al. 2018) and large burdens of nematodes can cause malnutrition as they interfere with the host's capacity to assimilate nutrients (Hlaing et al. 1991). Although there is ample evidence of the impacts of over-abundant parasites (Watson 2013), hosts can evolve a tolerance to parasites resulting in them having little-to-no cost to fitness (Paterson and Blouin-Demers 2020; Sanchez et al. 2018) and abundant parasites can actually be a sign of healthier individuals (Comas et al. 2014; Sanchez et al. 2018).

We found parasite presence and intensity differed among sites, positive relationships among parasite species, and nematodes were the only measured parasites to have a significant (but weak and positive) relationship with body condition. Seemingly unintuitively, the two most historically urbanised sites—Herdsman Lake and Bibra Lake—had snakes with the lowest parasite burdens. Most tiger snake parasites have

multi-host lifecycles (Jacobson 2007). For example, the trematode *D. macalpini* life cycle involves freshwater molluscs then tadpoles and frogs before infesting tiger snakes (Johnston and Angel 1940). Since urbanisation-caused habitat isolation, modification and homogenisation often reduces species diversity (Delgado-V and French 2012; Fenoglio et al. 2021), lower presence and intensity of parasites at these sites may be the result of a loss of intermediate hosts, such as molluscs.

Of the parasites we did record, trematodes, skin worms and ticks are likely the least pathogenic (Jacobson 2007; Ladds 2009; Natusch et al. 2018). The ascarid nematodes, however, cause abscesses from burrowing into the stomach wall, acquiring host nutrients, and can occur in intensities of > 150 individuals (Lettoof et al. 2020c). Thus, of the measured parasites, the ascarid nematodes have the greatest potential to impact snake health. The weak positive relationship with nematode presence and body condition probably reflects a host-tolerance (Mayer et al. 2015); however, large burdens could influence the body condition calculations. Parasite infections in already pollution-stressed hosts can have additive or synergistic effects (Marcogliese and Pietrock 2011; Rohr et al. 2008), consequently large nematode burdens may significantly impact populations of chronically polluted snakes. Unfortunately, the potential true relationship between nematodes, pollution and tiger snake health is masked by our presence–absence data. To assess these interactions properly we would need to sacrifice a large number of snakes.

7.5.4 Other health parameters

Given the appearance of the ventral dermatitis, we suspected a fungal aetiology. Onygenalean fungi (order: *Onygenales*) have commonly been associated with dermatitis in reptiles (Paré and Sigler 2016), specifically *Ophidiomyces ophidiicola* in free-ranging snakes in North America (Allender et al. 2015; Lorch et al. 2015) and Europe (Franklinos et al. 2017). Additionally, *O. ophidiicola* and *Paranannizziopsis australasiensis* have both been reported in Australian aquatic snakes (Sigler et al. 2013). None of our samples were PCR-positive for *O. ophidiicola* and testing for *P. australasiensis* was unavailable to this study. We did not detect *Nannizziopsis barbatae* on tiger snakes, despite its known presence on shingleback skinks (*Tiliqua rugosa*) from the same peri-urban area. We were not able to determine the cause of

the ventral dermatitis; however, we found no difference in ventral scarring between sites, nor was ventral scarring an importance predictor for body condition.

Frequent and severe tail loss is known from Herdsman Lake tiger snakes (Aubret et al. 2005). We found a similar degree of tail loss in populations from Herdsman and Bibra Lake, the two most urbanised sites. However, tail loss was not an important predictor of body condition or any other parameter of health. The drivers of tail loss in these populations of tiger snakes are still unknown, but could be caused by non-lethal bird or introduced rodent bites (Webb and Whiting 2005).

The health parameter profile highlights how the broader health of a population can be strongly influenced by the structure of its ecosystem. Snakes from the Herdsman Lake population had the most homogenous health profile, characterised by low body condition, high degree of tail loss, and fewest parasites. Compared to the other sites, Herdsman Lake snakes are isolated (Chapter 6) and live in the most polluted and urbanised wetland. In contrast, Yanchep snakes occupy the most superficially natural wetland and had the most heterogeneous health profile, probably reflecting health diversity of a more heterogeneous natural population. Interestingly, male snakes had a more heterogeneous health profile, characterised by more parasites and scarring/wounds, than did females. This may be due to a male-biased home range size and reproductive effort (Bonnet et al. 1999). Despite the variation of health profiles among sites, the most pertinent evaluation of fitness—body condition—was poorest in snakes from more polluted sites, irrespective of all the other health and environmental factors we assessed.

7.6 Conclusion

Using western tiger snakes as a bioindicator species, we confirmed that populations across the urban matrix exhibited lower body condition seemingly in response to environmental pollution, rather than parasites or other injuries. Although we did not detect any effect of landscape urbanisation on snake health, we found site-specific differences, which are likely indirect impacts of urbanisation. Most interestingly, sites at the polar ends of the urbanisation spectrum sustained populations of tiger snakes with the poorest body conditions and this appears to be associated with higher levels of metal(loid) pollution at these sites. Vertebrate ecotoxicological research remains understudied in Australia (Death et al. 2019), and we provide the first evidence to

suggest that environmental pollution can be a strong predictor of body condition of a reptilian wetland top predator. Even though tiger snakes appear abundant in these wetlands, our detection of poor population health at polluted sites could be a sign that populations of these predators are suffering chronic effects from anthropogenic pollution. More sensitive species may be unable to tolerate these conditions. A cautionary approach should be used to assess the resilience of populations based purely on metrics of abundance and diversity. Assessing individual health will profit the assessments of population health. In addition, metal(loid) and body condition metrics could be used as early warning signs of environmental deterioration, informing environmental management before ecological integrity reaches a point of non-return. Future research should aim to identify and disentangle the effects of chronic metal(loid) pollution on the physiology, behaviour and energy budgets of bioindicator snakes.

7.7 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Chapter 7 addendum

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Additional details for measuring landscape variables.

Each wetland site was delineated at the urban/vegetation boundary. An 800m buffer was applied to account for the potential for snakes to move through this area. This value was decided as the maximum home range diameter recorded in an Eastern tiger snake (Butler et al. 2005). All four landscape variables were then measured within this buffered boundary.

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The mean growth rate for all recaptured snakes (sites and sex pooled) was 0.07 ± 0.01 SD mm/day (n = 49).”

Chapter 7 Supplementary material

S7.1 Parasite identification methods

A sample of all parasites were collected and preserved in 70% ethanol. Parasites were examined under a dissector microscope and identified into groups based on morphology: ticks, cestode, nematode and trematode.

Nematodes were placed on slides in lactophenol and examined. Nematodes were identified as *Ophidascaris pyrrhus* based on Sprent (1988). Cestodes were determined to be larval *Spirometra* sp. based on location in the host (subcutaneous) and overall morphology. Trematodes were stained with aceto carmine and mounted in Canada balsam, following standard techniques, and examined. Trematodes were determined to be *Dolichoperoides macalpini* based on Nicoll (1918). The ticks were identified as *Amblyomma albolimbatum* by morphological descriptions (Roberts 1970).

S7.2 PCR and histological assessment of ventral dermatitis

From a selection of snakes with obvious ventral dermatitis, the affected skin was swabbed at various locations along the body. Skin was swabbed using dry FLOQSwabs®. Swab tips were stored within their respective casing at – 20ºC and submitted to Murdoch University to look for selected onygenalean fungi by PCR. Additionally, samples of scales were clipped using 90% ethanol sterilised scissors and stored at – 20ºC, and a skin/scale biopsy was collected from a euthanised snakes and fixed in formalin. These samples were used for PCR testing and histological assessment, respectively.

S7.3 PCR testing methods

DNA was extracted from skin swabs and freshly-frozen skin scrapings as previously described in Peterson et al. (2020). PCR testing for *Nannizziopsis* spp. (Peterson et al. 2020) and the ITS region of *Ophidiomyces ophidiicola* (Bohuski et al. 2015) was performed as described in the respective studies. DNA from *N. barbatae* and a synthetic oligonucleotide were used as positive controls for the *Nannizziopsis* and *O. ophidiicola* PCRs, respectively (Peterson et al. 2020). Water was used as a negative control for both PCRs.

Table S7.1 Western tiger snake (*Notechis scutatus occidentalis*) sites and samples used for PCR testing for *Nannizziopsis* spp. and *Ophidiomyces ophidiicola*. BL = Bibra Lake, HL = Herdsman Lake, JL = Lake Joondalup, YC = Loch McNess in Yanchep National Park.

Snake ID	Site	Sample	PCR Result	
			***Nannizziopsis* spp.1**	***Ophidiomyces ophidiicola* – ITS gene2**
B73	BL	Fresh frozen scale clipping	NEGATIVE	NEGATIVE
H10	HL	Fresh frozen scale clipping	NEGATIVE	NEGATIVE
H89	HL	Fresh frozen scale clipping	NEGATIVE	NEGATIVE
H88	HL	Fresh frozen scale clipping	NEGATIVE	NEGATIVE
Y39	YC	Fresh frozen scale clipping	NEGATIVE	NEGATIVE
Y42	YC	Fresh frozen scale clipping & Skin swab	NEGATIVE	NEGATIVE
B49	BL	Skin swab	NEGATIVE	NEGATIVE
B43	BL	Skin swab	NEGATIVE	NEGATIVE
B51	BL	Skin swab	NEGATIVE	NEGATIVE
B65	BL	Skin swab	NEGATIVE	NEGATIVE
B88	BL	Skin swab	NEGATIVE	NEGATIVE
H124	HL	Skin swab	NEGATIVE	NEGATIVE
H127	HL	Skin swab	NEGATIVE	NEGATIVE
H153	HL	Skin swab	NEGATIVE	NEGATIVE
H168	HL	Skin swab	NEGATIVE	NEGATIVE
J37	JL	Skin swab	NEGATIVE	NEGATIVE
J56	JL	Skin swab	NEGATIVE	NEGATIVE
J57	JL	Skin swab	NEGATIVE	NEGATIVE
J63	JL	Skin swab	NEGATIVE	NEGATIVE
J46	JL	Skin swab	NEGATIVE	NEGATIVE
Y47	YC	Skin swab	NEGATIVE	NEGATIVE

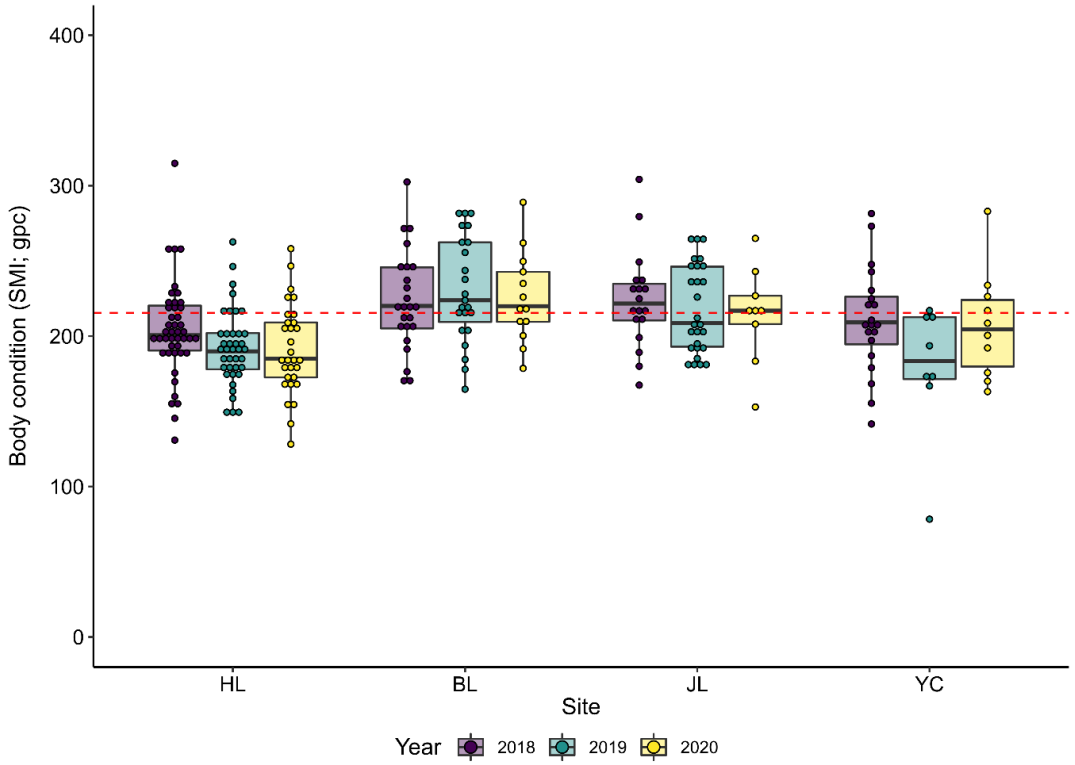

Figure S7.1 Western tiger snake (*Notechis scutatus occidentalis*) body condition across sites and years. Red dashed line is the population mean. Dots represent individual snakes. HL = Herdsman Lake, BL = Bibra Lake, JL = Lake Joondalup, YC = Loch McNess within Yanchep National Park.

S7.4 Histology methods

Three sections were made from one large skin biopsy that incorporated five scales. The tissue was processed in standard fashion and 4 µm sections were stained with haematoxylin and eosin (for routine examination), Gram's (for bacteria), and periodic acid-Schiff (for fungi).

S7.5 Histology results and interpretation

Histology of one skin lesion confirmed the presence of fungi in superficial debris and the underlying superficial epidermal keratin layer in affected scales (Fig S7.1). Fresh-frozen affected skin from this snake was PCR-negative for *Nannizziopsis* spp. and *O. ophidiicola*. While the morphology of the fungal hyphae in the skin is consistent with that of Onygenales such as *O. ophidiicola*, it is not specific for it, and histology alone is not considered a definitive diagnostic technique for fungal speciation (Paré and

Sigler 2016). Skin infection with onygenalean fungi is characterised by masses of superficial arthroconidia (Lorch et al. 2015; Paré and Sigler 2016), and these were not seen in this case. Onygenalean skin infection in snakes may cause lesions of varying severity, but is typified by severe necrotising lesions due to deep invasion into the epidermis (Bertelsen et al. 2005; Lorch et al. 2015; Paré and Sigler 2016). In contrast, the skin lesions in this case were mild with fungal hyphae limited to the superficial keratin layer. Thus, overall, the skin lesions in this biopsy are not particularly suggestive of onygenalean infection.

