Utility of the Little Penguin (*Eudyptula minor*) as a bioindicator of coastal metal pollution

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"We live on the veranda of the world's greatest island and it's our birthright to have a clean ocean, to catch a feed, to interact with nature.

And, like any birthright, we have to safeguard it."

— Tim Winton, ABC interview 2012

"*Felix believed that the answer to every problem involved penguins; but it wasn't fair to birds, and I was getting tired of teleporting them back home. Somewhere in Antarctica, a whole flock of Magellanic penguins were undergoing psychotherapy.*"

— Rick Riordan, The Throne of Fire

"Eudyptula minor" - song written by Kiana Day (age 12)

- Verse 1-
Fish, squid and krill - that's what they all kill,
'cause they're the kings of the sea
(not including you and me, and of course the sharks!).

- Chorus -
Eudyptula minor - you're not small to me,
you're the king of the sea and if you disagree,
I can prove it to you - just listen!

- Verse 2-
Who is so small, but packs a fight?
Who can kill fish with just one bite?
Who lives on our doorstep and just won't mind?

- Chorus -
Eudyptula minor - you're not small to me,
you're the king of the sea and if you disagree,
I can prove it to you - just listen!

- Verse 3-
Some people say you can't fly,
but through the waters you glide.
and there's no more majestic sight -
Eudyptula minor!

Summary

Trace metals are present in all aquatic systems, originating both from natural and anthropogenic sources. With concerns over the environmental impacts of metals, particularly in semi-enclosed aquatic systems such as the Port Phillip Bay, Victoria, Australia, it is important to be able to monitor the degree of pollutant exposure to local organisms. Little Penguins (*Eudyptula minor*) nesting at St Kilda, only 3 km from the centre of the 3.3 million people metropolis of Melbourne, are a potentially useful bioindicator species for toxicant exposure within Port Phillip Bay because they are resident and feed exclusively within the bay all year around. This study investigated metal and metalloid concentrations in Little Penguins at St Kilda and two other locations with different levels of anthropogenic impact: the penguin parade at Phillip Island and the remote off-shore Notch Island.

Non-essential metal and metalloid concentrations in the blood and feathers of Little Penguins during the moult season 2012 were strongly linked to the level of industrialisation adjacent to the respective foraging zones. This trend was more distinct in blood than moulted feathers. Metal and metalloid concentrations in penguin feathers, while potentially biased in this study by external contamination, are a low-impact sampling method, and correlated with whole blood concentrations of mercury, lead and iron. Notably, penguins from the semi-rural Phillip Island colony contained metal concentrations closer to those found in penguins from the remote Notch Island colony, and significantly different from those found in St Kilda's penguins. This indicated that Phillip Island may be a suitable reference colony for studies investigating

long-term variation and trends of metal and metalloid pollution within Port Phillip Bay.

Next, 157 blood samples were collected from Little Penguins at St Kilda and Phillip Island, over three and two years, respectively, and during three distinct annual seasons (breeding, moulting and non-breeding). Mean blood metal and metalloid concentrations at St Kilda and Phillip Island differed between years and seasons, but were predominantly influenced by location. St Kilda penguins' mean blood non-essential metal and metalloid concentrations were significantly higher than those found in Phillip Island penguins. Little Penguin body mass and flipper length also differed between the two colonies sampled, with penguins at St Kilda being on average heavier but having shorter flippers. Interestingly, mean blood mercury concentrations showed a negative correlation with penguin flipper lengths. An investigation of blood metal and metalloid concentrations during the different stages of moult provided novel insights into the complex mechanism of non-essential metal mobilisation from internal organs and depuration into new feathers. Most notably, mean mercury concentrations in the blood of St Kilda Little Penguins exhibited an increasing trend over the three years of sampling, whilst decreasing in penguins at the Phillip Island colony over part of that time frame. These variations may reflect differences in temporal metal bioaccumulation or seasonal exposure through prey.

To elucidate the link between predator body burden and prey, Little Penguin primary fish prey items were sampled within weeks of penguin blood and faeces collection. This allowed for the comparison of the relative composition of arsenic, mercury, lead and selenium in those matrices.

Mercury concentrations were highest in the blood, confirming the propensity of this pervasive toxicant to biomagnify in marine trophic chains. Fish and faecal samples contained similar concentrations of arsenic and lead, suggesting faeces as a primary route of detoxification in Little Penguins for those elements. Metal and metalloid concentrations in the sampled fish were generally within the expected limits, except for arsenic and mercury, which were higher than reported elsewhere. Mercury was highest in Australian anchovy (*Engraulis australis*), which is a primary prey species for St Kilda's Little Penguins. This raises concern for the welfare of this urban dweller, as chronic exposure to even moderate levels of mercury may contribute to sub-lethal effects at a population level. Correlations between paired penguin blood and faecal samples were only significant for selenium, excluding faecal samples as an appropriate sampling matrix for any other element. A preliminary investigation of paired stable isotope ratio and mercury data in penguin blood indicated that observed temporal changes in penguin blood mercury concentrations were not exclusively linked to penguins' trophic changes in their diet. This suggests that there are other sources of variation of bioavailable mercury within Port Phillip Bay, possibly linked to the bay's recent undergoing of capital dredging activities.

The findings presented in this thesis establish the Little Penguin as an ideal species for the monitoring of contamination in Port Phillip Bay. The results highlighted provide rare baseline data for bioavailable metals and metalloids within the bay and a non-destructive and effective multi-matrix method to monitor a potentially negative pollution impact through trophic exposure. In particular, the temporal trends observed for mercury warrant

long-term surveying in this resident seabird, to ensure the penguins' continued health, conservation and management, as well as to inform future environmental impact investigations.

Declaration

"I, Annett Finger, declare that the declare that the PhD thesis by Publication titled 'Utility of the Little Penguin (*Eudyptula minor*) as a bioindicator of coastal metal pollution' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work."

Signature: Date: 3rd of November 2016

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
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Declaration by [candidate name]: **Signature:** [redacted] **Date:** 3/11/2016

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Details of publications and conference presentations

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Interviews / Media / Internet:

- Victoria University Press Release, 2015, "Little penguin mercury levels echo bay health", https://www.vu.edu.au/news-events/news/little-penguin-mercury-levels-echo-bay-health

- ABC radio interview, 2015, "Little penguin mercury levels reflect health of the bay"

- The Port Phillip Leader, 2015, "High levels of mercury, arsenic and lead found in St Kilda penguins", http://www.heraldsun.com.au/leader/inner-south/high-levels-of-mercury-arsenic-and-lead-found-in-st-kilda-penguins/news-story/7c27726f2744b6e0888eb446e193f8ba#load-story-comments

- Dredging Today, 2015, "Hg Levels Reflect Health of Bay, http://www.dredgingtoday.com/2015/11/09/mercury-levels-reflect-health-of-bay/

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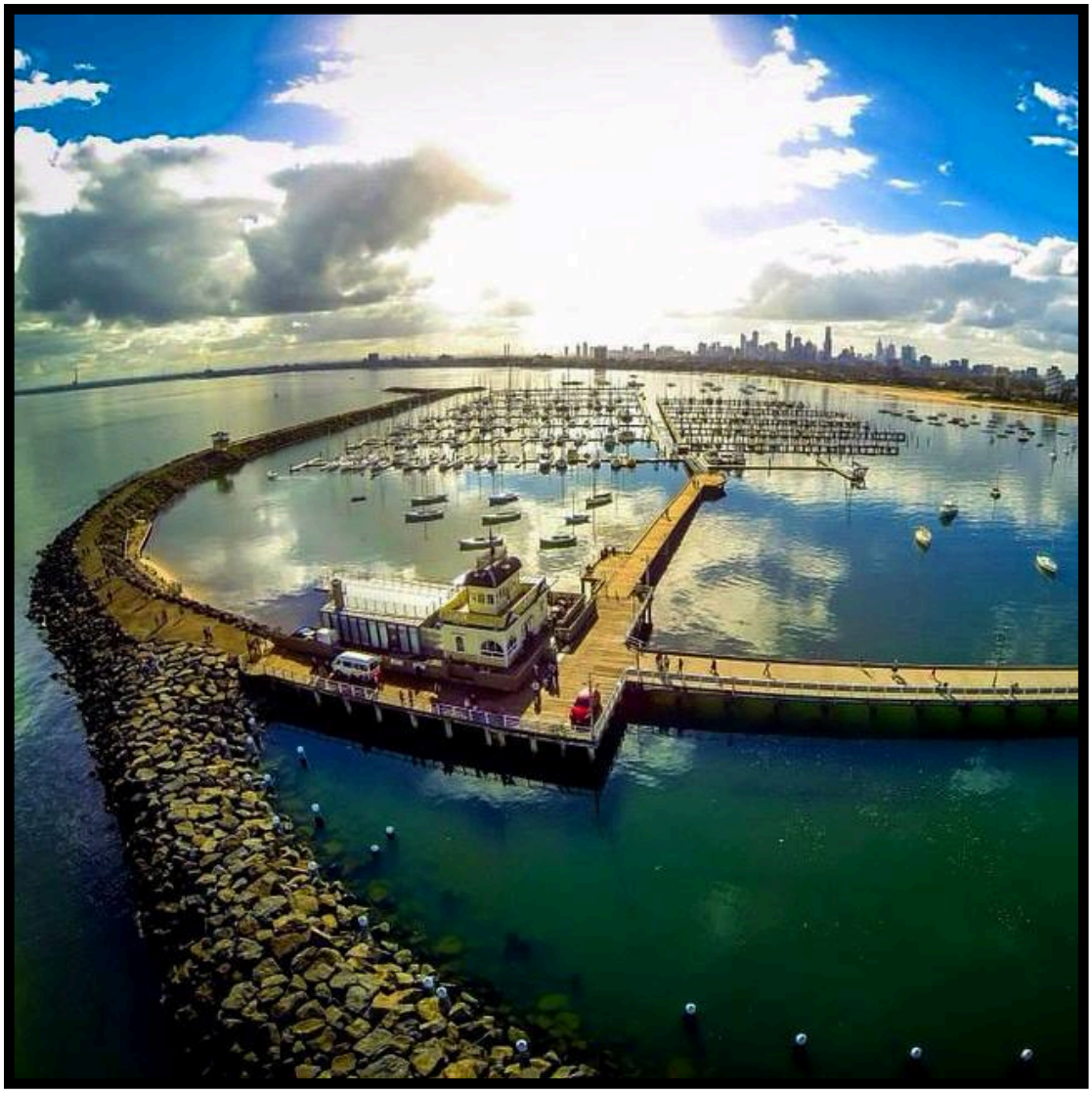
Chapter 1