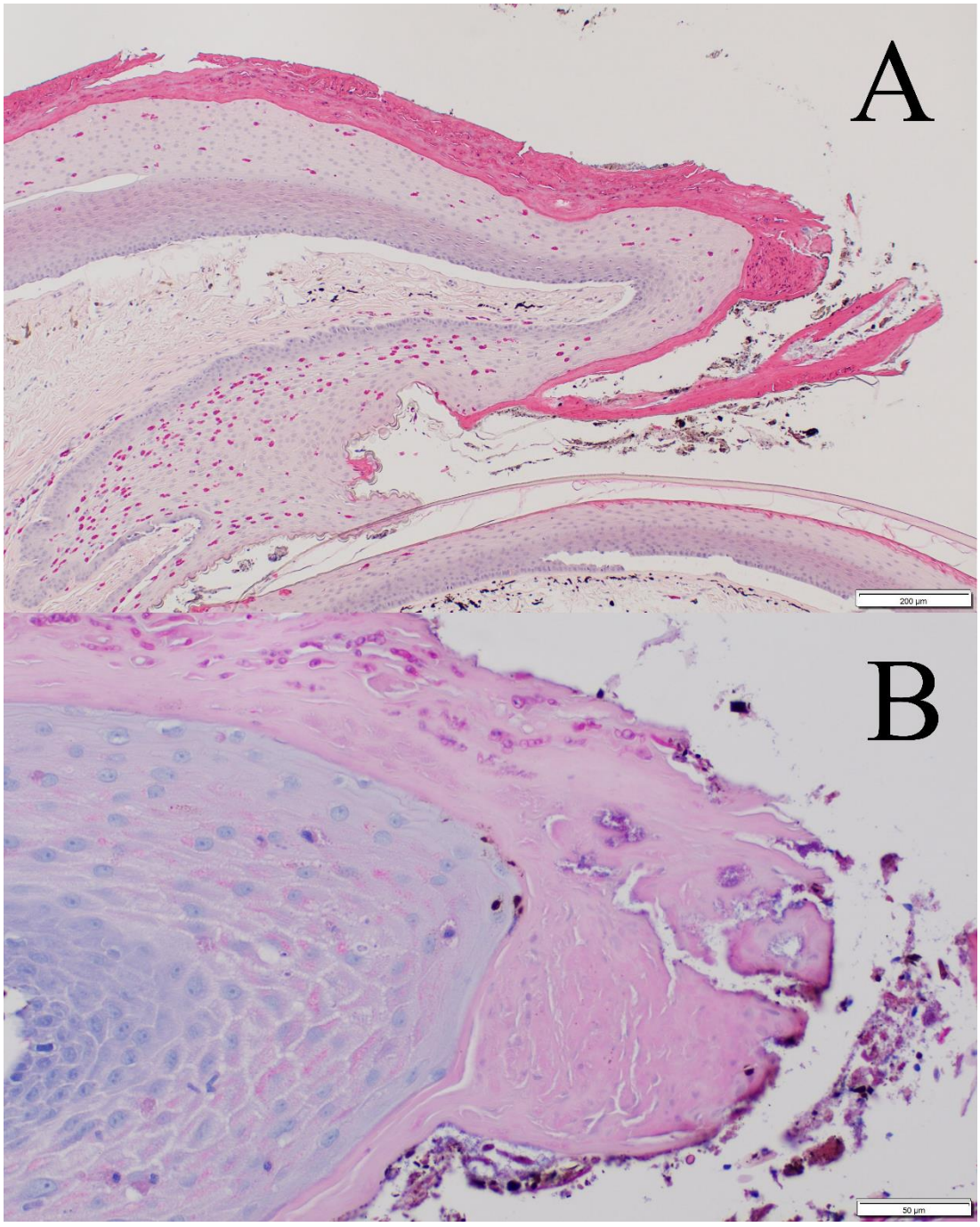

Figure S7.2 Histology photomicrographs of tiger snake skin lesion. A: Low power image depicting irregular thickening and fissuring (dark pink) of the superficial keratin layer of the dorsal surface and edge of the scale. There is variegated lightly adherent debris at the fissured keratin at the edge of the scale. There is mild (dorsal scale) and moderate (ventral scale) heterophil infiltration of the epidermis. Haematoxylin and eosin stain. Bar = 200 um. B: High power image showing magenta staining fungal hyphae in the superficial keratin (top of the image), and thickened, fissured keratin intermingled with foreign debris and bacteria at the edge of the scale (right side of the image). Periodic acid-Schiff stain. Bar = 50 um.

Table S7.2. Exploratory generalised linear mixed models comparing site, sex and year differences of health parameters in western tiger snakes (*Notechis scutatus occidentalis*).

Health parameter	Site	Sex	Year
Dorsal scars (#)	$X^2_{3,390}$ = 17.95, p < **0.01**	$X^2_{1,390}$ = 0.05, p = 0.83	$X^2_{2,390}$ = 0.08, p = 0.96
Ventral scars (#)	$X^2_{3,390}$ = 3.72, p = 0.29	$X^2_{1,390}$ = 7.98, p < **0.01**	$X^2_{2,390}$ = 12.48, p < **0.01**
Nematode presence (yes/no)	$X^2_{3,390}$ = 0.42, p = 0.94	$X^2_{1,390}$ = 0.81, p = 0.37	$X^2_{2,390}$ = 0.05, p = 0.83
Skin worms (#)	$X^2_{3,390}$ = 97.05, p < **0.01**	$X^2_{1,390}$ = 1.76, p = 0.18	$X^2_{2,390}$ = 3.70, p = 0.16
Trematodes (#)	$X^2_{3,390}$ = 171.37, p < **0.01**	$X^2_{1,390}$ = 7.25, p < **0.01**	$X^2_{2,390}$ = 19.38, p < **0.01**
Tail loss (%)	$X^2_{3,390}$ = 38.15, p < **0.01**	$X^2_{1,390}$ = 1.27, p = 0.26	$X^2_{2,390}$ = 1.38, p = 0.50
Body condition (SMI; gpc)	$F_{3,264}$ = 15.66, p < **0.01**	$F_{1,264}$ = 2.22, p < **0.01**	$F_{2,264}$ = 1.49, p = 0.23

Table S7.3. Site variation selection table, and top models identifying the strongest predictors of tiger snake dorsal scars based on ΔAICc and weight. Covariates reported as estimate (standard error).

Site variation global models (Negative binomial error structure)	**AIC**
MPI + v.scar + nema + sk.wrm + tick + tail + SMI	366.28
Site + v.scar + nema + sk.wrm + tick + tail + SMI	368.98
PC1 + v.scar + nema + sk.wrm + tick + tail + SMI	371.05

Dredged top global model (<2 ΔAICc)

	MPI	**v.scar**	**nema**	**Sk.wrm**	**tick**	**tail**	**Intercept**	**logLik**	**AICc**	**Δ**	**weight**
Mod.1	-7.79 (3.46)	0.12 (0.07)	-	-	0.30 (0.09)	-	-0.17 (0.61)	-173.98	360.28	0.00	0.10
Mod.2	-7.97 (3.42)	-	-	-	0.29 (0.08)	-	0.28 (0.62)	-175.10	360.44	0.16	0.08
Mod.3	-7.71 (3.48)	-	0.38 (0.35)	-	0.31 (0.09)	-	-0.11 (0.72)	-174.44	361.21	0.93	0.06
Mod.4	-7.58 (3.50)	0.11 (0.07)	0.36 (0.39)	-	0.31 (0.09)	-	0.20 (0.60)	-173.42	361.28	1.01	0.06
Mod.5	-8.03 (3.46)	0.13 (0.07)	-	-	0.30 (0.09)	0.07 (0.12)	0.20 (0.60)	-173.82	362.07	1.79	0.05
Mod.6	-8.10 (3.55)	0.12 (0.07)	-	-0.05 (0.13)	0.30 (0.09)	-	0.22 (0.62)	-173.91	362.25	1.97	0.05

Table S7.4. Site variation selection table, and top models identifying the strongest predictors of tiger snake skin worms based on ΔAICc and weight. HL = Herdsman Lake, BI = Bibra Lake, JL = Lake Joondalup, YC = Loch McNess within Yanchep National Park. Covariates reported as estimate (standard error).

Site variation global models (Negative binomial error structure)	**AIC**
Site + d.scar + v.scar + nema + trema + tick + tail + SMI	730.46
MPI + d.scar + v.scar + nema + trema + tick + tail + SMI	776.12
PC1 + d.scar + v.scar + nema + trema + tick + tail + SMI	812.37

Dredged top global model (<2 ΔAICc)

	Site (vs. HL)	**v.scar**	**nema**	**trema**	**tick**	**SMI**	**Intercept**	**logLik**	**AICc**	**Δ**	**weight**
Mod.1	**BI:** 3.25 (0.45)	-	0.93	-	0.16	-0.14	-2.92	-353.44	725.59	0.00	0.08
	JL: 3.12 (0.45)		(0.27)		(0.06)	(0.09)	(0.49)				
	YC: 3.51 (0.45)										
Mod.2	**BI:** 3.09 (0.44)	-	0.84	-	0.16	-	-2.78	-354.58	725.72	0.13	0.07
	JL: 3.09 (0.45)		(0.27)		(0.06)		(0.48)				
	YC: 3.41 (0.45)										
Mod.3	**BI:** 3.14 (0.27)	-	0.83	0.01	0.14	-	-2.79	-353.71	726.12	0.52	0.06
	JL: 2.96 (0.45)		(27)	(0.01)	(0.06)		(0.48)				
	YC: 3.29 (0.45)										
Mod.4	**BI:** 3.27 (0.45)	-	0.90	0.01	0.14	-0.13	-2.91	-352.77	726.41	0.82	0.05
	JL: 3.08 (0.46)		(0.27)	(0.01)	(0.06)	(0.09)	(0.49)				
	YC: 3.39 (0.46)										
Mod.5	**BI:** 3.14 (0.44)	0.06	0.80	0.01	0.13	-	-2.77	-353.04	726.95	1.36	0.04
	JL: 2.93 (0.45)	(0.04)	(0.27)	(0.01)	(0.07)		(0.48)				
	YC: 3.23 (0.46)										
Mod.6	**BI:** 3.09 (0.44)	0.05	0.82	-	0.16	-	-2.76	-354.13	726.97	1.38	0.04
	JL: 2.97 (0.45)	(0.04)	(0.27)		(0.06)		(0.48)				
	YC: 3.37 (0.45)										
Mod.7	**BI:** 3.24 (0.45)	0.04	0.91	-	0.15	-0.13	-2.89	-353.15	727.17	1.58	0.03
	JL: 3.10 (0.45)	(0.04)	(0.27)		(0.06)	(0.09)	(0.49)				
	YC: 3.48 (0.45)										

Table S7.5. Site variation selection table, and top models identifying the strongest predictors of tiger snake oral trematodes based on ΔAICc and weight. HL = Herdsman Lake, BI = Bibra Lake, JL = Lake Joondalup, YC = Loch McNess within Yanchep National Park. Covariates reported as estimate (standard error).

Site variation global models (negative binomial error structure)	**AIC**
Site + d.scar + v.scar + nema + trema + sk.wrm + tail + SMI + (1|sex)	749.88
PC1 + d.scar + v.scar + nema + trema + sk.wrm + tail + SMI + (1|sex)	848.71
MPI + d.scar + v.scar + nema + trema + sk.wrm + tail + SMI + (1|sex)	865.80

Dredged top global model (<2 ΔAICc)

	Site (vs. HL)	**d.scar**	**v.scar**	**nema**	**ticks**	**Sk.wrm**	**tail**	**Intercept**	**logLik**	**AICc**	**Δ**	**weight**
Mod.1	**BI:** -1.89 (0.78)	-	-	0.54 (0.32)	0.24	0.24	-	-0.58	-362.86	746.58	0.00	0.08
	JL: 2.04 (0.34)				(0.09)	(0.07)		(0.51)				
	YC: 2.71 (0.35)											
Mod.2	**BI:** -1.90 (0.78)	-	-0.09	0.58 (0.32)	0.24	0.25	-	-0.64	-362.01	747.07	0.49	0.06
	JL: 2.04 (0.34)		(0.08)		(0.07)	(0.09)		(0.50)				
	YC: 2.75 (0.35)											
Mod.3	**BI:** -1.82 (0.78)	-0.13	-	0.63 (0.34)	0.26	0.23	-	-0.70	-362.11	747.27	0.68	0.06
	JL: 2.09 (0.34)	(0.11)			(0.07)	(0.08)		(0.52)				
	YC: 2.77 (0.35)											
Mod.4	**BI:** -1.80 (0.78)	-	-	-	0.18	0.24	-	-0.12	-364.35	747.40	0.82	0.05
	JL: 2.04 (0.35)				(0.09)	(0.09)		(0.40)				
	YC: 2.71 (0.36)											
Mod.5	**BI:** -1.82 (0.78)	-	-	0.52 (0.32)	0.24	0.24	0.12	-0.63	-362.43	747.90	1.32	0.04
	JL: 2.11 (0.35)				(0.07)	(0.09)	(0.13)	(0.51)				
	YC: 2.83 (0.37)											
Mod.6	**BI:** -1.84 (0.78)	-0.11	-0.08	0.65 (0.34)	0.26	0.24	-	-0.73	-361.50	748.23	1.65	0.04
	JL: 2.08 (0.34)	(0.11)	(0.08)		(0.07)	(0.08)		(0.51)				
	YC: 2.78 (0.35)											
Mod.7	**BI:** -1.80 (0.78)	-	-0.08	-	0.18	0.24	-	-0.14	-363.71	748.28	1.70	0.04
	JL: 2.05 (0.35)		(0.08)		(0.06)	(0.09)		(0.39)				
	YC: 2.74 (0.36)											
Mod.8	**BI:** -1.74 (0.78)	-0.14	-	0.61 (0.34)	0.26	0.23	0.13	-0.77	-361.57	748.39	1.80	0.03
	JL: 2.17 (0.35)	(0.08)			(0.07)	(0.08)	(0.12)	(0.52)				
	YC: 2.90 (0.37)											
Mod.9	**BI:** -1.84 (0.78)	-	-0.09	0.57 (0.32)	0.24	0.25	0.12	-0.69	-361.61	748.45	1.87	0.03
	JL: 2.11 (0.35)		(0.08)		(0.07)	(0.09)	(0.13)	(0.50)				
	YC: 2.86 (0.37)											
Mod.10	**BI:** -1.73 (0.78)	-	-	-	0.18	0.24	0.14	-0.19	-363.81	748.48	1.90	0.03
	JL: 2.13 (0.35)				(0.06)	(0.09)	(0.13)	(0.41)				
	YC: 2.86 (0.39)											

Table S7.6. Site variation selection table, and top models identifying the strongest predictors of tiger snake tail loss based on ΔAICc and weight. Covariates reported as estimate (standard error).

Site variation global models (Tweedie error structure)	**AIC**
Site + d.scar + v.scar + nema + trema + sk.wrm + tail + SMI	172.30
PC1 + d.scar + v.scar + nema + trema + sk.wrm + tail + SMI	179.01
MPI + d.scar + v.scar + nema + trema + sk.wrm + tail + SMI	181.21

Dredged top global model (<2 ΔAICc)

	Site	**d.scar**	**v.scar**	**trema**	**SMI**	**Intercept**	**logLik**	**AICc**	**Δ**	**weight**
Mod.1	**BI:** -0.47 (0.21) **JL:** -0.76 (0.23) **YC:** -1.44 (0.31)	-	-	-	-	-1.64 (0.12)	-73.34	161.11	0	0.09
Mod.2	**BI:** -0.56 (0.24) **JL:** -0.82 (0.24) **YC:** -1.45 (0.31)	-	-	-	0.08 (0.09)	-1.61 (0.12)	-72.94	162.45	1.33	0.05
Mod.3	**BI:** -0.52 (0.22) **JL:** -0.78 (0.24) **YC:** -1.45 (0.31)	0.06 (0.09)	-	-	-	-1.63 (0.12)	-73.07	162.70	1.58	0.04
Mod.4	**BI:** -0.45 (0.22) **JL:** -0.74 (0.24) **YC:** -1.42 (0.31)	-	-0.09 (0.14)	-	-	-1.66 (0.12)	-73.09	162.74	1.63	0.04
Mod.5	**BI:** -0.46 (0.21) **JL:** -0.82 (0.25) **YC:** -1.55 (0.36)	-	-	0.08 (0.12)	-		-73.10	162.77	1.66	0.04

S7.5 References

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Chapter 8. General discussion

8.1 Summary of findings

In this thesis, I demonstrate how the health of a wetland top predator snake reflects, and is influenced by, wetland metal(loid) contamination. In addition, I show the impact that encapsulation of remnant wetland 'islands' by urbanisation can have, and the potential outcomes for isolated populations of tiger snakes taking refuge within. As tiger snakes are an excellent bioindicator of wetland health, the findings in this thesis could be applicable to syntopic fauna, which warrants investigation. In the following discussion, I outline the broad findings of this thesis, indicate limitations, and propose what I consider important future directions for reptile and urban ecotoxicological research.

Gastric nematodes can negatively impact the health of their hosts (Koski and Scott 2001), and urbanisation can increase parasite abundance and intensity (Giraudeau et al. 2014). In Chapter 2, I used museum specimens to conduct a temporal and landscape-scale assessment of gastric nematode parasitism in western tiger snakes across their species distribution. I found no significant relationship between nematode infection and distance to urban centres; however, infection was positively associated with proximity to wetlands, and areas with lower rainfall and topographic wetness index; nematode abundance showed a weak positive correlation with landscape mean maximum temperature. From these results, I suggest nematode infection comes from frog prey and if urbanisation increases landscape temperatures, future urban development has the potential to influenced nematode parasitism. In addition, this chapter's research highlights the value of museum specimens, but was limited by the lack of collection replication and the inability to assess the relationship between nematode infection and body condition (due to specimen storage techniques). To thoroughly assess the relationship between contemporary urbanisation and nematode parasitism in more detail, I recommend that a much larger sample size of tiger snakes needs to be systematically collected across their range and from wetlands differing in degree of urbanisation.

Urbanisation degrades and contaminates wetlands with a plethora of elemental and chemical pollutants (Paul and Meyer 2001), and top predator snakes are susceptible to bioaccumulating such persistent substances (Bauerle et al. 1975; Stafford et al. 1977).

Ecotoxicological research on terrestrial vertebrates in Australia is, however, relatively rare, compared to other developed countries (Death et al. 2019). In Chapter 3, I conducted a broad-scale screening of 52 contaminants in wetland sediment and in resident tiger snake livers, from four wetlands differing in degree of urbanisation. Of these contaminants, I found no evidence of organochlorine pesticide or polycyclic aromatic hydrocarbon accumulation; however, there was evidence of accumulation of nine different metal(loid)s in tiger snakes. I also identified sediment contamination that breached government guideline values in protected and important local wetlands. The relatively high concentrations of Cd, As, Hg and Se in the Yanchep National Park wetland – likely caused by excessive draining of the groundwater and sediment erosion – was both surprising and concerning, and demonstrates that pollution can result from indirect anthropogenic disturbance. Due to limited funding and ethical considerations, the results in Chapter 3 represent a snap-shot of contamination and bioaccumulation; a much larger sample size of both sediment and snake tissue would be needed to comprehensively assess wetland contamination and patterns of bioaccumulation in tiger snakes. In addition, sampling a larger scale of snake body sizes, including juveniles (if possible), would be beneficial for assessing the relationships between contaminant concentrations, body size, and trophic level shifts.

Plasma biochemical profiles can be an important measure of physiological health (Eatwell et al. 2014), as well as indicate subtle changes in health from various stressors (Campbell 2006; Villa et al. 2017). In Chapter 4, I caught tiger snakes from the most urbanised study site (Herdsman Lake) and the least disturbed study site (Loch McNess in Yanchep National Park), and compared their plasma profiles. I kept these snakes in captivity for six months to monitor their body condition and change in plasma profiles. I found the plasma profiles were similar between sites both before and after captivity, but significantly changed throughout the captive period. Body condition (a) was significantly lower in the urban wetland snakes upon capture, (b) significantly increased for all snakes throughout captivity, and (c) was statistically similar between snakes from both populations at the end of captivity. In this chapter I created the first baseline plasma biochemical profile for wild, and captive, tiger snakes and identified a change in both profiles and body condition over the captive period. Without sufficient controls, I could not determine if plasma profile and body condition changes were attributed to season, a change in diet, or toxicant depuration; therefore, I

recommend this study be repeated on more snake populations, fed a captive diet that's similar a wild diet, and with repeated plasma profile measurements of wild snakes in parallel with the captive snakes.

Quantifying numerous contaminants in organism tissue is expensive (Chapter 3), thus small sample sizes are often a limiting factor of ecotoxicological research. As accumulated metal(loid)s can sequester in snake scale keratin (Burger et al. 2017), in Chapter 5 I explored the use of laser ablation-ICP-MS to measure a suite of metal(loid)s in tiger snake scales. I found LA-ICP-MS could accurately measure 19 of the 26 target metal(loid)s in snake scales. Mn, As, Se and Sb concentrations were correlated between scale and liver tissue, and concentrations in scales from different snake populations reflected the contamination of their wetlands. LA-ICP-MS shows promise as a cheap method to measure metal(loid) concentrations in small volumes of non-lethally sampled tissue; however, further research is needed to determine the distribution of metal(loid) abundances in snake scales, and the relationship between scale contamination (as an indicator) and other tissues.

The expansion of urbanisation often isolates habitat patches and resident fauna therein, disrupting gene flow between populations while also introducing novel stressors. Over time, isolated populations can experience a loss in genomic diversity and subsequently individuals within the population can suffer from poor health, fitness (Reed and Frankham 2003), and reduced adaptive potential (Miles et al. 2019). With urbanisation continuing to encroach upon western tiger snake habitat, in Chapter 6 I investigated the contemporary population genomics of seven tiger snake populations across Perth. I found populations north of the major river system had lower heterozygosity than the southern populations, and signals of inbreeding were higher in populations isolated by urbanisation. Although I found no correlation between individual body condition and heterozygosity, the northern population's exhibit relatively low body condition and genomic diversity (adaptive potential); thus they are most at risk of extirpation from urban growth. As Perth is near the northern extremity of western tiger snakes distribution, a loss of these populations would reduce the overall species range. To further assess tiger snake genomic health and fitness, I recommend sampling more populations both across their entire distribution and across urban gradients to thoroughly evaluate urban elements that restrict dispersal and identify genetic markers that potentially play a role in urban persistence.

Western tiger snakes are exposed to, and accumulate, a suite of metal(loid)s in anthropogenically-disturbed wetlands around Perth (Chapters 3 & 5). In Chapter 7, I assess eight parameters of tiger snake health from my four study populations. I then model associations among health parameters, mean liver metal(loid) burden (pollution index) of populations, and degree of wetland urbanisation. I found differences in parasite abundances, degree of tail loss, and body condition of populations. Low population body condition was not associated with any parasite, scars or tail loss, but was significantly associated with higher mean liver pollution. I also found that the population at Herdsman Lake – the most urbanised and degraded wetland – had the most homogenous health profile, which was characterised by low body condition, low parasite presence and abundance, and high degree of tail loss. Despite observing broad health differences in snakes from the urban wetlands, I found no association between any health parameter and the urbanisation score, suggesting site differences among health parameters are probably influenced by additional variables I had not measured. Without sacrificing snakes, I could not thoroughly assess nematode intensity, nor the relationship between nematodes and snake pollution levels. The negative association between population pollution levels and body condition is concerning, and warrants further investigation to ascertain which metal(loid)s induce toxicity, and their toxicodynamics in snakes.

Reptile ecotoxicology, and the use of snakes as bioindicators of ecosystem health is a developing research area (Haskins et al. 2019; Hopkins 2000; Stafford et al. 1977), of which this thesis makes a substantial contribution. In this thesis I demonstrate (a) wetlands across the urban matrix of Perth are subjected to urban disturbance and contamination, although these two factors are not necessarily correlated; (b) western tiger snakes are exposed to, and accumulate, a suite of contaminants; (c) tiger snake populations north of the dividing rivers in Perth have the lowest genomic diversity (adaptive potential) and may be under threat of extirpation from urbanisation; (d) tiger snake body condition is negatively associated with wetland pollution by metal(loid)s. Results from this thesis would be complemented by identification of the toxicokinetics and toxicodynamics of metal(loid)s in reptiles. This was outside of my thesis scope, however, as I first needed to identify the biotic and abiotic differences among wetlands and determine if there was measurable associations between these and the health of tiger snakes. I have now shown this is a system in need of exploring, both from a

conservation and scientific perspective. Below, I discuss the areas I consider important to improve our understanding of reptile ecotoxicology and use of snakes as bioindicators.

8.2 Conclusions and future direction

As the human population grows, natural habitats are increasingly degraded by urbanisation and the inevitable introduction of contaminants. Despite the surge of research into the toxicological effects of environmental pollution, ecotoxicology is still underrepresented in Australian terrestrial vertebrates (Death et al. 2019), and in reptiles globally (Sparling et al. 2010). As I demonstrated in Chapter 3, wetlands are contaminated regardless of whether they are within or outside the urban matrix of a rapidly growing city, and these contaminants are accumulating in a reptilian top predator. As far as I am aware, the contamination of these wetlands is not being regularly monitored, neither are there sufficient attempts to rehabilitate or restore these sites nor further toxicant pollution. In addition, the impacts of chronic environmental contamination on wildlife and humans are largely unknown (Schmitt et al. 2021). Therefore, further research is urgently needed to assess the presence and abundance of a broad suite of contaminants in many more functional urban wetlands, in conjunction with bioaccumulation assessments and population health outcomes in resident bioindicator species such as fish, frogs and reptiles.

The life-history traits of higher trophic tier snakes (e.g. carnivorous diets, relatively long lifespan and high site fidelity) make them an ideal bioindicator taxon. My thesis highlights the value of using a top predator snake to indicate wetland health and contamination in an urban ecosystem, whereas similar species could be utilised as bioindicators of non-urban polluted ecosystems. Energy industries (e.g. fracking, coal burning powerplants), mining (especially in Australia) and agricultural industries often introduce a suite of contaminants to their local, natural environments. I recommend exploring the health and bioaccumulation of contaminants in snake species and populations in proximity to these non-urban polluting industries, where the impact on health may not be confounded by the additional stressors (e.g. road mortality, intentional killing, introduced predators, habitat loss, etc.) introduced from urbanisation.

Research suggests reptiles can accumulate – and show resistance to – high concentrations of toxicants (Finger et al. 2016; Mauldin et al. 2019; Weir et al. 2015), and as a result, health and population responses may reflect the more pernicious effects of chronic environmental contamination. However, the standard one-to-six month duration for laboratory ecotoxicological studies is probably not enough time to elicit a reaction from reptiles, due to their low metabolism and energy expenditure (Linder et al. 2010). Therefore, I recommend that toxicant-exposure laboratory experiments over a much longer term are required to comprehensively determine reptilian ecotoxicological responses, and the toxodynamics of common environmental toxicants. In addition, the monitoring of tiger snake (and other contaminated wetland snakes) population sizes and stability is needed to assess long-term outcomes from wetland degradation.

8.3 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Appendix 1. Author attribution and copyright statements

Chapter 2: Investigating the role of urbanisation, wetlands and climatic conditions in nematode parasitism in a large Australian elapid snake

I, Damian Christopher Lettoof, in conjunction with BvT and FA, conceived the idea and designed the methodology; I collected the data; I analysed the data with the guidance from BvT and FA; I wrote the manuscript and all authors contributed to the revisions of the manuscript for the following publication:

Lettoof, D. C., von Takach, B., Bateman, P. W., Gagnon, M. M., Aubret, F. (2020). Investigating the role of urbanisation, wetlands and climatic conditions in nematode parasitism in a large Australian elapid snake. Int J Parasitol Parasites Wildl. 11:32-9. doi: 10.1016/j.ijppaw.2019.11.006.

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Chapter 3: The Broad-Scale Analysis of Metals, Trace Elements, Organochlorine Pesticides and Polycyclic Aromatic Hydrocarbons in Wetlands along an Urban Gradient, and the Use of a High Trophic Snake as a Bioindicator

I, Damian Christopher Lettoof, in conjunction with MMG, conceived the idea and designed the methodology; I collected and analysed the data; I wrote the manuscript and all authors contributed to the revisions of the manuscript for the following publication:

Lettoof, D. C., Bateman, P. W., Aubret, F., Gagnon, M. M. (2020). The Broad-Scale Analysis of Metals, Trace Elements, Organochlorine Pesticides and Polycyclic Aromatic Hydrocarbons in Wetlands along an Urban Gradient, and the Use of a High Trophic Snake as a Bioindicator. Arch Environ Contam Toxicol. 78(4):631-45. doi: 10.1007/s00244-020-00724-z.

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Chapter 4: Plasma Biochemistry Profiles of Wild Western Tiger Snakes (Notechis Scutatus Occidentalis) before and after Six Months of Captivity

I, Damian Christopher Lettoof, in conjunction with FA, PWB, JH and MMG conceived the idea and designed the methodology; I collected the data; I analysed the data with guidance from FS; I wrote the manuscript and all authors contributed to the revisions of the manuscript for the following publication:

Lettoof, D. C., Aubret, F., Spilsbury, F., Bateman, P.W., Haberfield, J., Vos, J., Gagnon, M. M. Plasma Biochemistry Profiles of Wild Western Tiger Snakes (Notechis Scutatus Occidentalis) before and after Six Months of Captivity. (2021). J Wildl Dis. 57(2):253-63. doi: 10.7589/JWD-D-20-00115.

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Chapter 5: Snake scales record environmental metal(loid) contamination

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Chapter 6: Top predator snake shows genomic signatures of natural and anthropogenic barriers to gene flow

I, Damian Christopher Lettoof, in conjunction with BvT, conceived the idea and designed the methodology; I collected the data with contribution from VAT, JC and FA; I analysed the data with guidance from BvT; I wrote the manuscript and all authors contributed to the revisions of the manuscript for the following publication:

Lettoof, D. C., Thomson, V. A., Cornelis, J., Aubret, F., Bateman, P. W., Gagnon, M. M., von Takach, B. (2021). Bioindicator snake shows genomic signatures of natural and anthropogenic barriers to gene flow. PLoS One, 16: e0259124. doi: 10.1371/journal.pone.0259124.

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Chapter 7: Metal(loid) pollution, not urbanisation nor parasites predicts low body condition in a wetland bioindicator snake

I, Damian Christopher Lettoof, conceived the idea and designed the methodology; I collected the data with contribution from JC and TH; I analysed the data with guidance from CJ; I wrote the manuscript and all authors contributed to the revisions of the manuscript for the following publication:

Lettoof, D. C., Cornelis, J., Jolly, C., Aubret, F., Gagnon, M. M., Hyndman, T., Barton, D., Shilton, C., Bateman, P. W. Metal(loid) pollution, not urbanisation nor parasites predicts low body condition in a wetland bioindicator snake (2021). Environ Pollut,118674. doi: 10.1016/j.envpol.2021.118674.