General introduction



St Kilda breakwater with Melbourne CBD in the background
(Photo source: www.snapabove.com)

1. General introduction

1.1. Aquatic toxicology

The ocean acts as a sink for anthropogenic compounds and elements. Metals occur naturally in the ocean, but are also frequent waste products of industrial processes and due to their high molecular weights, often accumulate in marine sediments. Since the industrial revolution there has been a steady increase in metals and other pollutants in the ocean. For instance, mercury (Hg) levels in surface waters have increased three-fold since pre-anthropogenic times (Lamborg *et al.*, 2014). In Australia, more so than in many other countries, human settlements are concentrated along the coast. About 80% of Australians reside near the sea (Harvey & Woodroffe, 2008). Human and industrial wastes contribute to the majority of chemicals released into the marine environment. In this regard, coastal areas with tributaries near large human settlements, mining or farming operations are most affected by pollution (Long *et al.*, 1995; Islam & Tanaka, 2004).

Through wave action and microbial processes, these pollutants may become bioavailable and be transported up the food chain (trophic transfer) (Neff, 2002). Biomagnification occurs when the concentration of an element increases from one trophic level to another (Cui *et al.*, 2011). As a result, high-level trophic feeders such as sharks and piscivorous seabirds are most at risk of experiencing adverse effects (Phillips, 1977; Scheuhammer, 1987). However, this also makes them excellent candidates for biomonitoring the toxic levels in the marine environment (Monteiro & Furness, 1995; Becker, 2003). To paraphrase Paracelcus (1493 - 1541), "*All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a*

thing poison", meaning any chemical element can be deleterious to an organism if it exceeds a certain threshold of concentration. This is referred to as the 'window of essentiality" (Fränzle *&* Markert, 2007). Monitoring pollution levels in high trophic orders is important with respect to the conservation of species and ecosystems, as well as to human health.

Many studies reporting on metals in seabirds aim to establish a baseline and determine adverse effect levels for the most toxic elements, such as Hg, lead (Pb) and cadmium (Cd) (Burger, 1993; Thompson *&* Hamer, 2000; Becker, 2003). As seabirds are often prevalent in large numbers, many studies have utilised the carcasses of animals that have died or have sacrificed animals to determine metal and other concentrations in internal tissues (Honda *et al.*, 1986; Savinov *et al.*, 2003; Smichowski *et al.*, 2006). The problem with using carcasses is that it is often not known where they come from or what is the underlying cause of death. Thus these samples do not represent a random cross-section of the study population and cannot easily be used to draw conclusions for that population. Sacrificing animals, on the other hand, presents ethical issues and cannot be used in good conscience for species of moderate or high conservation concern or public interest. Increasingly, non-destructive (to the animal) methods are being used to assay metal concentrations in seabirds. For example, seabird feathers, collected non-destructively, are often used to determine metal concentrations (Burger, 1993). Metal concentrations in moulted feathers represent the concentration of metals present in the blood at the time the feather was grown, which in turn, is thought to be a combination of what was ingested in the weeks leading up to the moult and what was mobilized from internal

organs (liver, kidney, fat and muscle) during the moult fast (Furness *et al.*, 1986; Bearhop *et al.*, 2000). As such, feathers represent long-term indicators of exposure. However, external contamination is a common, and often considerable, bias in feather analysis (Dauwe *et al.*, 2003; Jaspers *et al.*, 2004).

Chemical analysis of inorganic components in organic samples progressed dramatically in the 1980s and 90s as advances in analytical instrumentation were made. For example, highly accurate trace metal analysis such as atomic absorption spectrometry (AAS) and inductively-coupled plasma spectrometry (ICP) promoted non-destructive cross-sectional sampling, e.g. the taking of a small sample of blood (~0.5 mL) from a free-ranging bird without sacrificing it (Becker, 2003). These advances enabled a series of new studies, using non-destructive sampling. Blood, in particular, is a sampling matrix that is of ecological importance to monitor. The successes of such sampling techniques and instrumental analysis are evident in the literature (e.g. ospreys: DesGranges *et al.*, 1998; oystercatchers: Thompson *&* Dowding, 1999; sea ducks: Wayland *et al.* 2008).

In a comprehensive study that established the validity of non-destructive sampling matrices, Eagles-Smith *et al.* (2008) measured Hg in blood, feathers and internal tissues of four species of waterbirds (American avocets [*Recurvirostra americana*], black-necked stilts [*Himantopus mexicanus*], Caspian terns [*Hydroprogne caspia*], and Forster's terns [*Sterna forsteri*]) in San Francisco Bay, California, USA. The authors destructively sampled in large numbers (n = 20 to 100), to be able to calculate robust conversion factors between blood, liver, kidney, muscle and feathers, and

presented robust equations for converting Hg between tissues. Importantly, Eagles-Smith *et al.* (2008) concluded that blood, more so than feathers, accurately approximated internal tissue contaminant concentrations.

In a terrestrial study, small passerine birds were investigated for heavy metals along a pollution gradient in Belgium using only blood and feathers, collected non-destructively (Geens *et al.*, 2010). The authors found blood to be a more reliable and consistent sampling matrix to detect geographical differences in local environmental pollution than feathers. Blood represents dietary exposure over a relatively short window (Monteiro & Furness, 2001) and enables seasonal changes to be considered. Because blood can be collected non-destructively, it can be combined with other related directions of enquiry and thus provide insights into the wider influence of contamination. In some cases, it enables replicate measurements on individuals, recording of behavioural effects or measures of breeding success, which can give insights into the population-wide impact of contaminants. For example, recent published papers have been able to establish a direct link between Hg concentrations in birds and the effect on population levels, such as black-legged kittiwakes (*Rissa tridactyla*) skipping breeding (Tartu *et al.*, 2013), reduced population growth in common loons (*Gavia immer*) (Schoch *et al.*, 2014), the breeding success of two species of skuas (*Catharacta* sp.) (Goutte *et al.*, 2014), the size of eggs of little auks (*Alle alle*) (Fort *et al.*, 2014) and higher incidences of egg neglect behaviour in snow petrels (*Pagodroma nivea*) (Tartu *et al.*, 2015).

1.2. Penguins as sentinels

Penguins (Family Spheniscidae) are flightless seabirds that, by the grace of their numbers, prevalence and foraging ecology have been coined "Marine Sentinels" (Boersma, 2008). They profess a sensitivity to their environment, which when observed and studied, can provide invaluable insights into variations of regional oceanographic conditions. We live in a time of unprecedented changes in our oceans. Increasing fishing pressures, climate change and pollution have caused population responses in a number of penguin and other seabird species (Croxall *et al.*, 2002; Boersma, 2008; Grémillet & Boulinier, 2009). Penguins forage at sea but conduct moulting and breeding on land, and are exposed to anthropogenic disturbances on both fronts. Of the 18 penguin species, three are classified as "least of concern", five are considered "near threatened", five are "vulnerable" and the remaining five are listed as "endangered" on the IUCN Red List (www.iucnredlist.org, accessed 24/09/2016).