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A1.1 International Journal of Parasitology: Parasites and Wildlife

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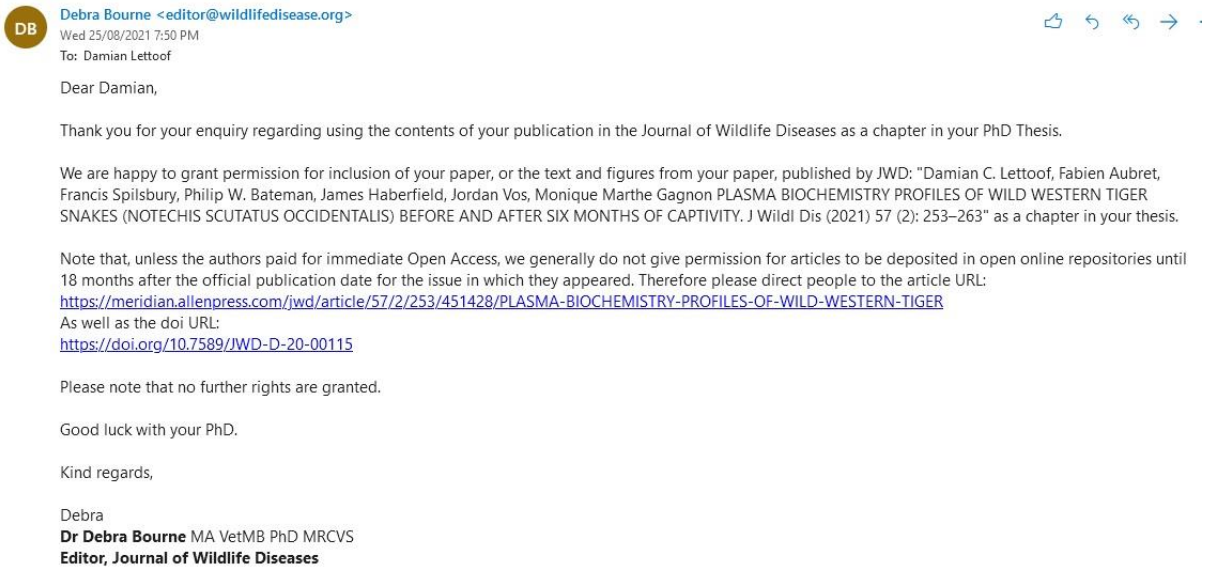

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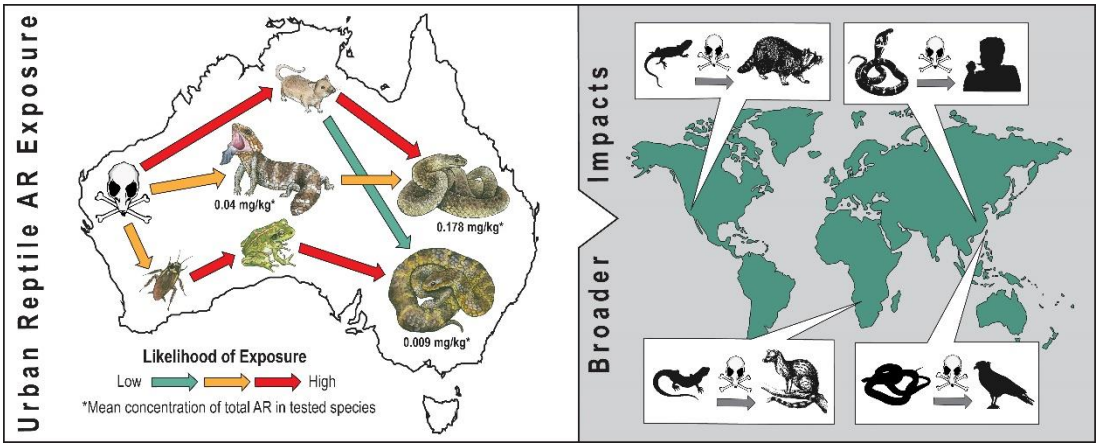
Appendix 2. Toxic time bombs: Frequent detection of anticoagulant rodenticides in urban reptiles at multiple trophic levels

The study presented in Appendix 2 was accepted in the peer-reviewed journal '*Science of the Total Environment*' on 24 March 2020.

Lettoof, D. C., Lohr, M. T., Busetti, F., Bateman, P. W., Davis, R. A. (2020). Toxic time bombs: Frequent detection of anticoagulant rodenticides in urban reptiles at multiple trophic levels. *Sci Total Environ*. 724:138218. doi:10.1016/j.scitotenv.2020.138218.



A2.1 Abstract

Anticoagulant rodenticides (ARs) are regularly used around the world to control pest mammals. Second-generation anticoagulant rodenticides (SGARs) are highly persistent in biological tissue and have a high potential for bioaccumulation and biomagnification. Consequently, exposure and poisoning of non-target organisms has been frequently documented, especially in countries with unregulated AR sales and usage. Most of this research has focussed on rodent-predators, usually raptors and predatory mammals, although exposure has also been documented in invertebrates and insectivorous fauna. Few studies have explored non-target exposure in reptiles, despite species sharing similar trophic positions and dietary preferences to other exposed fauna. We tested three abundant urban reptile species in Perth, Western Australia that differ in diet and trophic tiers for multiple AR exposure, the dugite *Pseudonaja affinis* (rodent-predator), the bobtail *Tiliqua rugosa* (omnivore) and the tiger snake *Notechis scutatus occidentalis* (frog-predator). We found frequent exposure in all three species

(91% in dugites, 60% in bobtails and 45% in tiger snakes). Mean combined liver concentrations of ARs of exposed individuals were 0.178 mg/kg in dugites, 0.040 mg/kg in bobtails and 0.009 mg/kg in tiger snakes. High exposure frequency and liver concentration was expected for the dugite. Exposure in the other species is more surprising and implies widespread AR contamination of the food web. We discuss the likelihood of global AR exposure of urban reptiles, highlight the potential for reptiles to be important vectors of ARs in the food web and highlight implications for humans consuming wild reptiles.

A2.2 Introduction

Anticoagulant rodenticides (ARs) are used globally to control pest rodent populations (Shore and Coeurdassier 2018). ARs are applied in a wide variety of land use settings including commercial and residential areas, agricultural and silvicultural land, and islands with threatened ecological communities (Lohr 2018; Lopez-Perea et al. 2019; Pitt et al. 2015). Baiting is the standard method for delivering ARs, as the baits can be easily dispersed across a landscape and modified to suit particular target species (Hoare and Hare 2006b). To avoid bait consumption from non-target species, baits are often deployed inside stations intended to minimise access from other fauna; for example, access holes to the stations may be raised above the ground and sized appropriately for target species (Bettink 2015). Even so, many non-target species are poisoned or exposed to ARs, either as a result of direct bait consumption or through consumption of target and non-target fauna which have eaten bait (Elliott et al. 2014; Hong et al. 2019; Pitt et al. 2015). Such secondary poisoning events have been documented in avian and mammalian predators, particularly rodent-predators and scavengers (Colvin et al. 1988; Cox and Smith 1990; Eason and Spurr 1995; Hindmarch et al. 2019; Hosea 2000; Lopez-Perea et al. 2019; Sanchez-Barbudo et al. 2012). Only more recently has the true saturation of ARs throughout the food web been identified: when baits are accessible, they are consumed directly by a suite of invertebrates (Alomar et al. 2018; Elliott et al. 2014), birds (Masuda et al. 2014), lizards (Wedding et al. 2010) and small mammals (Brakes and Smith 2005). Non-target primary consumers of ARs are potentially important vectors for ARs to organisms in higher trophic levels; for example, insectivorous hedgehogs (*Erinaceus europaeus*) in Britain have a similar prevalence of AR exposure to predatory birds (Dowding et al. 2010), and Stewart Island robin (*Petroica australis*) nestlings have

died from AR poisoning linked to being fed poisoned invertebrates (Masuda et al. 2014).

ARs work by blocking the recycling of vitamin K in the liver, disrupting the normal blood clotting mechanisms in vertebrates (Park et al. 1984). Commonly used ARs, such as brodifacoum, are classed as second generation anticoagulant rodenticides (SGARs) according to their chemical structure and period of development. SGARs are a significant risk to the vertebrate food web due to their long periods of persistence in liver tissue. In rodent livers, the mean half-life of SGARs can range from 108 days for difethialone to 220 days for flocoumafen (Eason et al. 2002), and up to 307.4 days for brodifacoum (Vandenbroucke et al. 2008). Despite long half-lives and the potential for environmental contamination for some ARs, currently only the United States, Canada and the UK impose substantial restrictions on AR use through permits, best-practice guidelines and limiting the more toxic ARs to indoor use (Bradbury 2008; Health Canada 2010; Tosh et al. 2011). In Australia, for example, nine first and second generation ARs can be legally sold to the public, resulting in exposure for humans and wildlife (Lohr and Davis 2018). It has been estimated that this leads to 1400 human AR exposures per year (Australian Pesticides and Veterinary Medicines Authority 2017) and high exposure for urban wildlife with 72.6% exposure recorded in Australian Boobooks (*Ninox boobook*) in Perth, Western Australia (Lohr 2018).

Detection of AR exposure in non-target vertebrates is increasingly documented in the published scientific literature, but previous research on vector and indicator species has focussed on invertebrates, birds and mammals (Lopez-Perea et al. 2019; Serieys et al. 2019; Thomas et al. 2017). Reptiles have largely been ignored despite observations of direct bait consumption (Hoare and Hare 2006a) including one instance where bait consumption is suspected to have directly caused mortality (Bettink 2015). In addition to evidence of direct AR exposure, reptiles have potential to be vectors of high AR loads by virtue of their longevity, occupying multiple trophic levels and apparent resistance to anthropogenic contaminants (Hopkins 2000). Recently, Lohr & Davis (2018) reviewed the literature on interactions between reptiles and ARs and identified the role of reptiles as a vector for ARs as a research priority. Here, we address this recommendation by analysing AR concentrations in the livers of three large-bodied and abundant reptiles in Perth, Western Australia, an urban landscape where secondary exposure to ARs has already been documented in

predatory birds (Lohr 2018). Our study species – the dugite (*Pseudonaja affinis*) a rodent-predator snake, the bobtail (*Tiliqua rugosa*) an omnivorous lizard and the tiger snake (*Notechis scutatus occidentalis*) a wetland snake with a dietary preference for frogs – were chosen as they give insight to AR exposure in reptiles and trophic transfer. Each of our study species differs in dietary preference and trophic tier, and therefore should exhibit different frequencies and concentrations of ARs. We predicted that the dugite would have the highest concentration of ARs, the tiger snake would have limited or no exposure to ARs, and the bobtail would fall between these two.

A2.3 Materials and methods

A2.3.1 Study area and species

The urban footprint of Perth, the capital city of Western Australia, covers over 1050 km^2 with a population of over two million (MacLachlan et al. 2017) . The primary land uses of urban Perth are residential and industrial intersected with parks of remnant native vegetation. Currently all ARs available in Australia, with the exception of pindone, are sold directly to the public without requiring a license and there are no records on the volume of sales or frequency of use (Lohr and Davis 2018). Anecdotal accounts of increased baiting in winter corresponding with increased exposure in Australian Boobooks during winter suggest some degree of seasonality in the use of AR baits (Lohr 2018) but substantial deployment of ARs has been observed by the authors year-round in and outside of residential and commercial buildings.

We tested AR exposure in three large (200-1100g) reptile species frequently found within urban Perth and demonstrating differences in diet and trophic tier. Dugites (*Pseudonaja affinis*: Elapidae) are a large snake (>1.7m) that ontogenetically shift their diet from reptiles to mammals (Cipriani et al. 2017; Wolfe et al. 2018). Bobtails (*Tiliqua rugosa*: Scincidae) are a large (~0.4m) omnivorous lizard known to eat primarily vegetation as well as a variety of invertebrates and anthropogenic scraps such as pet food and rubbish, in urban areas (Dubas and Bull 1991; Norval and Gardner 2019). Both dugites and bobtails occupy the same open woodland and heath habitats, and are frequently found in urban gardens. West Australian tiger snakes (*Notechis scutatus occidentalis:* Elapidae) are a large (~1m) snake that have a diet comprising of mostly frogs, but occasionally reptiles, mammals and birds (Lettoof et

al. 2020b). All three study species spatially overlap in wetland habitats, but tiger snakes are rarely found outside wetlands in Perth. Bobtails can suffer predation from dogs (*Canis lupus sp.*), cats (*Felis catus*), foxes (*Vulpes vulpes*), wedge-tailed eagles (*Aquila audax*) and a range of snake species including dugites (Norval and Gardner 2019). Evidence of predation on Australian snakes is rare but predators of smaller individuals include carnivorous birds (raptors, kingfishers, corvids), dogs, cats, foxes, monitor lizards (*Varanus sp.*) and other snakes (Shine 1995). As Australia doesn't have many large-bodied predators, predation on larger snakes is likely to be rare, especially in urban environments with fewer predators present. For this study we only tested adult dugites (>1.2m) and tiger snakes (>0.7m).

A2.3.2 Specimen collection

Dugites (n = 11) and bobtails (n = 10) were collected opportunistically as road kill or non-rotten carcasses donated by wildlife care centres between 2014 and 2018, and tiger snakes (n = 11) were wild caught and euthanised between 2018 and 2019 (morphological data presented in online Table A.1). Carcasses were stored within 12h of collection frozen at -20^{o}C until the liver was extracted and analysed for AR residues. All specimens were collected within 30 km from the centre of Perth, Western Australia, and within 250m of residencies or other urban infrastructure (Fig. A2.1). Specifically, dugites and bobtails were collected from areas surrounded by residential or industrial infrastructure. Tiger snakes were collected from four urban wetlands surrounded by residential infrastructure, with one wetland being partially bordered by an industrial area.

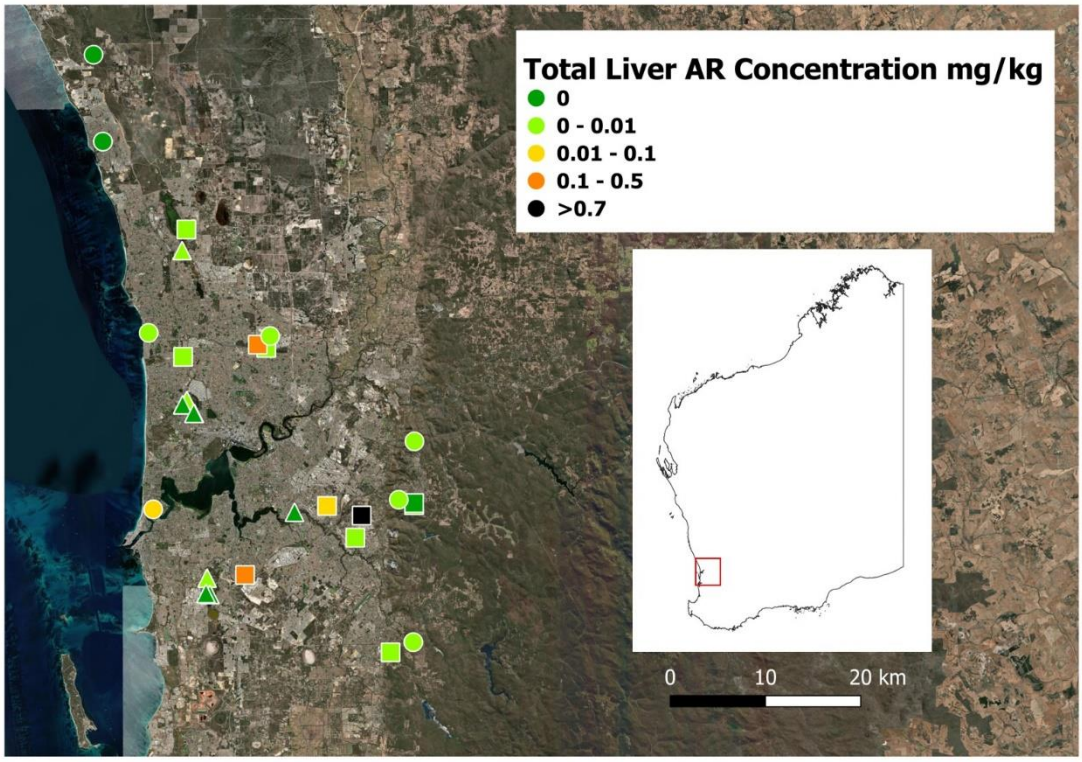


Fig. A2.1 Locations of individual reptiles screened for ARs in Perth, Western Australia. Squares = dugites (*Pseudonaja affinis*), circles = bobtails (*Tiliqua rugosa*) and triangles = tiger snakes (*Notechis scutatus occidentalis*).

A2.3.3 Samples extraction and purification

Liver samples aliquots of 1 gram (wet weight, *w.w.*) were accurately weighed, frozen at -80 ºC and then freeze-dried using a SubliMate 2 Bench Top laboratory Freeze dryer (EscoGlobal, Singapore). Freeze dried samples were homogenised in stainless steel vessels using a MM 400 milling system (Retsch GmbH, Germany). Homogenised samples were transferred into centrifuge plastic tubes (15 mL) and two aliquots of 5 mL of acetonitrile were pipetted into the tubes added with a 10 µL (10 ng/ µL) solution containing the deuterated surrogates. Analytes were extracted using a sonication bath (15 min sonication for each aliquot). After extraction, samples were centrifuged at 4400 rpm for 5 minutes using a Heraeus Megafuge 8 centrifuge from ThermoFisher Scientific (Sydney, Australia) and the supernatant was transferred into a new plastic tube added with 2 mL of n-hexane. Samples were then vortexed for 5 minutes and then centrifuged at 4400 rpm for 5 min and the supernatant discarded. Samples extracts were evaporated near to dryness under a gentle nitrogen stream and then reconstituted

in 400 µL of a 50:50 ACN/H_2O solution. The final extracts were transferred in 2 mL Teflon-lined screw cap amber glass vials stored at 0-4 °C until analysis. Bias (average percentage recovery) and precision (percentage relative standard deviation of recoveries, % RSD), determined by processing through the entire analytical procedure spiked samples of organic chicken liver supplied by a local butcher (South Perth, WA). Samples were spiked with a solution containing all AR and surrogate standards to give a final concentration of either 10 ng/g or 75 ng/g of each AR and 100 ng/g of deuterated standards. Unspiked chicken liver samples (n=3) were used as a negative control (i.e., blanks). These samples were extracted and analysed along with the batch of samples. Details regarding chemicals, analytical standards, solutions and calibration standards are summarised in the Online Supporting Information.

A2.3.4 UHPLC MS/MS analysis

Chromatographic separation was achieved with an UltiMate 3000 UHPLC system (Thermo Fisher Scientific Corporation, US) coupled to an Agilent InfinityLab Poroshell 120 SB-C18 column (100 x 2.1 mm, 2.7 µm using acetonitrile and water containing 10mM ammonium acetate at pH 5.7 at 25 ºC and 0.250 mL/min flow rate. Rodenticides were detected using a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific Corporation, US). Analytes ionisation was achieved using an Ion Max NG API source operated in negative mode. The mass spectrometer was operated in multiple reactions monitoring (MRM) mode. UHPLC, Max NG API source and mass spectrometry settings are summarised in the Online Supporting Information (Table S1-S3). Data was processed using Xcalibur 4.1.31.9 and Tracefinder 4.1software packages.

A2.3.5 Statistical analysis

We compared the differences in AR concentration between each reptile using a Kruskal-Wallis for each AR that was detected in more than one species, as well as for the total (sum) concentration of ARs for individuals exposed to multiple ARs. For statistical analysis, samples that were recorded below detectable limits were entered as half the detection limit. A Dunn post-hoc test with Benjamini-Hochberg adjusted p-values was performed to identify which species differed significantly (p <0.05) from each other. We used chi-squared tests to compare differences between species for detection frequency (% of individuals with any AR) and the mean number of ARs

detected per exposed individual. All statistical analyses were conducted in R Studio (R Core Team 2021).

A2.4 Results

ARs were detected in all three species (Table A2.1). All dugites and seven of ten bobtails were killed by vehicle collisions, the remaining three bobtails were euthanised by wildlife care centres and contained no ARs. 91% of 11 dugites were exposed to ARs and 73% were exposed to more than one AR, 60% of 10 bobtails were exposed to ARs and 40% were exposed to more than one AR, and 45% of 11 tiger snakes were exposed to brodifacoum only. The highest combined AR concentration was detected in dugites, which were three times higher compared to bobtails, and 51 times higher compared to tiger snakes (Fig. A2.2). The most commonly detected AR was the SGAR brodifacoum. The FGAR warfarin was only detected in dugites, which were generally exposed to the most ARs. Flocoumafen was detected only in a single bobtail, and pindone and coumatetralyl were not detected in any samples. Total AR concentrations in livers were significantly higher for dugites than for tiger snakes ($p = 0.008$) and approached significance between bobtails and dugites ($p = 0.069$). Bromadiolone and difenacoum concentrations in livers were significantly higher for dugites than for tiger snakes ($p = 0.048$). There was no significant difference in detection frequencies between species, and the difference in number of ARs in exposed individuals exposed individuals for dugites and tiger snakes approached significance ($p = 0.05$).

Dugite and bobtail carcasses varied in damage and condition which limited our ability to conduct necropsies or identify AR toxicity symptoms such as haemorrhaging, or determine the sex of most individuals (Online Table A.1). No tiger snakes exhibited any haemorrhaging. Of the best condition carcasses, the livers of two bobtails and one tiger snake were enlarged and pale or mottled, which can be symptoms of AR poisoning; however, none of these individuals contained ARs above detectable limits.

Table A2.1 Concentration of reptile livers exposed to ARs from greater Perth, Western Australia (mg/kg). *n* values are the number of each individual exposed to each compound. Values presented as: mean ± SE (range).

Species (*n* = exposed vs. tested)	Total conc. of all ARs for exposed	Brodifacoum	Bromadiolone	Difenacoum	Flocoumafen	Warfarin
Dugite *Pseudonaja affinis* (10/11)	0.178±0.074 (0.003 – 0.704)	0.096±0.046 (0.010 – 0.330)	0.202±0.137 (0.002 – 0.700)	0.019±0.012 (0.003 – 0.053)	NA	0.007±0.002 (0.004 – 0.011)
		n = 7	*n* = 5	*n* = 4		*n* = 4
Bobtail *Tiliqua rugosa* (6/10)	0.040±0.031 (0.007 – 0.182)	0.025±0.017 (0.006 – 0.109)	0.020±0.018 (0.001 – 0.073)	0.002	0.004	NA
		n = 6	*n* = 4	*n* = 1	*n* = 1	
Tiger snake *Notechis scutatus* (5/11)	0.009±0.002 (0.006 – 0.014)	0.009±0.002 (0.006 – 0.014)	NA	NA	NA	NA
		n = 5				

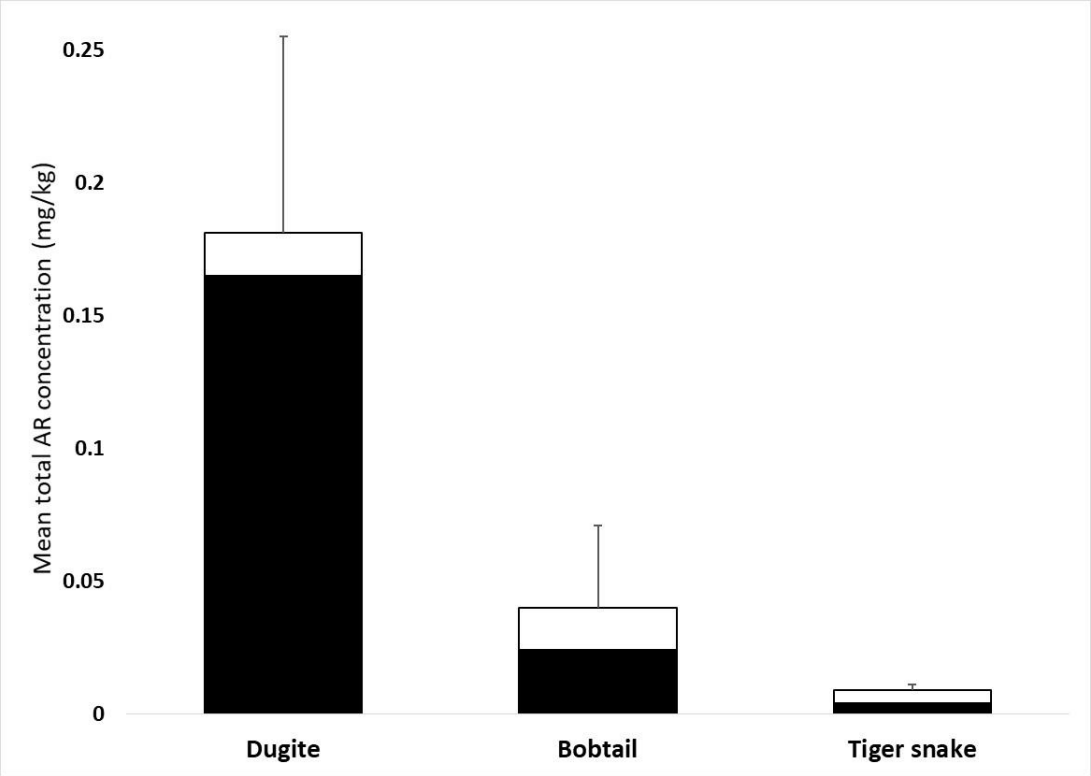


Fig. A2.2 Mean total AR liver concentration of exposed reptile species tested in Perth, Western Australia. Bar fill represents frequency of exposure (dugites 91%, bobtails 60% and tiger snakes 45%). Error bars = SE.

A2.5 Discussion

All three reptile species tested were exposed to ARs, and this is the first study to report ARs in wild reptiles not associated with rodent eradications on oceanic islands. The impacts of AR on reptiles are relatively unknown and what is known has been thoroughly summarised in (Lohr and Davis 2018). Although we cannot infer any toxicological effects on reptiles from this study, we present insight into AR exposure of multi-trophic reptile species in an urban ecosystem. As predicted, adult dugites (common urban rodent-predators) had the highest frequency of exposure and concentration of ARs, and was the only species exposed to warfarin. In Australia, warfarin is available at nearly all hardware and grocery stores and is not associated with any particular land use. We predict it's detection in dugites is a function of higher exposure from rodent predation. Also, as predicted, adult tiger snakes had the lowest exposure and bobtails fell between the two snakes. The frequency and concentration of ARs in bobtails, and the trace amounts in tiger snakes is concerning; however, not surprising given the public availability of ARs through retail sales (Lohr and Davis 2018). Bobtails are probably exposed to ARs both directly and indirectly: Perth urban bobtails are commonly found throughout residential gardens and have been observed inside AR bait boxes (Ashleigh Wolfe, pers. comm.). As bobtails are known to eat anthropogenic food scraps we suspect they, like many other large omnivorous lizards (Bettink 2015; Merton 1987), are likely to eat baits found in residential backyards. Bobtails have also been recorded consuming mice (Norval and Gardner 2019) and thus may be secondarily exposed to ARs from predating poisoned rodents or scavenging carrion, as well as contaminated invertebrates (Alomar et al. 2018; Elliott et al. 2014).

Tiger snakes in Perth predominately eat frogs and rarely eat rodents (Damian Lettoof, unpublished data), and yet we detected a relatively high (45%) prevalence of a single SGAR (brodifacoum) at trace concentrations. The four wetlands from which tiger snakes were collected are surrounded by residential areas, and we suspect several routes of AR exposure for tiger snakes: (1) traces of SGARs may be detectable for years after a single predation of a poisoned rodent as a consequence of persistence in liver tissue (Rueda et al. 2016); (2) urban wetlands are contaminated by storm water run-off (Lettoof et al. 2020a) which may include residentially used ARs (Kotthoff et al. 2019) and result in direct exposure from contaminated water; and (3) the primary prey of tiger snakes, frogs, may be exposed to ARs. Amphibians have never been

tested for AR exposure despite being very susceptible to accumulating pesticides via direct contact with contaminated water (Bruhl et al. 2011), or through consumption of exposed invertebrates (Alomar et al. 2018; Elliott et al. 2014). Any combination of these routes of exposure could explain the prevalence of ARs in urban tiger snakes, and probable exposure for each species is illustrated in our graphical abstract.

The liver concentrations we detected in our test species are similar to those found in other wild reptiles. A single whip snake (*Hemorrhois hippocrepis*) was found with a liver concentration of 0.54 mg/kg of flocoumafen, and wild lava lizards (*Microlophus duncanensis*) were found with brodifacoum concentrations ranging between 0.001 and 0.8 mg/kg as well as two individuals at 1.6 and 1.9 mg/kg (Rueda et al. 2016). Although we are unsure if the AR liver concentrations in our study species are lethal or sublethal, the prevalence and mean total AR concentration in dugites is concerning compared to other taxa (see Table 3 in Lohr 2018 and Table 9, 10 and 12 in Laakso et al. 2010). The few short-term laboratory and field studies on the acute toxicity or sub-lethal effects of ARs and other pesticides in reptiles have rarely detected physiological impacts (Mauldin et al. 2019; Pauli et al. 2010); however, considering the slow metabolism of reptiles, the testing time periods may have been too short to show acute clinical symptoms. The available literature, nonetheless, suggests that at least some reptile species are more resistant to pesticide toxicity than are other taxa (Pauli et al. 2010). The Western fence lizard (*Sceloporus occidentalis*), for example, required an acute oral dose of the first generation (FG) anticoagulant pindone three to five times the fatal dose for birds and mammals before mortality occurred (Weir et al. 2015).

If reptiles are truly more tolerant of AR toxicity, their potential to be toxic vectors poses a much greater threat to the food web than currently recognised. We frequently detected (45 – 91%) ARs in all three reptile species, despite the difference of their trophic tiers and diet, and we found a single dugite (9% of tested) with total AR liver concentration of 0.7 mg/kg (a concentration that is considered lethal in raptors (Kaukeinen et al. 2000)). This suggests at least two possibilities: (1) there is pervasive AR contamination throughout the food web (Pitt et al. 2015) in areas of high baiting e.g. urban landscapes, and (2) reptiles accumulating high AR concentrations may present a fatally toxic meal for predators or scavengers. This may not have serious implications for urban wildlife in our study system, as we suspect predation events for urban adult dugites and tiger snakes are rare. On a global scale, however, this has

serious implications for regions with higher AR use, biomass and biodiversity of reptiles, and more reptile predators than Perth. For example, North American racoons (*Procyon lotor*) are common scavengers of urban reptile road-kill (Antworth et al. 2005) and are likely to be exposed from eating poisoned reptiles. Urban genets (*Genetta sp.*) in Africa also predate on reptiles (Delibes et al. 1989; Widdows and Downs 2015), and have been found with AR exposure (Serieys et al. 2019). There is already emerging evidence of this scenario: An island-wide study of ten raptor species in Taiwan found a high prevalence of AR exposure in rodent-eating and scavenging species as well as snake-eating species (Hong et al. 2019), suggesting that snakes may be an important vector of ARs to other trophic levels in this area.

Reptile species richness is highest in tropical and arid regions of the world (Böhm et al. 2013), and the tropical bioregion is also densely occupied by humans. As AR exposure is highest in wildlife living in or in close proximity to urban or agricultural land (Lopez-Perea et al. 2019; Serieys et al. 2018), tropical urban reptile populations are highly likely to be exposed. This highlights AR toxicity as an additional threat to habitat loss and wild harvest (Böhm et al. 2013) for tropical reptile populations and biodiversity. Urban reptiles' high exposure to ARs has further implications for humans. Reptile meat is a common food resource in tropical and subtropical regions of the world (Klemens and Thorbjarnarson 1995), and is consumed on almost every continent. Although AR use and wildlife exposure has not been well studied in these regions, many reptiles persist in urban environments and would be harvested with close proximity to areas where anticoagulant rodenticides are used. Snakes, as rodent-predators, are of the highest risk of exposure, and thus localities where snakes are regularly eaten by humans, such as Vietnam (Magnino et al. 2009), China (Wang et al. 2014), Malaysia (Cantlay et al. 2017) and Africa (Taylor et al. 2015) are consequently also at high risk of exposure.