The Little Penguin (*Eudyptula minor*)*,* the smallest of the penguins, is a temperate species that is considered common, but has experienced population declines within its range (Dann *et al.*, 1992; Norman *et al.*, 1992; Stevenson *&* Woehler, 2007). Being an inshore species with a relatively small home range (Collins *et al.*, 1999; McCutcheon *et al.*, 2011) makes it easier for researchers to interpret the analysis of results and to pinpoint these to local contamination sources. Unlike most other seabirds, penguins undergo a 'catastrophic' moult once a year whereby they replace all their plumage while fasting on land (Reilly *&* Cullen, 1983). For ecotoxicological research, this has the advantage of being able to sample feathers from a large number of

moulting penguins and to utilise any feather on their body. Surprisingly, few such studies have been published (Muirhead & Furness, 1988; Brasso *et al.*, 2015; Carravieri *et al.*, 2016). Some toxicological research on penguins has investigated feathers and internal tissues from sacrificed or accidental kills (e.g. Szefer *et al.*, 1993; Ancora *et al.*, 2002; Jerez *et al.*, 2013). Notably, the research published as an outcome of this thesis represents the first reports of metals in blood for any penguin species.

Only a limited number of toxicological studies on the Little Penguin has been carried out to date (Table 1.1). Lock *et al.* (1992) reported on metals in liver, kidney and feathers of a small sample of Little Penguins found washed up on a beach, as part of an extensive study on seabirds in New Zealand. Gibbs (1995) investigated metals and organic pollutants in internal tissues of a large sample of Little Penguins at several locations. However, the samples differed in the way they were collected. Some were fox kills, some were washed-up carcasses of unknown origin and some were birds that died of various causes (e.g. heat exhaustion, starvation). The results, although biased by such sampling variation, indicate that the concentrations of some toxicants (e.g. cadmium in kidney and mercury in the liver) of these Little Penguins are higher than those reported for penguins from sub-Antarctic and Antarctic regions (Szefer *et al.*, 1993) but lower than those reported for other seabirds in the northern hemisphere (Walsh, 1990). Choong *et al.* (2007) measured a number of metals in muscle and liver in Little Penguins killed by foxes; two from Middle Island and three from Phillip Island, Table1.

Table 1.1: Concentrations of metals and metalloids in tissues of adult Little Penguins, *Eudyptula minor*. All results are expressed as mg/kg wet weight, except where indicated with * which represents mg/kg dry weight. Absence of data is shown as '-'. <ML represents a value determined that was below method detection limit.

Region, Year	Tissue	n	Cu	Cr	Fe	Zn	As	Cd	Hg	Pb	Source	Method
Phillip Island, 2013	Feather	19	-	-	-	-	-	-	2.00*	-	Brasso *et al.*, 2015	Collected at moult
St Kilda, 2008	Feather	18	-	-	-	-	-	-	5.01*	-	S. Caarels, unpublished data	Collected at moult
Middle Isl, 2005	Muscle	2	2.95	0.79	125	9.8	1.44	0.99	0.55	0.07	Choong *et al.* 2007	Fox kill
Middle Isl, 2005	Liver	2	6.0	0.2	210	36.4	0.93	1.7	0.68	<ML	Choong *et al.* 2007	Fox kill
Phillip Isl, 2005	Muscle	3	2.7	0.04	220	10.2	0.91	0.07	0.08	<ML	Choong *et al.* 2007	Fox kill
Phillip Isl, 2005	Liver	3	5.9	0.06	885	42.5	1.5	1.65	1.38	<ML	Choong *et al.* 2007	Fox kill
Phillip Isl, <1994	Liver	40	6.8	0.4	-	38	1.2	1.25	1.2	<ML	Gibbs 1995	Fox, road kill
Sydney, <1994	Liver	34	8.7	0.3	-	47	1.8	0.8	1.3	<ML	Gibbs 1995	Sick, starved
Bowen Isl, <1994	Liver	19	10.3	0.6	-	54	1.5	1.4	0.75	<ML	Gibbs 1995	Sick, starved
NZ, 1970s	Liver	6	6.4	-	-	57.5	-	2.9	1.4	-	Lock *et al.* 1992	Washed up
NZ, 1970s	Kidney	7	-	-	-	-	-	11.2	-	-	Lock *et al.* 1992	Washed up
NZ, 1970s	Feather	5	29.4	-	-	99.2	-	0.4	3.4	1.7*	Lock *et al.* 1992	Washed up

More recently, Brasso *et al.* (2015) reported on Hg in feathers of ten species of penguins, collected at varying sites for each species, including Little Penguins at St Kilda and Phillip Island, Table 1.1.

1.3. Port Phillip Bay

Port Phillip Bay is located in Victoria, Australia, encased by the 4 million people metropolis of Melbourne (ABS, 2011, Figure 1.1). The bay is relatively shallow (mean depth of 13.6 m), with a shoreline of approximately 264 km and encompasses an area of 1900 km^2 that connects to Bass Strait via a narrow 3 km channel (Sampson *et al.*, 2014). Due to the semi-enclosed nature of Port Phillip Bay, wave action is restricted and its water takes approximately one year to be replenished (EPA, 2011). Several rivers and creeks feed into the bay and two large wastewater treatment plants exist to service the population of Melbourne. The Yarra River, the most significant river flowing into Port Phillip Bay, is 242 km long and has about 50 tributaries feeding into it (Sampson *et al.*, 2014). Some of the waters and sediments of the tributaries contain historical contamination from the late 19^{th} century when gold mining and other industries occurred in the upper regions of the Yarra and other rivers (Harris *et al.*, 1996). An increase of industry and population in the 1970s and 80s caused a marked deterioration of water quality. A major fish kill occurred in 1984 (Gibbs *et al.*, 1986) and concerns were raised for the health status of Port Phillip Bay when Phillips *et al.* (1992) reported elevated concentrations of Cd and Hg, among other metals, in fish and biota. This led to the implementation of stricter controls and regulations for the disposal of industrial effluents into the bay (Harris *et al.*, 1996). A comprehensive

environmental impact study in the late 1990s, designed and managed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and funded by Melbourne Water and Melbourne Parks and Waterways, included measurements of contaminant concentrations in water, soil and biota throughout the bay (Harris *et al.*, 1996). The results showed that despite high annual inputs of metals and organic pollutants into Port Phillip Bay, with the Yarra River contributing more than half, concentrations were not excessive and well within accepted water quality guidelines (Fabris *et al.*, 1999). Some sediment near the shipping port areas, the Yarra River, as well as near the main industrial area, Corio Bay, still contained excessive levels of some metals (Fabris *et al.*, 1999). Localised high concentrations were found in fish, mussels and other biota collected near the point sources (Fabris, 1995) but, overall, the study did not detect any significant effects from the present nutrient and toxicant loads, and the ecology of Port Phillip Bay was assessed as "still healthy" (Harris *et al.*, 1996). The study recommended five-yearly monitoring of toxicants in sediments and "ecosystem components", to look for "signs of biomagnification and test for low-level chronic effects" (Harris *et al.*, 1996).

Port Phillip Bay is Australia's largest shipping port and underwent substantial dredging works to deepen its shipping channels in 2008 and 2009 (PoMC, 2010). A total of 22.97 million m^3 of marine sediments, including 1.39 million m^3 of contaminated soft silts and clays from the Yarra River and Hobsons Bay, were dredged up (PoMC, 2010). The environmental impact statement prepared prior to the channel deepening considered the impact of highly contaminated particles being disturbed, re-suspended and thus,

entering the food chain as "unlikely to have ecological consequences", and further stated "no coastal ecosystem will be affected by any contamination bound to sediments" (PoMC, 2004).

A suite of nine baywide monitoring programs was conducted to inform operations and management of the channel deepening project (PoMC, 2010), two of which are relevant to this thesis. Firstly, the "Contaminants in Fish" monitoring program executed a baywide contaminant study and measured metals, as well as organic pollutants, in mature Black bream (*Acanthopagrus butcheri*) in January 2009 (EPA, 2009), eight months before the dredge project concluded (PoMC, 2010). They found no elevated concentrations of any pollutant compared to an investigation on the same species of fish at Port Phillip Bay in 2006 (EPA, 2007). The authors' conclusion, that the dredge works did not cause any increase in bioavailable contaminants within the bay is, however, contestable. The physiochemical mechanisms of contaminant mobilisation and trophic uptake are complex and dependent on many factors (Lavoie *et al.*, 2013). Yet, a timeline from Hg poisoning at Minamata Bay (Harada, 1995) suggests it would take years, rather than months for an effect to be measurable in large predatory fish, such as the one monitored for this study (length > 26 cm, EPA, 2009).