Besides tolerance to toxicity, reptiles may be particularly good reservoirs of ARs due to: a) the slow decomposition rate of ARs (Eason and Spurr 1995), and b) a much lower rate of elimination and depuration of accumulated ARs from reptiles due to slower metabolism compared to other taxa (Campbell et al. 2005; Davenport et al. 1990). There is one excellent example of extreme persistence of ARs in a wild population of lizard: after a heavy baiting program was implemented on Pinzon Island, Galapagos to eradicate rats, a high prevalence of ARs was detected 100 - 850 days

post-baiting in the livers of lava lizards (*Microlophus duncanensis*) (Rueda et al. 2016). Although no population-level poisoning was observed in the lizards, there was an unexpected outcome for the island birds of prey: 22 Galapagos hawks (*Buteo galapagoensis*) and a short-eared owl (*Asio flammeus*) were found dead from brodifacoum poisoning, presumably from predation of toxic lizards, 12-773 days after the baiting event. Lava lizards were found with liver concentrations of brodifacoum between 0.001 and 0.8 mg/kg at 400 days post-baiting, and at 800 days post baiting lizards were still found to have liver concentrations between >0.001 and 0.2 mg/kg. If elimination rates are similar for our test species then it's possible that some of our individuals have been retaining ARs for years. ARs are primarily shed from the body through faeces which has important implications for reptiles as vectors: a) insectivorous reptiles may be recursively exposed to toxic invertebrates which feed on contaminated reptile faeces; and b) reptiles that feed, and subsequently defecate, infrequently, i.e. snakes, should retain ARs substantially longer than mammals and birds.

Reptiles' sensitivity to AR toxicity is fundamentally unknown. Western fence lizards survived oral dosages of brodifacoum up to 1750 mg/kg, a concentration thousands of times higher than the LD50s recorded for most birds and mammals (Laakso et al. 2010). Laboratory experiments also found no observable effect on gopher snakes (*Pituophis catenifer*) that were fed mice that died from a lethal dose of the anticoagulants warfarin and diphacinone (Brock 1965); while one of 19 iguanas (*Iguana iguana*) orally administered brodifacoum died and showed signs of intoxication with blood in the body cavity, although oddly this individual was from the lowest concentration treatment (Mauldin et al. 2019). There is speculation on why reptiles are relatively more resistant to AR toxicity than are other taxa; suggestions include a difference in blood coagulation chemistry (Merton 1987) and naturally slower clotting mechanisms (Dessauer 1970). In raptors, despite variation between species (Thomas et al. 2011), a liver threshold of 0.1 mg/kg is considered a minimum for toxicity (Rattner et al. 2014). We detected a mean total AR liver concentration above 0.1 mg/kg in exposed dugites (91%), and a single exposed bobtail (17%). As SGARs are more toxic than diphacinone, we logically expect a liver concentration of 0.1 mg/kg to have at minimum a sublethal effect. We also found exposure to multiple rodenticides was relatively common in 80% of exposed dugites and 67% of exposed

bobtails (maximum 3 ARs), and suggests accumulation from multiple prey items. Multiple ARs may have a synergistic effect rather than a cumulative effect (Lohr 2018). For example, laboratory studies have demonstrated rat sensitivity to warfarin vastly increased after chronic exposure to brodifacoum (Mosterd and Thijssen 1991), and American kestrels (*Falco sparverius*) exposed to the FGAR chlorophacinone experienced prolonged prothrombin times if they were previously exposed to brodifacoum (Rattner et al. 2020).

Sublethal concentrations have been shown to impact both the physiology and behaviour of exposed individuals. General symptoms of intoxicated animals shortly before mortality are anorexia, weakness, lethargy and dyspnea (shortness of breath) (Fitzgerald and Vera 2006), and thus any reduction in mobility could increase the likelihood of mortality from other causes (Brakes and Smith 2005). As with other taxa, exposed reptiles may be vulnerable to increased predation (Cox and Smith 1992), increased mortality from vehicle collisions when on roads (Lohr 2018; Mendenhall and Pank 1980; Serieys et al. 2015), and a disruption of their thermoregulation routine (Merton 1987). Thus, we recognise the potential for livers from dugites and bobtails which were collected as roadkill, or from wildlife rehabilitators, to be biased towards higher AR concentrations, compared to livers of tiger snakes that were collected alive and euthanised. However, if AR exposure does interfere with a reptile's normal behaviour then we also acknowledge that the easily hand-caught tiger snakes could be biased towards higher AR concentrations.

A2.6 Conclusions and future direction

This study offers convincing evidence that urban reptiles of different trophic tiers and diet are exposed to residentially used ARs, and suggests the surrounding food web is more contaminated than previously assumed. We predict a similar AR exposure in reptiles of the same ecological niche in cities where the purchase of ARs are unrestricted and retail available. Based on their probable resistance to toxicity, low elimination rates, and multi-trophic positions, we consider reptiles in proximity to AR sources (i.e. urbanisation) to be good indicators of food web contamination. Our data highlight a novel threat faced by reptile predators and humans consuming wild reptiles captured near human habitation – particularly if liver or fat tissues are consumed.

To further assess the contamination of reptiles in the food web, we suggest investigating AR exposure of small insectivorous species, such as geckos, that have been detected living in bait boxes and are frequently eaten by other wildlife taxa known to be susceptible to AR toxicity. To address the concerns of human exposure we suggest screening for ARs in livers from reptiles sold in meat markets, particularly in countries of Eastern Asia. The frequent AR exposure in a snake that mostly eats frogs suggests frogs may also be contaminated. To the best of our knowledge ARs have never been detected or screened for in an amphibian, thus we recommend testing wild urban amphibian populations in AR exposed areas. Additional research is urgently needed to determine the scope and severity of AR exposure in reptiles in order to mitigate risks to humans and non-target wildlife.

A2.7 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Appendix 3. Evidence and patterns of maternal transfer of metals and trace elements in Western tiger snakes (*Notechis scutatus occidentalis*) – a pilot study

The study presented in Appendix 3 was accepted in the peer-reviewed journal '*Austral Ecology*' on November 2020.

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A3.1 Abstract

Urban wildlife are regularly exposed to a variety of anthropogenic contaminants that have the potential to bioaccumulate in body tissues. As a consequence, developing embryos and offspring can be at risk from exposure to maternally-accumulated contaminants, yet this has rarely been reported in reptiles. We opportunistically collected one pregnant Western tiger snake (*Notechis scutatus occidentalis*) from each of three wetlands with differing sediment metal contamination around Perth, and analysed maternal snake livers and three foetuses per litter for a suite of 17 elements representing either alkaline earth metals, transition metals or metalloids. We detected 14 elements, and compared their concentrations in maternal livers to foetus whole-bodies to determine preliminary patterns of maternal transfer. Our results suggest antimony, arsenic, manganese, mercury, molybdenum and zinc are maternally transferred in Western tiger snakes. We urge further research to further quantify patterns of contaminant maternal transfer in viviparous snakes, and determine their impacts on the development and health of contaminated offspring.

A3.2 Introduction

Urban wildlife are often chronically exposed to anthropogenic contaminants, such as metals and pesticides (Murray et al. 2019). For terrestrial vertebrates, diet is usually the most significant route of contaminant exposure; however, developing embryos can

be exposed to maternally transferred contaminants. Currently, research on maternal transfer of contaminants in reptiles is mostly limited to the oviparous taxa: turtles (Ehsanpour et al. 2014) and crocodilians (Rauschenberger et al. 2004); whereas squamates, particularly viviparous species, have received little attention. In addition, most of this research has focused on organic contaminants due to their lipophilic properties (Rowe 2008), despite many metals being shown to transfer to offspring during development (Ehsanpour et al. 2014). The knowledge of maternal transfer of elements in snakes specifically, is limited to mercury in the viviparous wetland species *Nerodia sipedon* (Chin et al. 2013a; Cusaac et al. 2016) and selenium in the oviparous terrestrial species *Lamprophis fuliginosus* (Hopkins et al. 2004).

During an ecotoxological study in Perth, Western Australia (Lettoof et al. 2020), Western tiger snakes (*Notechis scutatus occidentalis*) were collected and euthanised to analyse element concentration and potential bioaccumulation in their livers. Of these snakes, one pregnant female from each of three wetlands was collected. We took this opportunity to screen three foetuses from each litter to compare concentrations of 14 elements (five non-essential metals and nine trace elements) to element concentrations in their mothers' livers and explore patterns of maternal transfer. Herein we present a preliminary study on the first evidence of maternal transfer in a viviparous snake. Our results justify further research on this system and enrich the global knowledge of maternal transfer of metals and trace elements in snakes.

A3.3 Methods

Between March and April 2019, one pregnant tiger snake was hand collected from each of three wetlands (Herdsman Lake and Bibra Lake within urban Perth, and Loch McNess within Yanchep National Park) that differed in their degree of urbanisation and contamination with metals and other trace elements (Lettoof et al. 2020). Specifically, Herdsman Lake generally had the highest sediment concentration of all screened elements including arsenic, copper, lead and zinc concentrations exceeding Australian government guidelines. Bibra Lake and Loch McNess differed in element concentrations; however, selenium exceeded Australian government guidelines in both wetlands (Loch McNess four times higher than Bibra Lake). Snakes were humanely euthanised by blunt force trauma, and we removed their livers, foetuses, and infertile ova. After removing the foetuses and ova, we measured the snout-vent length

(SVL) and body mass of females and foetuses. We randomly selected three foetuses per litter to analyse for whole-body concentrations of metals. Maternal livers and foetuses were screened for 17 elements (i.e. metals and trace elements) by ChemCentre (Perth, Western Australia); these elements were chosen based on the lack of historical data from these wetlands and regular screening of this suite for environmental monitoring (Leif Cooper, ChemCenter, *pers. comm.*). We screened livers only as an index of the snakes' accumulation of the elements that could be compared across sites. Whole livers and foetuses were homogenised, extracted with nitric and hydrochloric acid, and analysed for metals using a combination of inductively coupled plasma-atomic emission spectroscopy (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS). For more detailed descriptions on sites and chemical analysis see (Lettoof et al. 2020). For statistical analyses, samples that were recorded below detectable limits were entered as half the detection limit (Zeghnoun et al. 2007). We compared the relationships between maternal liver concentrations (independent variable) and foetus total body concentrations (dependent variable) using a separate mixed-effects regression model for each metal with maternal ID as a random effect, which grouped foetuses by litter to avoid pseudoreplication. All data were log-transformed. Due to small sample sizes we also assessed the relationships visually using scatter plots. Snakes were collected under Western Australia's Department of Biodiversity, Conservation and Attractions Permit No. 08-002624-1. Curtin University's Animal Research Ethics Committee approved the use and handling of snakes for this research under Approval No. ARE2018-23.

A3.4 Results

Sixteen elements were detected in foetuses (Table A2.1; see Chapter 3 supplementary material for limits of detection (LODs)). Beryllium was not found above detection limits in any maternal liver or foetus, tin and lead were not detected in most samples hence these metals are excluded from further discussion. Foetuses from the Bibra Lake and Yanchep snakes were smaller, and had less pigmentation, indicating they were a slightly earlier stage of development. Due to small tissue samples, elements could only be quantified as wet weight.

Table A3.1 Morphometrics and metal concentrations (wet weight) of wild-caught pregnant *Notechis scutatus occidentalis* livers and whole body concentrations of three randomly chosen foetuses (mean ± SD) per litter. Mothers mass was measured after extraction of foetuses and infertile ova. SVL = snout-vent length, N = no. of foetus/infertile ova, < = below detection limit, # likely process acting between mother and foetus, *indicates statistical positive relationship between maternal liver and foetuses concentrations.

	Herdsman Lake		**Bibra Lake**		**Yanchep National Park**		
	Mother	Foetus (*n* = 10/0)	Mother	Foetus (*n* = 8/5)	Mother	Foetus (*n* = 8/4)	**Process#**
SVL (mm)	776	20.3 ± 0.6	704	16.4 ± 1.0	671	17.7 ± 1.9	
Mass (g)	213.8	6.8 ± 0.2	211.9	3.5 ± 0.5	208.3	3.7 ± 0.9	
Non-essential metals (µg/Kg)							
Ag	13	0.8 ± 0.3	2	1.3 ± 0.6	11	<1	?
Ba	150	937 ± 57	60	1233 ± 58	140	807 ± 107	Accumulation
Cd	35	0.8 ± 0.3	12	0. 8 ± 0. 3	73	<1	Exclusion
Hg	150	<10	80	<10	240	12 ± 8	Exclusion
Pb	<5	<5	<5	<5	13	<5	NA
Sb	40	7 ± 10	16	<1	3	<1	Exclusion
Trace elements – lower (µg/Kg)							
As	430	63 ± 6	110	40 ± 0	440	50 ± 10	Exclusion
Co	59	6 ± 1	31	9 ± 5	60	10 ± 2	Exclusion
Cr	150	90 ± 20	<50	393 ± 353	130	353 ± 339	NA
Ni	60	263 ± 180	030	220 ± 260	260	150 ± 79	NA
Sn	<50	<50	<50	<50	<50	53 ± 6	NA
Trace elements – higher (mg/Kg)							
Cu	9.8	0.5 ± 0.1	3.1	0.4 ± 0.1	15	0.43 ± 0.04	Exclusion
Mn*	0.9	0.8 ± 0.1	0.8	0.6 ± 0.1	0.93	0.8 ± 0.1	Equivalence
Mo*	5	0.03 ± 0.01	1.3	0.01 ± 0	1.4	0.01 ± 0.01	Exclusion
Se	1	0.30 ± 0.03	1.3	0.21 ± 0.01	1.5	0.28 ± 0.02	Exclusion
Zn	32	23.7 ± 2.1	21	19.7 ± 1.2	25	16 ± 1	Equivalence

There were significant positive relationships between Western tiger snake maternal liver and foetus concentrations of manganese ($r^2 = 0.61$, $F_{1,6} = 14.19$, $p = 0.009$) and molybdenum ($r^2 = 0.57$, $F_{1,6} = 9.28$, $p = 0.023$), while barium showed a near-significant negative correlation ($r^2 = 0.75$, $F_{1,6} = 4.78$, $p = 0.07$). Relative to maternal liver concentrations, foetus concentrations of antimony, arsenic, mercury, and zinc appeared to increase, while foetus concentrations of cadmium and silver appeared to decrease, but all of these relationships were non-significant, possibly due to small sample sizes (Fig. A3.1). Foetus concentrations of cobalt, copper, chromium, nickel and selenium remained relatively constant as maternal liver concentrations increased.

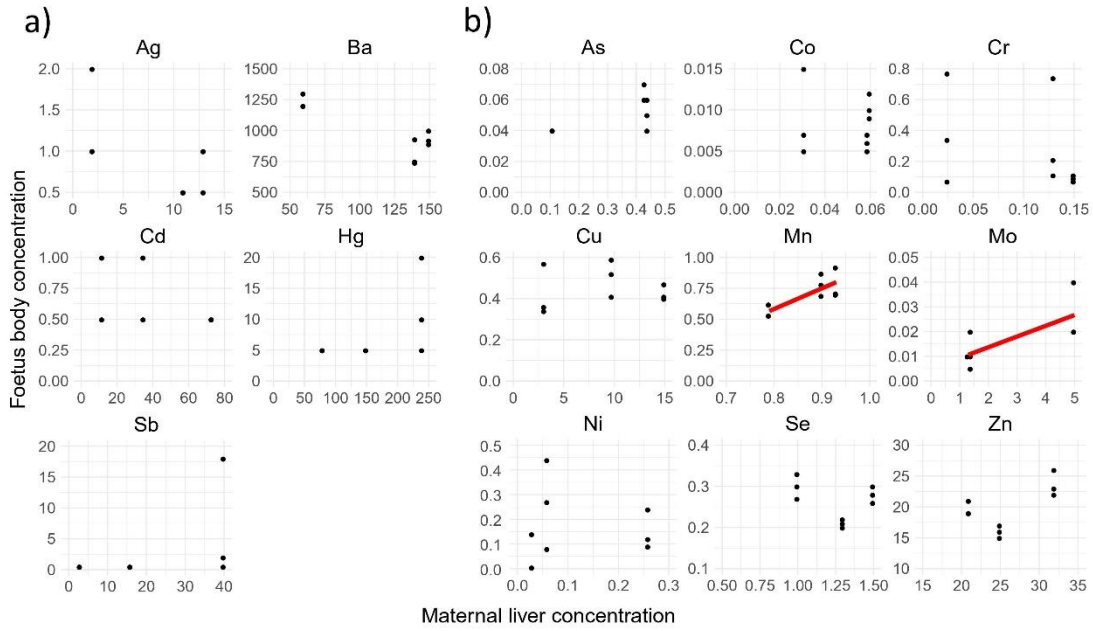

Fig. A3.1 Relationships between Western tiger snake (*Notechis scutatus occidentalis*) maternal liver and foetus whole-body concentrations (wet weight) of a) non-essential metals, and b) trace elements. Regression line indicate a statistical significant (p = <0.05) relationship. Concentration units are µg/Kg for non-essential metals (a), and mg/Kg for trace elements (b). Axis ranges are unique for each metal.

A3.5 Discussion

Our data indicate statistical evidence of maternal transfer of manganese and molybdenum between contaminated female Western tiger snakes and their foetuses, and potential maternal transfer of antimony, arsenic, mercury and zinc. Evidence for maternal transfer of arsenic, mercury and zinc exists in other reptiles, however this is limited to turtles and one snake (Chin et al. 2013a; Ehsanpour et al. 2014; Van Dyke et al. 2014). We could not find any published literature on maternal transfer of antimony, manganese or molybdenum in reptiles. Although these elements have been shown to maternally transfer in humans (Krachler et al. 1999), we present here the first evidence of maternal transfer in a reptile. We acknowledge that the element concentrations lacking statistical relationships or correlative patterns are not indicative of a lack of maternal transfer, and may suggest partial maternal elimination. We also acknowledge that these patterns could change or be clarified with more data; and thus, our preliminary results indicate that further studies exploring maternal transfer of

metals and trace elements in snake populations likely to be exposed to pollution are warranted.

Interpreting patterns of maternal transfer from small data sets and single points in time is difficult in viviparous squamates, as they potentially rely on two periods of nutritional allocation: vitellogenesis prior to ovulation and placental transport during pregnancy (Van Dyke and Griffith 2018). These two potential periods of reproductive allocation could transfer the same maternal metals to developing foetuses differently, depending on the stage of development when the mother is exposed to the elements. For example, if a mother accumulates a contaminant during or before vitellogenesis, it should be incorporated in the yolk during vitellogenesis (Van Dyke et al. 2012), from where it would expose developing embryos. As vitellogenesis takes place in the liver, the element concentrations of foetuses should reflect that of their mother's liver if they were incorporated into yolk during vitellogenesis. A similar pattern would be present if the same mother was exposed to the element post-ovulation and it was transported via the placenta. However, most viviparous squamates are not highly placentotrophic (Van Dyke and Griffith 2018), so the placenta may be incapable of transporting metals that were accumulated post-ovulation. Alternatively, the placenta may exclude elements bound to metallothionein as is shown in placental mammals (Yoshida et al. 2002). If no placental transport occurred, the data would show higher maternal liver concentration and lower foetus body concentration for elements the mother accumulated during gestation. This would suggest maternal transfer does not occur for said element across a placenta, which could be reflected by the absent or negative correlations that our data show for barium, cadmium and silver (Fig. 1). Our small sample size and single-point in time testing (during late development) do not allow us to confidently deduce whether these elements are transferred during vitellogenesis or placentation, but provide some indication that either or both mechanisms might be possible in viviparous squamates like tiger snakes.

Regardless of the mechanism, manganese and molybdenum's significant positive correlations between maternal livers and foetuses suggest these metals were maternally transferred to the developing foetuses, while antimony, arsenic, mercury, and zinc all exhibit patterns supporting the potential for maternal transfer. Arsenic, manganese, molybdenum and zinc are trace elements essential for biological functions, and therefore some degree of maternal transfer of these is expected.

Maternal liver and foetus manganese concentrations are similar and potentially reflect essential developmental requirements, whereas molybdenum concentrations are much lower in foetuses and therefore this element may not be as important during this stage of tiger snakes lifecycle. The non-essential metals, i.e. antimony, cadmium, mercury etc. are known to maternally transfer due to their lipophilic properties and affinity for metalloproteins (Guirlet et al. 2008).

Although trace elements are usually regulated by the body, over exposure can lead to accumulation and toxicity, and non-essential metals usually bioaccumulate more easily and are toxic at much lower concentrations. Some of the sampled wetlands have sediment contaminated with arsenic, zinc and mercury concentrations exceeding Australian and New Zealand Environment and Conservation Council (ANZECC) sediment quality guidelines; and antimony, arsenic, mercury, molybdenum and zinc bioaccumulate in tiger snakes at these wetlands (Lettoof et al. 2020). The potential for these elements to maternally transfer in tiger snakes suggests they are not only cycling throughout the food-web of these wetlands, but have potential to impact vertebrates during embryogenesis, when they may be most at risk of biological effects of contamination (Wolfe et al. 1998).

Exposure to contaminants via maternal transfer may be more harmful to the foetus than exposure from diet in adults, as the organism is exposed during sensitive stages of development (Russell et al. 1999). The developmental consequences of maternally transferred contaminants in snakes are virtually unknown, as previous research is limited to three studies of a single non-essential metal and snake species. Maternally acquired mercury was shown to not have any effect on *Nerodia sipendon* reproductive output or offspring survival (Chin et al. 2013b), nor stress levels (Cusaac et al. 2016); however, it did reduce offspring strike efficiency and motivation to feed (Chin et al. 2013a). In other taxa, maternal transfer of selenium and strontium resulted in lower hatchling success, developmental abnormalities and abnormal swimming in *Gastrophryne carolinensis* toad larvae (Hopkins et al. 2006), while *Esox lucis* fry suffer higher frequency of deformities and edema when maternally exposed to selenium (Muscatello et al. 2006). Offspring starting life with such handicaps may experience lower survival, higher predation and ultimately reduce population replenishment. Eventually, exposure to contaminants during embryonic and foetal

development can have detrimental impacts on both the individual organism and population.

Data from this study, although limited, offer preliminary patterns of maternal transfer of a suite of metals and trace elements in Western tiger snakes. Tiger snakes in Perth may be particularly sensitive to population impacts from maternal transfer as they are restricted to wetlands both enclosed and chronically contaminated with various metals, metalloids and other organic compounds from urbanisation. We suspect tiger snakes maternally transfer antimony and mercury that they have accumulated in urban Perth wetlands, and arsenic, manganese, molybdenum and zinc as part of natural offspring development but may be susceptible to higher loads from over exposure and bioaccumulation. Thus, we urge further research into the mechanism of transfer and impacts on the development and health of offspring.

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Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Appendix 4. Evidence of plastic consumption by a tiger snake (*Notechis scutatus*) from a highly urbanised wetland

The study presented in Appendix 4 was published in the peer-reviewed journal '*The Western Australian Naturalist*' on January 2020.

Lettoof, D. C., Orton, K. (2020). Evidence of plastic consumption by a tiger snake (*Notechis scutatus*) from a highly urbanised wetland. *West Aust Natural*. 31: 3 (187-189).

On November 7, 2001 a female Tiger Snake (*Notechis scutatus*) was collected by Phil Stone as dead on the road alongside Herdsman Lake, Western Australia, and donated as a specimen to the Western Australian Museum. In September 2018 the specimen was examined and then dissected as part of a research project.

The snake was in a noticeably poor condition; measuring 602mm in length (snout to vent), emaciated and suffering tail loss, with only 48mm of its tail remaining. Tail loss is common for Tiger Snakes in urban wetlands but currently this phenomenon is poorly understood (Aubret et al. 2005). The snake's stomach bulged from an obvious nematode infestation and a second abnormal bulging was noticed at the end of its digestive tract. There was a small exit wound about one centimetre from the cloaca with what appeared to be hard plastic protruding from the body (Fig. A4.1).

During dissection of the digestive tract the protrusion was found to be a much larger piece of hard plastic, what appeared to be half a bottle cap (Fig. A4.2). Presumably the bottle cap had made its way through the snake's entire digestive system until reaching the point at which it was wider than the snake's body cavity and could not be passed. We deduced that the pressure from the muscles and digestive system forced the inflexible and artificially shaped foreign item by its hard corner out through the body cavity. The organs around the bottle cap appeared to be heavily damaged, it is unlikely the snake could have passed any waste from this blockage.

Fig. A4.1 Plastic protruding from the tiger snakes body cavity.

Fig. A4.2 Half a plastic bottle cap being removed from the lower end of the snake's digestive track.

Snakes are known to eat foreign non-prey items although throughout scientific literature this is not a commonly documented occurrence. A wild King Cobra (*Ophiophagus hannah*) died after ingesting a plastic bag (Strine et al. 2014) and a deceased wild Lesser Black Whip Snake (*Demansia vestigiata*) was found to have a foam ear plug in its stomach (Charlton 2019). Over the last three years in Australia the media has documented snakes eating many foreign items such as a slipper, tennis ball and a teddy bear (ABC News 2018; news.com.au, 2018; Telegraph.co.uk, 2018). It is presumed that snakes mistake these items as prey if the items are covered in the scent of the prey species (Stopford 1980), or by accidently consuming the item with adjoined prey. Although we are sceptical that a bottle cap could have been covered in enough frog scent to entice the tiger snake into eating it, this seems to be the most logical theory. An alternate hypothesis is that a large frog such as *Litoria moorei* or *Limnodynastes dorsalis* has accidentally eaten the bottle cap prior to being consumed by the snake. Although this seems unlikely considering the snakes' size, it was presumably too small to eat a frog large enough to swallow a bottle cap. As far as we are aware this is the first record of a tiger snake eating plastic.

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Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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Appendix 5. *Notechis scutatus occidentalis* (Western tiger snake). Diet.

The study presented in Appendix 5 was published in the peer-reviewed journal '*Herpetological Review*' on December 2020.

Lettoof, D. C., Cornelis, J., Harvey-Hall, J., Aubret, F. (2020). *Notechis scutatus occidentalis* (Western tiger snake). Diet. *Herpetol Review*. 51: 4 (873).

Notechis scutatus is a large, polymorphic elapid generally found in wetlands across Southern Australia, yet some populations exist on offshore islands in habitats not typically occupied by mainland conspecifics (Shine 1987. Herpetologica 43:233-240). The successful colonisation of these islands is a result of their behavioural and morphological plasticity (Aubret and Shine 2009. Curr. Biol. 19: 1932-1936). Mainland *N. scutatus occidentalis* primarily feed on frogs (Lettoof et al. 2020. IJP:PAW. 11:32-39); however, they will occasionally take lizards, and small mammals and birds (Shine 1987. Herpetologica 43:233-240; Aubret 2004. Amphibia-Reptilia 25:9-17).

On the 30th of September 2019, an adult male (SVL = 81.6 cm) *N. s. occidentalis* was observed and captured by hand at Bibra Lake (-32.090009, 115.824388, WGS 84). We collected morphological data under permits DBCA: FO25000149 and ARE2018-23 as part of a population health monitoring project. We partially palpate any snakes found with prey items until enough of the item is visible to determine what taxa it belongs to, and then the item is returned to the snakes' stomach. This snake had a particularly large prey item and when palpated we observed the back legs of a young *Isoodon fusciventer* (quenda; Fig. A5.1). We believe this is the first record of a *N. scutatus* consuming an *Isoodon sp*.

While some *N. scutatus* populations consisting of large individuals have been recorded to consume possums (Oliver et al. 2010. Herpetofauna. 40:119-122), mainland *N. s. occidentalis* found in the vicinity of Perth are particularly small (Aubret et al. 2006. Behav. Ecol. 17: 716-725) and feed on relatively smaller prey (mice, frogs and small skinks; Aubret 2004. *op. cit.*). However, Carnac Island counterparts off the coast from

Perth, reach greater sizes and feed on larger prey items (nesting sea-birds chicks; Bonnet et al. 2002. Austral Ecol. 27:442-450). Following their recent introduction on Carnac Island (Ladyman et al. 2020. J. R. Soc. West. Aust. 103:39-42), *N. s. occidentalis* have seemingly successfully adapted to the island environment, notably via the expression of high levels of adaptive plasticity in response to prey size, allowing them to exploit large and abundant prey items, and thrive (Bonnet et al. 2002. *op. cit.*). While plasticity levels were shown to be minimal in mainland WA populations of *N. s. occidentalis* (Aubret et al. 2004, 2009. *op. cit.*), they were not nil. We suggest that occasional feeding opportunity of large prey items such as young quendas or possums may foster the maintenance of minimal levels of adaptive plasticity in swallowing performances in mainland *N. s. occidentalis*. Island colonisation, or introduction, may then generate bottlenecks and increase plasticity levels to significant levels, as was previously described in several *N. scutatus* populations across Australia (Aubret et al. 2004, 2009 *op. cit.*; Aubret 2015. Heredity 115:349-356).

Fig. A5.1 *Notechis scutatus occidentalis* regurgitating a young quenda (*Isoodon fusciventer*) characterised by its feet.

Appendix 6. *Notechis scutatus occidentalis* (Western tiger snake). Reproduction/Unfertilised ova post-parturition.

The study presented in Appendix 6 was published in the peer-reviewed journal '*Herpetological Review*' on December 2020.

Cornelis, J., **Lettoof, D. C.** (2020). *Notechis scutatus occidentalis* (Western tiger snake). Reproduction/Unfertilised ova post-parturition. *Herpetol Review*. 51: 4 (873).

At 1122 h on 8 May 2020, at Yanchep National Park, Western Australia, Australia (31.54727°S, 115.68182°E; WGS 84), we encountered an adult female Notechis scutatus occidentalis in a basking position and beginning to shed. An unfertilized ovum lay beside the snake's tail, presumably passed post-parturition (Fig. 1). The snake was identified as an individual being monitored in an on-going study that was normally in very good body condition; the snake had been released in April 2019 after spending six months in captivity and had not been recaptured since (D. Lettoof, *unpubl*.). However, at the time of encounter the snake appeared relatively emaciated, suggesting the snake has recently given birth (Naulleau and Bonnet 1996. Oecologia 107:301–306).