Secondly, the "Little Penguins" monitoring program aimed to "detect changes in Little Penguin body mass (an indicator of health) outside expected variability" (PoMC, 2010). To that end, weights of Little Penguins were recorded and daily average population weights calculated for female and male penguins. However, these measures were taken from the Phillip Island colony and not the resident penguin population at St Kilda. While Phillip Island Little

Penguins do visit and forage in Port Phillip Bay during part of the year (Chiaradia *et al.*, 2012), they would have been less affected by the dredging than their St Kilda conspecifics, which remain in the bay all year and feed predominantly in Hobsons Bay (Kowalczyk *et al.*, 2015), where most of the dredge works occurred.

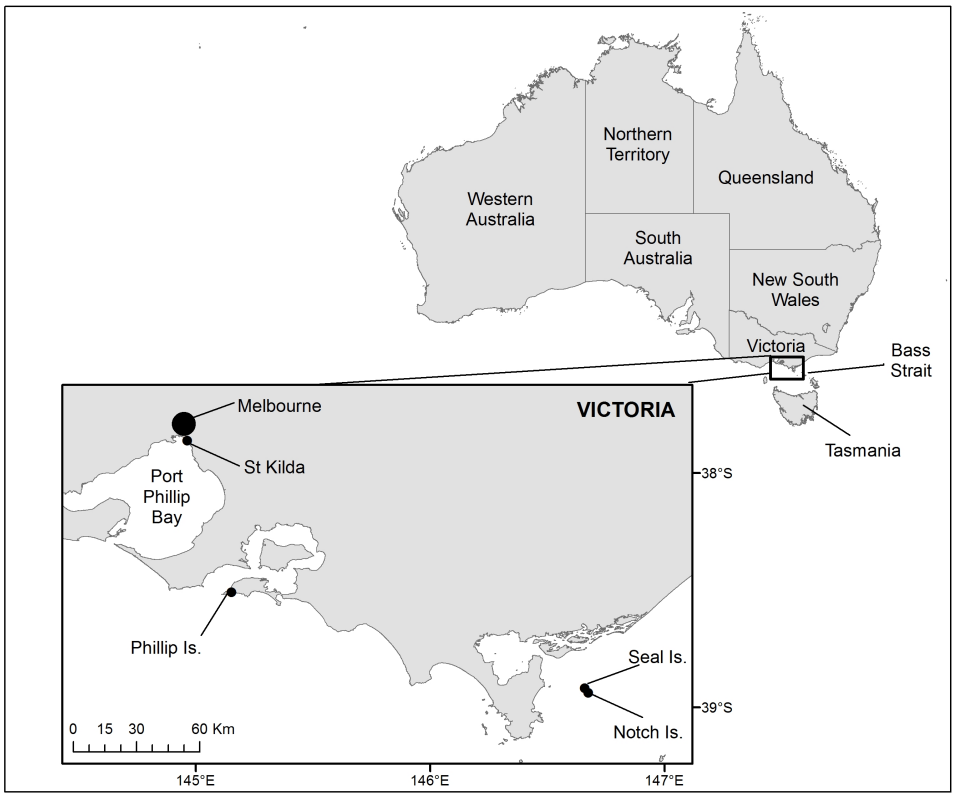


Figure 1.1: Sampling locations visited during this study

1.4. Knowledge gaps

As previously indicated, knowledge of the toxicology of penguins has thus far been limited to internal tissues and feathers, Table 1. This thesis is the first research to report on blood metal concentrations in any penguin species. While there is some data on metal concentrations in faeces (guano) of some penguin species (Sun & Xie, 2001; Ancora *et al.*, 2002; Celis *et al.*,

2014; Celis *et al.*, 2015), there is no data of metals in faeces of the Little Penguin, and correlation factors between blood - feathers and blood - faeces in penguins hasn't been reported. Furthermore, the metal concentrations in the penguin's main prey items within Port Phillip Bay are essentially unknown and, indeed, only very sparse data exist for metal concentrations in biota of the Bass Strait (Walker, 1988).

1.5. Study species - Little Penguins

1.5.1. Distribution and colony specifics

The Little Penguin breeds at offshore islands and several small mainland colonies in southern Australia and New Zealand (Marchant & Higgins, 1990, Figure 1.2). While considered common in Bass Strait, Little Penguin populations in Victoria have experienced large fluctuations in the past, which have been linked to food availability and predation (Norman *et al.*, 1992; Dann *et al.*, 2000; Sutherland *&* Dann, 2014). Phillip Island is the largest colony sampled in this study, Figure 1.1, and it also is the largest colony of Little Penguins worldwide, with approximately 32,000 birds (Sutherland *&* Dann, 2014). It is thought that this number is close to the maximum carrying capacity, based on available fish resources in the Bass Strait (Dann *&* Norman, 2006). The colony is situated along the beaches, sandy hills and scrubland on the southwestern end of the peninsula. Penguins breeding at Phillip Island undertake short foraging trips of ~15 km radius from their burrows during the breeding season (Weavers, 1992), but in winter often travel ~100 km to exploit more reliable foraging grounds within Port Phillip Bay (McCutcheon *et al.*, 2011; Chiaradia *et al.*, 2012).

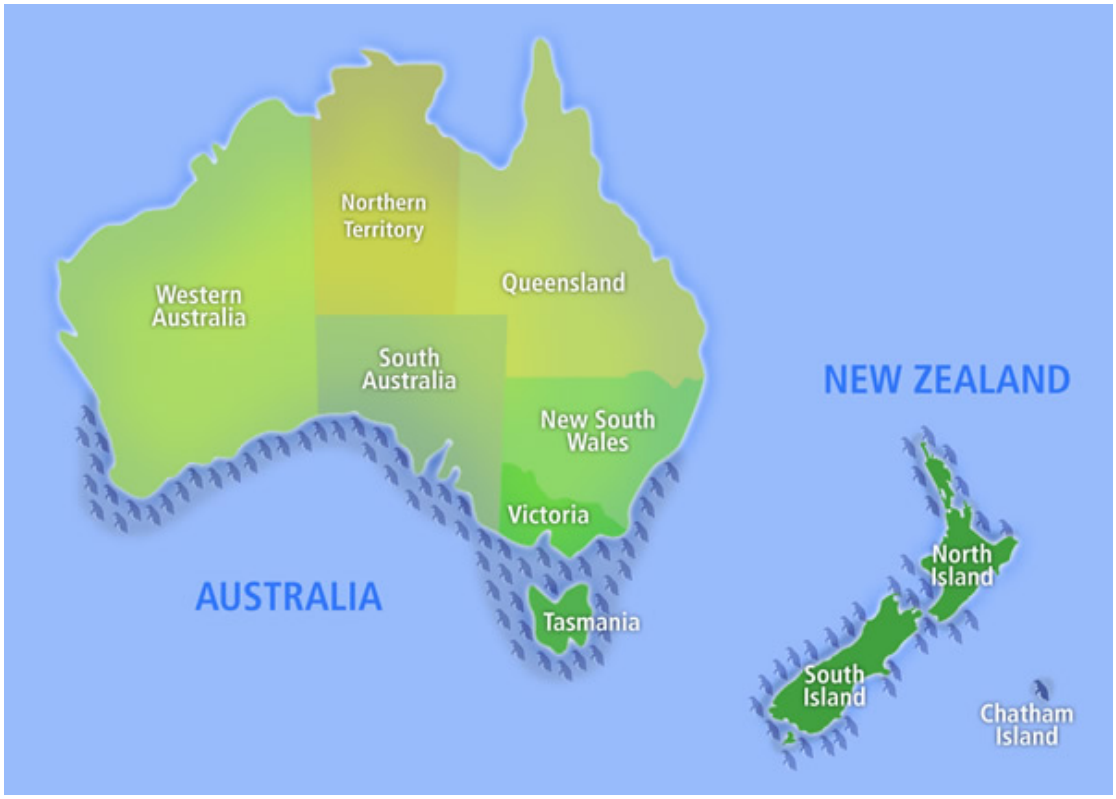


Figure 1.2: Little Penguin (*Eudyptula minor*) breeding range (Source: http://www.abc.net.au/tv/penguinisland/images/little_penguin_distribution_map.jpg)

The colony at St Kilda, Melbourne, Australia, is one of only a few penguin colonies utilising a man-made structure in an urban environment (Preston *et al.*, 2008, Figure 1.1). The breakwater was built to accommodate the sailing competition event during the 1956 Olympics. In the early 1970s, the first sightings of Little Penguins were reported (Eades, 1975) and, currently, approximately 1300 adult penguins nest within the rocks of the breakwater (Z. Hogg, unpublished data). Little Penguins at St Kilda have one of the smallest foraging ranges among seabirds (< 20 km, Collins *et al.*, 1999; Preston *et al.*, 2008; Kowalczyk *et al.*, 2015). The other two colonies sampled during this study are Notch Island and Seal Island, which are two small uninhibited islands situated close to each other, about 20 km east of Wilsons

Promontory, Figure 1.1. Penguin populations at Notch Island fluctuated between ~660 in the breeding season of 2012/2013 and ~2100 during the non-breading season of 2013 (Schumann *et al.*, 2014). Seal Island experienced a wildfire that almost entirely wiped out the local penguin population in 2005 (Chambers *et al.*, 2009). Penguins have continued to breed on the island since its recovery, but no survey has been conducted yet to establish numbers. The EPA, however, did visit the island and declared it 'recovered' in 2011 (EPA, pers. communication) and granted permission for this project to sample there in 2013.