Notechis scutatus have been recorded breeding throughout the year (Shine 1977. Aust. J. Zool. 25:647–653), although spring and early summer (October–December) tends to be the main breeding season for live-bearing Australian elapids, and parturition occurs at the end of summer (February–April; Shine 1977. Aust. J. Zool. 25:655–666). In captivity, snakes depositing unfertilized ova during parturition is a common observation (Lourdais et al. 2005. Biol. J. Linn. Soc. 84:767–774; Madsen et al. 1992. Nature 355:440–441; Aldridge et al. 2008. In Hayes et al. [eds.], The Biology of Rattlesnakes, pp. 403–412. Loma Linda University Press, Loma Linda, California); however, we believe this is the first time this phenomenon has been observed in a wild N. scutatus and potentially the first record for a snake in the wild.

Fig. A6.1 *Notechis scutatus occidentalis* unfertilized ovum from Western Australia.

Appendix 7. *Notechis scutatus occidentalis* (Western tiger snake). Defensive behaviour.

The study presented in Appendix 7 was published in the peer-reviewed journal '*Herpetological Review*' on September 2020.

Cornelis, J., **Lettoof, D. C.** (2020). *Notechis scutatus occidentalis* (Western tiger snake). Defensive behaviour. *Herpetol Review*. 51: 3 (70-71).

Notechis scutatus occidentalis is a large, polymorphic elapid found in a variety of habitats across south-western Australia, but it is particularly abundant in wetlands (Mirtschin et al. 2017. Australia's Dangerous Snakes: Identification, Biology and Envenoming. CSIRO Publishing, Clayton, Australia. 100 pp.) due to its preference for feeding on frogs (Aubret et al. 2006. Behav. Ecol. 17:716–725; Lettoof et al. 2020. IJP:PAW 11:32–39). Although N. s. occidentalis is mainly terrestrial, individuals are known to occasionally forage in the aquatic environment (Aubret and Shine 2008. Am. Nat. 171:524–531). Across their range *N. scutatus* have also been observed fleeing into the water (Mirtschin and Bailey 1990. SA Nat. 64:53–61) and swimming underwater has been recognized as a behaviour to escape predation (Aubret 2004. Aust. J. Zool. 52:357–368). Aubret (2004, op. cit.) reported that under laboratory conditions, *N. s. occidentalis* from the urban wet-land Herdsman Lake, Western Australia, Australia, can hold their breath for over 20 min and suggested this could be advantageous to escape predation.

At 1249 h on 23 September 2019, at Herdsman Lake (31.92023°S, 115.80448°E; WGS 84), we released a small (72.3 cm SVL) adult *N. s. occidentalis* after recording morphometric data, and observed it fleeing into the water. We have released hundreds of *N. s. occidentalis* and the usual response is to hide in vegetation, but occasionally some will choose the water and swim away. This individual swam straight down to the bottom of the water (ca. 50 cm deep) and proceeded to hide under debris. We remained quiet and motionless while recording the time the snake spent under the debris. After a total of 18 min 36 s the snake surfaced and took its first breath of air (Fig. A7.1), this is not incorporating a ca. 2 min delay before we initiated the

recording. This is not the longest apnoea record for the species (Aubret 2004, op. cit.), but to our knowledge it is the first known in situ record of apnoea, as well as the first record of a wild *N. s. occidentalis* selecting to hide beneath submerged debris for an extended period of time as opposed to swimming away and resurfacing, as an antipredator behaviour.

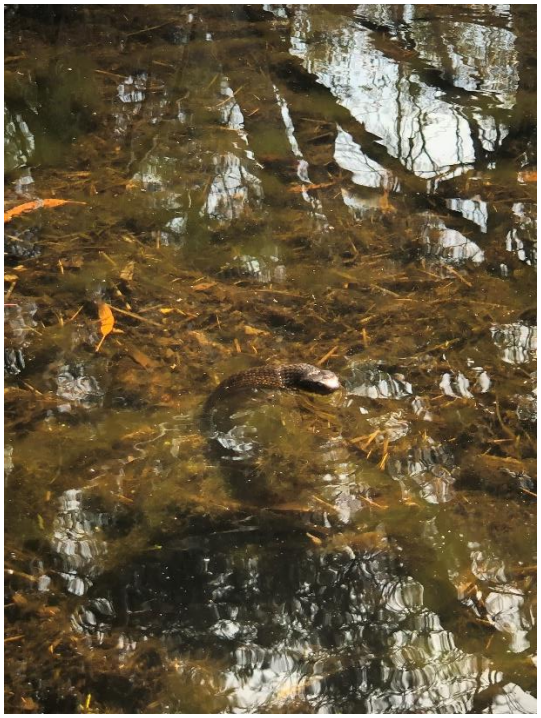

Fig. A7.1 *Notechis scutatus occidentalis* surfacing to breathe with the rest of the body still hidden under debris.

Appendix 8. First record of predation of a hatchling turtle by the Western tiger snake (*Notechis scutatus occidentalis*).

The study presented in Appendix 8 was published in the peer-reviewed journal '*Australian Zoologist*' on June 2021.

Lettoof, D. C., Santoro, A., Swinstead, C. VL., Cornelis, J. (2021). First record of predation of a hatchling turtle by the Western tiger snake (*Notechis scutatus occidentalis*). *Aust. Zool.* 11: 3. doi: 10.7882/az.2021.017.

A8.1 Abstract

Snake-Turtle interactions have been rarely documented. We recorded a hatchling *Chelodina oblonga* within the stomach contents of a Western tiger snake (*Notechis scutatus occidentalis*). This is the first recorded observation of an interaction between snakes and hatchling freshwater turtles in Western Australia. Field based palpitation failed to detect the hatchling, suggesting that without dissection, turtle hatchling predation by snakes more generally could be higher than commonly reported. Snake predation of hatchlings could be placing additional pressure on threatened populations of freshwater turtles in Australia, warranting further investigation.

A8.2 Introduction

Freshwater turtles are imperilled globally (Stanford et al. 2020) being among the most endangered vertebrate groups, with approximately 61% of species at risk of extinction or already extinct (Lovich et al. 2018). Australian freshwater turtles are in a similar position with approximately half of the 25 extant species currently listed as vulnerable, endangered, or critically endangered, with declines documented in multiple species in recent decades (Santoro et al. 2020; Spencer et al. 2018; Van Dyke et al. 2019). Causes of decline include mortality of nesting females through predation (Spencer 2002; Thompson 1983) and wildlife-vehicle incidents (Santori et al. 2018; Spencer 2002), destruction of nests through predation (Giles et al. 2008; Spencer 2002; Thompson 1983), habitat decline (Santoro et al. 2020) and novel diseases (Spencer et al. 2018).

Freshwater turtles rely on both the aquatic and terrestrial habitats of wetlands to complete their life cycles (Burke et al. 2000). While many species are either apex predators or high in the food chain in the aquatic environment, once in the terrestrial environment, turtles are highly vulnerable to predation (Segura et al. 2020; Spencer and Thompson 2003; Tucker and Janzen 1999). Freshwater turtle life histories are characterised by long generation times, high adult survivorship, late age maturation, low annual reproductive effort, as well as low and variable recruitment (Congdon et al. 1993; Iverson 1991; Spencer 2018). Therefore, the survival of adults is more influential on population persistence than any other stage (Heppell 1998); however, given the increased predation pressure placed on both adults and nests by invasive species in Australia (e.g. red foxes *Vulpes vulpes* (Giles et al. 2008; Spencer 2002; Thompson 1983)), understanding the threats to the survival and recruitment of hatchlings into populations is more critical than ever.

On the Swan Coastal Plain in south-west Western Australia, the Oblong turtle (*Chelodina oblonga*) persists in a number of urban wetlands (Santoro et al. 2020); however, relative abundances in most wetlands are alarmingly low. In addition, the latter study revealed that adults made up ~90% of captures and juveniles were absent from captures at ~40% of wetlands. Known predators of *C. oblonga* include foxes (*V. vulpes*) (Dawson et al. 2016), the Australian raven (*Corvus coronoides*; AS *pers. obs.*), Australian white ibis (*Threskiornis molucca*; JC *pers. obs.*) cormorants (*Phalacrocoracidae*) and White-necked herons (*Ardea pacifica*; (Cann and Sadlier 2017). A top predator of wetlands, Western tiger snakes (*Notechis scutatus occidentalis*), are abundant in at least seven of the wetlands surveyed by Santoro et al. (2020; DL and AS *pers. obs.*), yet the predation pressure by snakes is not understood in Australia, nor globally.

Across their Southern and Eastern Australian range, tiger snakes collectively show a generalist diet with a high degree of plasticity based on the population. For example, mainland tiger snakes found around wetlands dominantly eat frogs, and occasionally small birds, mammals and lizards (Lettoof et al. 2020; Shine 1987), island populations can specialise in only bird chicks or lizards (Aubret et al. 2004; Schwaner 1991), and Tasmanian tiger snakes have a large proportion of mammals in their diet (Fearn et al. 2012). Interestingly, Western tiger snake hatchlings from an island with only bird and lizards available have been shown to still preference the scent of frogs and mice

(Aubret et al. 2006). A summary of all known tiger snake diet records in Greer (2020) indicates that the most commonly detected dietary taxa is frogs. Snake-turtle predation events are seemingly rare globally, and within Australia; here we present the first known record of an Oblong turtle hatchling predation by a snake.

A8.3 Observation

On 17/09/20 an adult female Western tiger snake was hand caught from fringing vegetation at Bibra Lake, Perth, Western Australia (Figure A8.1; 32° 5′ 32 S, 115° 49′ 27 E) by DL and JC, and euthanised as part of an ongoing research project (Western Australia's Department of Biodiversity, Conservation and Attractions Permit No. FO25000149; Curtin University Animal Research Ethics Committee Approval No. ARE2020-15). Prior to euthanasia the snake was measured (Snout-vent length: 667 mm, body mass: 147.5 g), assessed for external condition and ectoparasites, and gently palpated for food items. Palpation of the stomach revealed the feet of a motorbike frog (*Litoria moorei*), and a large stomach burden of *Ophidascaris pyrrhus* nematodes. The snake was dissected on 27/10/2020 by DL and CS to assess endoparasite abundance and diet. Dissecting the stomach revealed the back legs and half-digested body of the motorbike frog, entangled and being fed on by nematodes. Beyond the nematode colony, at the posterior end of the stomach, was a hatchling long-necked turtle (Figure A8.2). The turtle was 55 mm total length, 25 mm carapace length, and 2.8 g body weight, and there was no evidence of feeding from the nematodes.

Fig. A8.1 (A) a Western tiger snake (Notechis scutatus occidentalis) from Bibra Lake, and (B) an example of a bank at Bibra Lake where Oblong turtles nest and tiger snakes shelter in fringing vegetation.

Fig. A8.2 The hatchling oblong turtle (Chelodina oblonga) found inside the Western tiger snake's (*Notechis scutatus occidentalis*) stomach.

A8.4 Discussion

Predation of freshwater turtle hatchlings by snakes has rarely been recorded in Australia. We could only find a single record of such an occurrence, which was the observation of a *Emydura krefftii* turtle hatchling being predated by a Mulga snake (*Pseudechis australis*) (Cann 1978). There has been a further observation of a dugite (*Pseudonaja affinis affinis)* attempting to a swallow an adult *C. oblonga* but could not get past the carapace (Maryan and Gaikhorst 2005). In the United States Eastern Diamondback rattlesnakes (*Crotalus adamanteus*), indigo snakes (*Drymarchon corais*) and Eastern coachwhips (*Masticophis flagellum*) are known to predate on young gopher tortoises (Butler and Sowell 1996; Perez-Heydrich et al. 2012), and larger African snakes have been found predating on small hingeback tortoises (*Kinixys spekii*) (Coulson and Hailey 2001). Nevertheless, snakes do not seem to be a common predator of turtles, as many Australian snakes do not forage in the water and the carapace is too large for a snake to swallow.

The turtle hatchling may have been scavenged, as hatchling turtles can die from desiccation while migrating (Janzen et al. 2000; Kolbe and Janzen 2002; Paterson et al. 2012), and tiger snakes are known to eat carrion (Aubret et al. 2005; Fearn 1993).

However, Oblong turtle nest are usually within 500m of the water's edge (Clay 1981), and a large portion the Bibra Lake population nests among fringing vegetation (Figure A8.1). We find it unlikely that a hatchling would have died due to desiccation with such close proximity to the lake.

Due to their small size, research on the aquatic movements and behaviour of freshwater turtle hatchlings is scarce. Rosenberg and Swift (2013) reported that hatchling western pond turtles (*Actinemys marmorata*) were always located in areas with woody debris and dense submerged vegetation, were within 1 m of the shore, and moved up to 38 m between observations (~1-3 days). When AS has observed hatchling *C. oblonga* in aquatic environments, they show similar behavioural traits, and can swim relatively quickly. We suggest a hatchling within the aquatic environment would be relatively difficult for a foraging snake to capture. There is significant overlap between peak movement periods of hatchling oblong turtle and Western tiger snakes. Oblong turtle hatchlings are recorded as emerging from their nests and migrating to the nearest waterbody between May and August (Burbidge 1967; Clay 1981), however AS has observed hatchlings emerging in late September in 2019 and 2020. Western tiger snakes in Perth show peak abundance in between August and September (DL and JC *pers. obs.*, surveying from August through to April) as they begin mating season. Therefore, we suspect the hatchling *C. oblonga* must have been predated on land or the shallow waters edge and was only swallowed because of its small size.

Chelodina oblonga populations are believed to be declining through a lack of recruitment (Santoro et al. 2020). Further research into *C. oblonga* populations currently occurring at wetlands within the Beeliar Regional Park by AS has revealed that *C. oblonga* populations face significant threats from terrestrial predation of nesting females and eggs by introduced species (such as red foxes (*Vulpes vulpes*)) and domestic dogs (*Canis familiaris*). Anecdotal reports have also identified the laughing kookaburra (*Dacelo novaeguineae*) as a predator of hatchling turtles (AS and DL, *pers. obs.*). Native species such as the Australian Raven (*Corvus coronoides*) have been identified as significant predators and observed attacking and killing nesting females, and both ravens and quenda (*Isoodon fusciventer*) have been observed destroying nests. Currently, aspects of the early life history of the oblong turtle such as survivorship rates of hatchlings and the relative impact of known predators remains unknown. However, observations from tracking hatchlings with UV powders during

migration to waterbodies after emergence from nests allude to high rates of suspected avian predation (AS *pers. obs.*). The finding of a hatchling oblong turtle within the stomach contents of a Western tiger snake suggests that they could be another native predator that is reducing the recruitment of juveniles to the population.

Traditionally, snake diet is assessed by manual palpation of prey items on live animals, or dissection of carcasses (Dorcas and Willson 2009). Both methods have limitations, for example: easily digestible soft-bodied prey such as amphibians can be underrepresented in museum snake specimens (Glaudas et al. 2017), and small prey items may be more difficult to detect by palpation (Dorcas and Willson 2009). The turtle hatchling was not detected during palpitation in the field, rather by dissection of the stomach. Although small prey items may be difficult to detect via palpation we regularly palpate metamorph frogs (DL and JC, *pers. obs.*), and rather dietary unknowns are usually a result of Western tiger snakes' common infection with gastric nematodes. Nematode colonies are attached to the stomach wall (Lettoof et al. 2020) and often entangle prey items when feeding; thus, prey can often be felt through palpation but cannot be removed. Specifically, near 500 tiger snakes have been palpated in the field by DL and JC, and in 43% of snakes with prey items cannot have them removed. We propose predation of hatchling turtles may be higher than detectable without dissection, especially in species with gastric nematodes, and may warrant further investigation.

A8.5 References

Every reasonable effort has been made to acknowledge the owners of the copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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