1.5.2. Foraging and breeding behaviour

Extensive research has been conducted on Little Penguin breeding and foraging ecology (Reilly & Cullen, 1979; Newman, 1992; Fortescue, 1999; Robinson *et al.*, 2005; Chiaradia *et al.*, 2007; Sidhu *et al.*, 2007). Penguins are generalist feeders, predominantly preying on small clupeoid fish, such as Australian anchovy (*Engraulis australis*) and pilchard (*Sardinops sagax*) (Chiaradia *et al.*, 2010; Preston, 2010). Cephalopods and small crustaceans make up a varying part of their diet, primarily in birds breeding on offshore islands (Schumann *et al.*, 2014). Penguins have been found to be highly reliant on certain prey. (Chiaradia *et al.*, 2003) attributed the Phillip Island penguin population decline in 1995/96 to the collapse of the local pilchard population (due to a virus). The penguin population at Phillip Island adapted by switching to other prey species and recovered within one year (Chiaradia *et al.*, 2010). The St Kilda penguins appear to prefer anchovy but can switch and exploit local resources as flexible generalist predators (Kowalczyk *et al.*,

2014). Little Penguins nesting on Bass Strait islands are less well researched. Limited information on the foraging ecology of Notch and Seal Island Little Penguins originates from a diet study (Schumann *et al.*, 2014) and a telemetry study (P. Dann, unpublished data). Schumann *et al.* (2014) found that penguins in the summer of 2009/10 fed predominantly on unidentified post-larval fish, Australian pilchard, barracouta (*Thyrsites atun*) and arrow squid (*Nototodarus sloanii*). All penguins tracked in the telemetry study foraged in open Bass Strait waters, rather than coastal areas (P. Dann, unpublished data).

The annual cycle of Little Penguins (henceforth called "seasons") is separated into breeding, moult and non-breeding periods. The start of the breeding season, July to September, is dependent on food availability, starting earlier in years when Bass Strait is warm in autumn (Cullen *et al.*, 2009), but also differs between colonies. At St Kilda, most penguins start laying eggs on average two months prior (July to August, Kowalczyk *et al.*, 2014) compared to penguins at Phillip Island (September to October, Cullen *et al.*, 2009). This has been contributed to more stable food resources within Port Phillip Bay (Kowalczyk *et al.*, 2014).

1.6. Study system

Little Penguins are ideal bioindicators (Becker, 2003) as they are long-lived (Dann *et al.*, 2005), high trophic feeders (Cullen *et al.*, 1992) and exhibit strong site fidelity (Robinson *et al.*, 2005). They are found in areas of varying degrees of industrialisation, their ecology is well researched (Trathan *et al.*, 2014) and they are robust and resilient to being handled. Moreover, St Kilda's

Little Penguin population is an ideal study species for ecotoxicological research because they remain within Port Phillip Bay throughout the year (Preston *et al.*, 2008; Kowalczyk *et al.*, 2015) and are the only high trophic level predator within Port Phillip Bay to meet all the monitoring criteria listed above.

1.7. Study aims and scope

This study aims to enhance our understanding of the ecotoxicology of the Little Penguin. Specifically, this research will elucidate geographical, seasonal and annual variation of metal concentrations in Little Penguins, as well as provide insights into mechanisms of metal biomagnification and detoxification. Conclusions from this study carry significance not only for the conservation of the Little Penguin, but also for the health of Port Phillip Bay.

Overall Research Aim: To investigate the toxicology of the Little Penguin and its utility as a bioindicator of local contamination.

Research Questions:

1. Can the Little Penguin nesting at St Kilda be utilised as an effective bioindicator for metal pollution in Port Phillip Bay?
2. Do Little Penguins at St Kilda carry significantly higher concentrations of metals than Little Penguins feeding at more pristine feeding grounds?
3. Which sampling matrices (blood, feathers or faeces) are preferential for the quantification of which metals?

4. What are the inter-annual and inter-seasonal variations in metal concentrations in Little Penguins at St Kilda and Phillip Island?
5. Which of the metals measured in this study are of most concern to the Little Penguin within Port Phillip Bay?
6. How do metal concentrations in the Little Penguin's main fish prey species relate to Little Penguin blood and faecal concentrations??
7. What sampling protocol would be suitable to survey long-term metal pollution within Port Phillip Bay?

1.8. Thesis structure

This thesis is structured as six chapters: An introduction, a methodology, three data chapters and a conclusions chapter. Each data chapter is self-contained and is either published or under review for publication in the peer-reviewed international literature. There is some overlap in the usage of data sets between chapters, as different aspects were explored and different research questions were addressed in each of the data chapters.

Chapter One presents an introduction to the thesis, current knowledge of aquatic toxicology and the Little Penguin, as well as outlines research questions and thesis structure.

Chapter Two details all methods, materials, facilities, equipment and techniques that have been used throughout this study. Descriptions of specific experiments are included in each data chapter.

Chapter Three has been published in *Environmental Pollution* and introduces the Little Penguin as bioindicator of coastal metal and metalloid

pollution. This publication represents the first investigation of metals and metalloids in blood of any penguin species. The data set used in this publication is a small subset of the 3-year data set, namely data from February and March 2012, where blood and feathers were collected from Little Penguins at three colonies: St Kilda, Phillip Island and Notch Island.

Chapter Four has been published in *Marine Pollution Bulletin* and assesses variations in trace element blood concentrations in Little Penguins between years, seasons, sex and body mass at two colony locations: St Kilda and Phillip Island. In this chapter, the majority of data collected in this thesis are analysed and information on long-term variation of bioavailable metals and metalloids within Port Phillip Bay is presented.

Chapter Five has been submitted for publication in *Environmental Pollution* and reports data on metals and metalloids in Little Penguin fish prey items, blood and faeces. This data chapter presents how metals and metalloids in fish, blood and faeces of Little Penguins relate by way of multi-dimensional scaling, reports on correlations between metal concentrations and fish-length data, penguin blood and faecal metal concentrations, as well as investigates the influence of trophic position of prey items on Hg concentrations in blood of Little Penguins at St Kilda.

Chapter Six integrates the findings from the three data chapters and assesses the implications for the management of Little Penguins at St Kilda, as well as Port Philip Bay as an ecosystem. Thus, this study is placed in context of broader ecotoxicological scenarios and suggestions for future research are made.

Appendices 1 to 5 contain the standard operating protocol for the sample preparation, supplementary materials for each of the data chapters, as well as details of the adverse incident report and investigation outcomes.

The writing style of the data chapters has followed the requirements of the journals to which they were submitted to, however, the format, section headings, numbering and referencing have been amended to be consistent across the thesis.

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Chapter 2

General methodology

St Kilda Little Penguin on author's lap (Photo courtesy: Debbie Lustig)

2. General methodology

The structure of this chapter follows the line of investigation pursued in this study (Figure 2.1). Choices made in each section are contextualised and discussed.

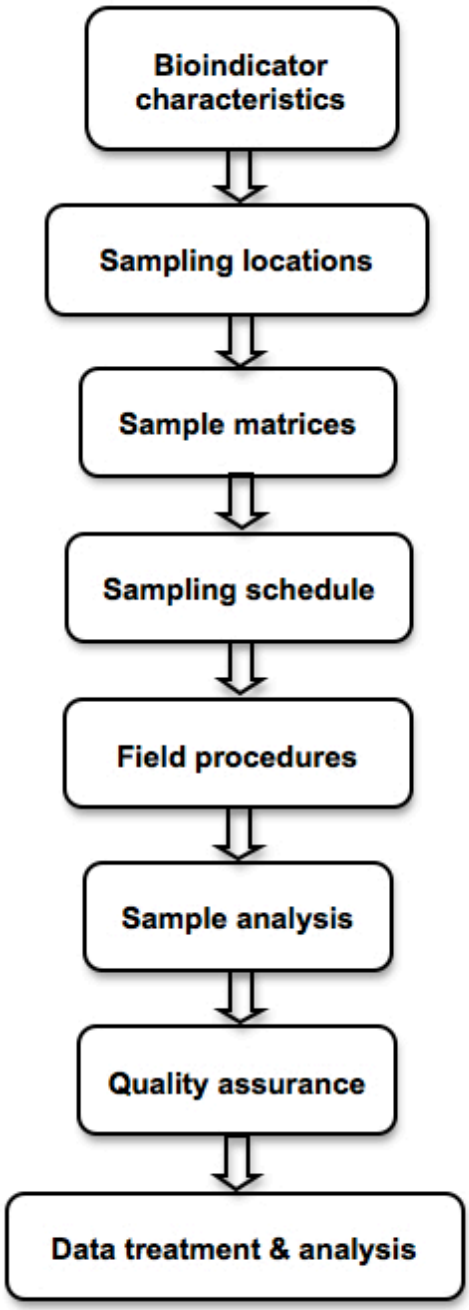

Figure 2.1: Line of investigation undertaken in this study

2.1. Bioindicator characteristics

Dmowski (1999) reviewed the use of bird as bioindicators of heavy metal pollution and summarised the required attributes for suitable bioindicator species as: (1) widespread occurrence, (2) clearly defined individual territory size and territory fidelity, (3) common occurrence and easy capture, (4) homogeneity of the material and standardising possibility, (5) individual size large enough for sampling, (6) well known biology of the species, (7) breeding possibility for laboratory tests, and (8) bioaccumulation of pollutants. The Little Penguin (*Eudyptula minor*) fulfils all of these criteria, plus additionally, it is (9) long-living and (10) a high trophic feeder. These last two aspects enable investigations into bioaccumulation (by age) and biomagnification (through trophic chains), which are an important part of assessing the health of an ecosystem (Burger & Gochfeld, 2001). Even with non-destructive sampling (e.g. plucking moulted feathers), it is important to select a species that is not threatened or endangered. Any handling of wildlife has an impact on that individual, however small, and it is crucial to consider such impact and judge the (11) robustness of the species to withstand invasive procedures. It is germane to point out that no one species can be used to answer all questions relating to the prominence and impact of metal and metalloid contamination within an ecosystem, however, I assess the Little Penguin to be a suitable bioindicator species for Port Phillip Bay.

2.2. Sampling Locations

In ecotoxicology, comparing metal concentrations in the target population with a ‘reference’ population provides a benchmark and allows for

a greater understanding of what can be considered ‘normal’ (Rand, 1995). True reference populations, where all things are equal except the toxicant levels, hardly ever exist in real life. Hence it is vitally important to know as much as possible about any other differences between the target and reference population and how such differences could influence any performance indicators, such as body mass and breeding success. In this study, the most important consideration for selection as a reference site was the foraging area of its penguin population. A seabird's metal load is predominantly determined by the diet it ingests (Monteiro & Furness, 2001). As such, it is crucial to know as much as possible about the foraging ecology of the target species. Unfortunately, there are no data available on penguin prey items in the Bass Strait. It is therefore presumed that fish growing up away from industrial and agricultural outlets will carry loads close to baseline levels.

Notch Island and Seal Island were selected because they house a sufficiently large population of penguins to enable study and there were some limited data available (Schumann *et al.*, 2014), suggesting penguins nesting there were foraging to the east of the islands, towards the open Bass Strait, rather than in Corner Inlet, which has shipping and agricultural influences. It is also an island that could be reached by boat at relatively low expense. Landing on and launching off Notch Island, even in fair weather, is difficult due to the topography of the island. In the second year of sampling, my volunteers and I sampled penguins on Seal Island, which has slightly better access. Both islands are in such close proximity (Figure 1.1, Harris & Deerson, 1980), that the same foraging ecology can be presumed.

The target colony, St Kilda, has easy access via a public pier. Most of the penguins nest in a gated section. The colony is relatively well studied. In particular, the foraging ecology, diet and breeding success of Little Penguins at St Kilda has recently been extensively investigated (Cullen *et al.*, 1996; Kowalczyk *et al.*, 2015a; Kowalczyk *et al.*, 2014; Kowalczyk *et al.*, 2015b; Kowalczyk *et al.*, 2015c; Preston, 2010; Preston *et al.*, 2008). Earthcare St Kilda, a local community group, carries out bi-monthly surveys collecting data such as penguin ID (via passive integrated transponders, Trovan Ltd., Australia), weight, sex and location within the colony.

The “penguin parade” at Phillip Island might be the largest Little Penguin colony in the world, and it certainly is the most researched (Dann, 2013). It is also a very popular tourist attraction, managed by the Phillip Island Nature Parks, which help fund penguin and other research on the island. This colony was chosen because previous ecotoxicological studies had been carried out there (Table 1.1), and the local research centre and its members provide outstanding logistical support.

Data were collected at four penguin colonies in Victoria, Australia, over three years (2011 - 2013). The four sites were the St Kilda Breakwater, Phillip Island, Notch Island and Seal Island (Figure 1.1). The St Kilda colony is located 5 km from the central business district of Melbourne, Australia (37˚51'S, 144˚57'E). Approximately 1,300 Little Penguins nest on the 650 m long man-made breakwater structure (Z. Hogg, unpublished data). Phillip Island (38˚30'S, 145˚10'E) is located 140 km southeast of Melbourne, Australia, and has approximately 32,000 breeding Little Penguins nesting on sandy dunes and coastal scrublands of the Summerland Peninsula

(Sutherland & Dann, 2012). Notch Island (38˚94'S, 146˚68'E) and Seal Island (38˚92'S, 146˚66'E) are neighbouring islands within the Seal Islands group and lie 19 km east of Wilsons Promontory, Australia. Notch Island is about 10 ha in size, uninhabited and has approximately 660 Little Penguins (Schumann *et al.* 2014). Seal Island lies 2 km northwest of Notch Island (Harris & Deerson, 1980) and has a similar size population of Little Penguins as Notch Island (EPA Victoria, personal communication).

The Victoria University Animal Ethics Committee approved all field and experimental procedures carried out on Little Penguins in this study, project number AEC 05/09. The Victorian Department of Environment and Primary Industries issued a scientific permit for this research (number 10005200) and Parks Victoria kindly granted permission to work along the St Kilda breakwater.

2.3. Sample matrices

Varying matrices have been used to assess metal and metalloid contamination in birds and mammals (see reviews in Das *et al.*, 2002; Eisler, 2009; Scheuhammer *et al.*, 2007). As an illustrative example of these matrices, a simplified model for mercury dynamics in seabirds, adapted from Monteiro & Furness (1995) is given in Figure 2.2. The most common non-destructive matrix sampled in birds is feathers (Becker, 2003; Burger, 1993). Feathers are easy to collect, with minimal impact on the animal and are easy and cheap to store. Contrary to other seabirds that undergo a sequence of moults throughout the year, penguins perform what has been termed a "catastrophic" moult over two to three weeks, during which the animals fast

and replace their entire plumage (Stonehouse, 1967). Due to this, and again contrary to most other seabirds, there is low intra-individual variation in penguin feather metal concentrations (Brasso *et al.*, 2013). However, metal concentrations in feathers in other seabirds have been found to be a poor indicator for internal tissues (Eagles-Smith *et al.*, 2008; Lavers *&* Bond, 2013) and results can be highly biased by external contamination (Jaspers *et al.*, 2004).

The collection of blood, in particular on smaller sized animals, requires a high level of skill and has larger potential impact on the bird. Hoysak *&* Weatherhead (1991) investigated different blood sampling techniques on small birds and assessed their effects. The authors found that the jugular vein was preferable to the wing vein. They also discuss maximum blood volumes to be safely extracted, based on a total blood volume of 6 - 8 mL per 100 g bird (Sturkie, 2012) and the American Ornithologists' Union (1988) guideline to not sample more than 10 - 20 % of total blood volume. For a 1 kg penguin, and applying the safer (for the bird) of both limits, this would translate into a maximum safe volume limit of 6 mL. However, I would caution against such a high limit and Whitworth *et al.* (2007) recommend a more conservative range of 0.3 to 0.6 mL per 100 g body weight.

Sergent *et al.* (2004) executed a three-year study into the health of Little Penguins from Bowen and Lion Island populations, New South Wales, Australia, to assess potential causes for recent decline. The authors investigated the hemotological values in the blood of 294 adult penguins over 2 years. They extracted 2.5 mL of blood from the medial metatarsal vein (caudal tibial), and while they did not find any cause for the decline, they

found that sampling blood quantities larger than 1 mL in this species was feasible and that the treated penguins appeared to cope well with the procedure (T. Rogers, personal communication).

Faecal material of seabirds has been proposed as a suitable metal bioindicator matrix (Yin *et al.*, 2008), but collection methods range from being "taken off the top of a mass of faeces deposited on the ground" (Celis *et al.*, 2015), "taken from unattended nests" (Bargagli *et al.*, 1998), to being collected freshly, meaning "that it's produced by seabirds or animals in the past couple of days" and pooled by species (Yin *et al.*, 2008), or not specified (Ancora *et al.*, 2002). Collecting faeces by placing the animal in a clean and safe container means a distinct identity to any deposited sample can be assigned. This, in turn allows for potential paired sample correlations to establish any relationships between the different sampling matrices.

Eggs are often used as a contamination bioindicator matrix, particularly in birds nesting in large colonies, or laying multiple clutches (Becker, 2003; Bond *&* Diamond, 2009; Brasso *et al.*, 2012; Braune, 2007; Burger, 2002; Burger *&* Elbin, 2015; Burger *et al.*, 2007; Burgess *et al.*, 2013; Evers *et al.*, 2003). In species with higher conservation concern and fewer eggs, addled eggs (no longer viable because the embryo has died) are sometimes favoured. These eggs are often collected at Phillip Island, as they are a common occurrence during the start of the breeding season and can be stored for future uses in scientific research. However, addled eggs are seldom encountered in the St Kilda colony. This is thought to be due to the nests not being as easily visible or accessible, often deep between the rocks of the breakwater, but also because the resident rakali (*Hydromys chrysogaster*), a

native water rat, feeds on unattended and abandoned eggs. In any case, there is an inherent bias in using addled eggs, as they do not represent a random cross-section of the total available pool of eggs, and there is the potential for the first egg to contain excessive contamination loads, which may in fact have caused the egg to become non-viable (Helander *et al.*, 1982; Thompson *et al.*, 1991).

Internal tissues harvested from carcasses can yield important information about pollutant body loads (Arcos *et al.*, 2002; Eagles-Smith *et al.*, 2008; Kenow *et al.*, 2007; Lewis *&* Furness, 1991). But as Bryan *et al.* (2007) discussed in an article on bottlenose dolphins (*Tursiops truncatus*), there are issues with sampling washed-up specimens: (1) sample size is often small as they are rare events; (2) it is hard to identify the origin of the animal (unless ID-ed); (3) there is bias added by using partly decomposed parts; (4) sample is not a good representation of the population as a whole. There is one more important point to be considered: (5) the changed lipid concentration in starving or sick animals may falsify metal concentrations in tissues. While all these points are pertinent, internal tissues of Little Penguins are not included in this study because they were an extreme rare occurrence at St Kilda, our target sample location.

Another popular method to determine the pollution load of a high-trophic feeder is to measure their concentration in dietary items (Bisi *et al.*, 2012; Bocher *et al.*, 2003; Cain *et al.*, 1983; DesGranges *et al.*, 1998; Dietz *et al.*, 2013; Reynolds *&* Perrins, 2010), although it is often difficult to determine with any certainty exactly which prey species were consumed at what percentage frequency or mass, especially in the field. While I was unable to

make this determination (Chapter 5), I saw merit in investigating metal and metalloid concentrations in three species of the main prey items of St Kilda Little Penguins. Firstly, because there were no local metal data of penguin prey species available and secondly, because I wanted to relate those to blood and faecal metal data collected within the same time periods.

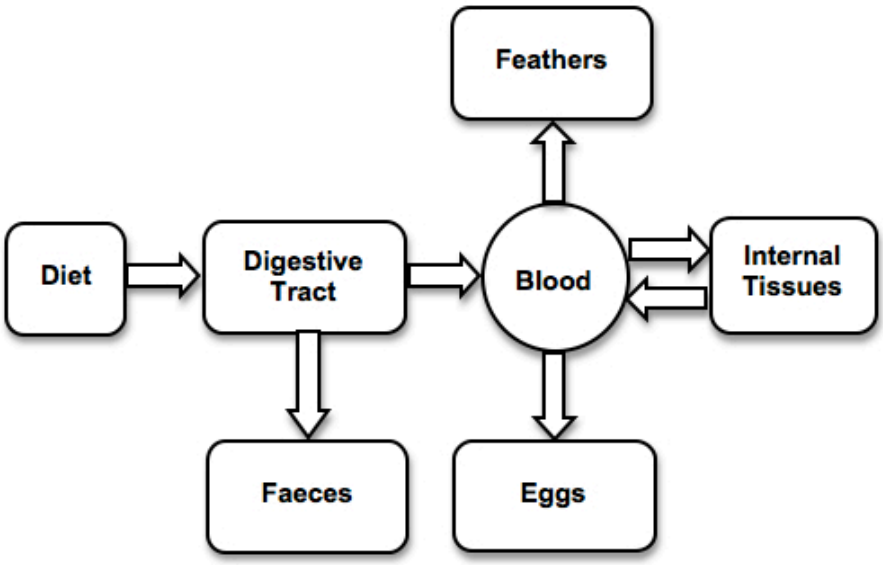

Figure 2.2: Simplified model for mercury dynamics in seabirds. Figure is adapted from Monteiro & Furness (1995).

2.4. Sampling schedule

Long-term studies of any fauna species need to consider and incorporate the life cycle of that species to be able to make comparisons with other locations, as differences between life cycle stages at times exceed geographic differences (Croxall *et al.*, 2002; Otsuka *et al.*, 2004; Walker *et al.*, 2005). Reilly & Cullen (1981, 1983) first described the life cycle of the Little Penguin. Breeding starts in late winter to early spring and is followed by moult in late summer, after which there is a brief period of non-breeding. The largest variation in these annual life cycle stages pertain to the onset of the breeding

season, which is dependent on sea surface temperature variations (Cullen *et al.*, 2009) and varies between colonies (Chiaradia *et al.*, 2012).

To account for those differences between locations, samples were collected during three sampling periods, henceforth called 'seasons': breeding (September - January), moult (February - March) and non-breeding (April - August). Blood collection training and feasibility study started at St Kilda, and after two sampling seasons, Phillip Island was added as a second study site. Penguins were usually caught at night, on their way to their burrows with the aid of trained wildlife volunteers. Samples were collected at St Kilda and Phillip Island for three and two consecutive years, respectively. Access to remote islands was restricted by the expense of hiring a boat and skipper, the difficulty of getting on and off the island in less than perfect weather conditions and the logistics of potentially being forced to overnight on remote islands. Thus, it was decided to sample at Notch Island (in 2012) and Seal Island (in 2013) only during moult, when Little Penguins could be caught in their burrows during the day.

The aim for each sampling period (breeding, moult and non-breeding) was to collect 10 to 15 blood samples larger than 1 mL from each sampling site. One mL is the minimum volume required to determine metals in blood using duplicate ICP-MS analysis. The number of sampling sessions required to achieve that aim for each sampling period varied (Table 2.1) and was mostly dependent on penguin presence at colonies, timing and weather conditions. For instance, as Notch Island and Seal Island are not monitored, the timing of moult had to be estimated. In 2012, sufficient numbers of penguins were encountered on Notch Island. However, in 2013 the trip to

Seal Island was delayed by two weeks due to unfavourable weather conditions and limited access to boat and skipper.

Table 2.1: Sampling effort and success of blood collection in Little Penguins for all locations combined (*success rate is expressed as percentage of viable blood samples per bird sampled, i.e. > 1 mL).

Year	Season	No. of sampling sessions	No. of birds caught	No. of birds sampled	No. of blood samples > 1 ml	Success rate [%] *
2011	Moult	4	16	15	10	67%
	Nonbreeding	3	28	25	14	56%
	Breeding	6	51	49	20	41%
2012	Moult	5	49	44	31	70%
	Nonbreeding	5	40	38	12	32%
	Breeding	5	44	42	31	74%
2013	Moult	3	24	21	11	52%
	Nonbreeding	2	24	23	14	61%
	Breeding	2	26	25	13	52%
TOTAL		**35**	**198**	**183**	**95**	

On the day of sampling on Seal Island, only four moulting Little Penguins were found, three of which were under the weight criterion for bleeding. The one individual that was bled yielded less than 1 mL of blood (Table 2.1). Also, the non-breeding sampling at Phillip Island in 2012 only yielded four viable blood samples due to severely cold winds. Additionally, moult sampling in 2013 at Phillip Island was temporarily suspended when a late-stage moulting female Little Penguin unexpectedly died during handling. The detailed report of the incident and the investigation outcomes are provided in Appendix 5, in the hope that researchers can learn from it and avoid such tragic outcomes.

2.5. Field procedures

2.5.1. Capture, weight and ID

Little Penguins were caught individually by hand from their burrows and weighed to the nearest 10 g using a hand-held spring balance (Figure 2.3). Animals that weighed less than 950 g were released without sampling for ethical and logistical reasons, as they were characterised as being of poor body condition and were unlikely to yield a valid blood sample larger than 1 mL. Different weight considerations applied for sampling during moult. Penguins will gorge themselves, nearly doubling their weight in preparation for moult, which lasts two to three weeks and during which time the animals fast (Reilly & Cullen, 1983).

Following consultation with research staff from the Phillip Island Nature Parks (PINP), the minimal weight criterion for bleeding was adjusted for each moult stage (Figure 2.5) as follows: M1 - 1400 g, M2 - 1300 g, M3 - 1100 g, M4 - 1000 g, M5 - 900 g. Following an incident where a penguin died during handling (Appendix 5), it was decided not to handle and collect blood from any penguin in the last stage of moult (M5).

Little Penguins were identified by passive integrated transponder (Trovan Ltd., Australia, Figure 2.3 right) or flipper band (Dann et. al 2014). Unidentified Little Penguins without passive integrated transponders or flipper bands were still processed for blood across consecutive sampling sessions on the condition that sessions at the location were at least four weeks apart. This criterion was followed to ensure no individual penguins were bled twice within a four-week period, as stipulated in the animal ethics permit.

Figure 2.3: Weighing of a Little Penguin (left) and scanning the passive integrated transponder of a Little Penguin (right).

2.5.2. Morphometrics

Standard morphometrical measurements were performed on each Little Penguin as follows:

- Total head length (THL): measurement of the length from back of the head to the tip of the beak using digital callipers (± 0.1 mm, Figure 2.4 left).
- Beak length (BL): measurement of the length of the beak from the posterior section to the tip of the beak using digital callipers (± 0.1 mm, Figure 2.4 middle).
- Beak depth (BD): measurement of the depth of beak taken just anterior to the nasal cavities using digital callipers (± 0.1 mm). This

measurement was used to determine the gender of the penguin (Arnould *et al.*, 2004).

- Flipper length (FL): length of the right flipper was measured by using a stopped ruler (± 1 mm) with the flipper extended at a 90˚ angle to the body (Figure 2.4 right).

Figure 2.4: Measuring total head length (left), bill length (middle) and flipper length on a Little Penguin (right).

M0 (pre-moult): old and worn feathers, but not standing up and flippers not swollen. Note the different appearance to penguins in stage M5.

M1: flipper swollen, feathers standing upright, but not coming out yet.

M2: old feathers begin to fall out.

M3: 1/3 to 2/3 new feathers.

M4: more than 2/3 of new feathers grown.

M5: all new feathers. Moult finished.

Figure 2.5: Moult stages (M0 to M5) of the Little Penguin with descriptions given beneath each picture (Photos courtesy of Flossy Sperring).

2.5.3. Blood collection

The technique used in this study closely follows the one described in (Sergent *et al.*, 2004). The penguin was placed upright into a lightweight

cotton bag, with the feet protruding out of the opening and the head with its beak pointing upward in one of the corners of the cotton bag. The cotton material was wrapped around the penguin to ensure its wings were tucked in close to its body and remained fairly immobile. A trained volunteer held the penguin in this position, continually monitoring the bird's breathing by feeling the thorax movements. One foot of the bird was tucked into the bag, near its body (for warmth), while the other foot was presented to the researcher to take blood. If needed, the foot was gently massaged to increase blood flow. In difficult weather conditions with low temperatures or strong winds, when this treatment failed to provide sufficient blood flow, the bird's foot was placed in a container with warm water (~ 30˚C) for a minute to promote blood circulation. The volunteer was trained to hold out the exposed foot while applying slight pressure on the metatarsal vein (Figure 2.6 top left). The blood was extracted from the medial metatarsal (caudal tibial) vein using a 25-gauge butterfly needle with a short cord and attached to a plastic 3 mL syringe (Sergent *et al.* 2004, Figure 2.6 top right). A maximum of 2 mL of blood was collected. The blood was transferred from the syringe into a 6-mL vacutainer (BD Diagnostics, trace element tube plus K_2EDTA with BD Hemogard™ safety closure, product number 368381), gently inverted eight to ten times, labelled and stored in a cool box. A cotton ball, dabbed in corn starch, was pressed onto the puncture site to stop the bleed and prevent the building of a painful haematoma (Owen, 2011; Whitworth *et al.*, 2007). One field blank was prepared during each sampling session with Milli-Q ultrapure water (Merck Millipore) that was transported into the field. The Milli-Q water was drawn up into 3 mL syringes (Figure 2.6 bottom left), transferred into 6 mL vacutainers

and treated henceforth the same as penguin blood samples. Blood samples and field blank were transferred to a -20˚C freezer within 12 hours of sampling (Figure 2.6 bottom right).

Figure 2.6: Blood sampling: entering the metatarsal vein of a Little Penguin (top left), collecting a blood sample (top right), drawing up a field blank (bottom left) and blood samples from one sampling session, including one field blank (bottom right).

2.5.4. Feather collection

During moult, a handful of readily available moulting feathers were plucked from the dorsal region and another from the ventral region of the penguin. Due to the lack of Little Penguins encountered on the Seal Island trip in 2013, fresh penguin feathers were also collected from burrows. A comparison of these burrow-collected samples were significantly different to feather samples collected off birds for six out of nine elements measured (aluminum, cadmium, copper, mercury, selenium and zinc: pairwise t-tests with 'Holm' correction, $p < 0.05$). I attribute these increased concentrations to external contamination and excluded these samples from further analyses and publication. All feathers were kept separate in labelled sterile, lab-grade press and seal bags, and stored at room temperature. The stage of moult was recorded following pictures and descriptions given in Figure 2.5.

2.5.5. Faeces collection

Little Penguins were placed inside thoroughly cleaned plastic boxes and topped with plastic lids with a sufficiently large breathing hole (Figure 2.7 left) for up to 30 minutes. Any faecal material left voluntarily by the penguin was transferred into sterile 70 mL yellow-top specimen containers by use of clean wooden spatulas. The plastic boxes were cleaned after each penguin with hot water and clean microfiber or paper towels. During each field sampling session, one faecal control sample was collected by emptying ~10 mL of Milli-Q ultrapure water (Merck Millipore) into the cleaned plastic box and collecting the control sample in the exact same manner as was done for faecal samples.

Occasionally, a penguin would defecate while being restrained for bleeding. If possible, an extra volunteer would "harvest" this material with a clean wooden spatula, if it was possible to do so without disturbing the bleeding process (Figure 2.7 right). All faecal and control sample specimen containers were labelled and stored in a cool box, together with the blood and field blank samples. All samples were transferred to a -20˚C freezer within 12 hours of sampling. Faecal field control samples were analysed along with the faecal samples from the same sessions. All control samples returned measurements below the limit of reporting.

Figure 2.7: Faecal collection: boxes used to house Little Penguins for voluntary faecal collection (left), opportunistic faecal collection while handling a penguin (right).

2.5.6. Fish collection

Three species of potential Little Penguin fish prey were donated by P. Mc Adams from VanCouver Fisheries, Williamstown, Victoria, Australia, who commercially catches baitfish. I gratefully received quantities of Australian Anchovy (*Engraulis australis*), Sandy sprat (*Hyperlophus vittatus*) and Pilchard (*Sardinops sagax*), which had been caught within Port Phillip Bay on recorded dates. Fish samples were received on several occasions and transported frozen from Williamstown to Victoria University's Werribee campus. The fish were allowed to partially thaw, until single fish could be safely (without breaking) separated from the bulk. Up to 15 fish were then individually stored in labelled sterile, lab-grade press and seal bags and kept frozen at -20˚C.

2.6. **Sample analysis**

2.6.1. Literature review

Measuring metal concentrations in seabird tissues has been widely applied since the 1970s (see review in Furness, 1993). Aided more recently by the development of analytical capabilities to affordably measure low concentrations in small quantities of biological sample materials, more and more studies conduct non-destructive sampling (Becker, 2003). The most effective way, in terms of utilising the limited sample material, to measure metals including Mercury, is to use a multi-element analyser. Inductively coupled plasma (ICP) spectrometry allows the measurement of a whole suit of elements at one time using a small of sample organic material. In this study, we used inductively coupled plasma mass spectrometry (ICP-MS) and

inductively coupled plasma atomic emission spectroscopy (ICP-AES). The use of such effective analytical instruments has galvanised ecotoxicological studies, and often researchers can measure a long list of metals and metalloids, both at trace and higher concentrations in a wide range of organisms (Bond & Lavers, 2011; Ciesielski *et al.*, 2006; Geens *et al.*, 2010; e.g. Gibbs, 1995; Ikemoto *et al.*, 2005; Kakuschke *et al.*, 2008; Smichowski *et al.*, 2006; Zhou *et al.*, 2001). We determined the following 13 metals and metalloids in Little Penguin blood: aluminium, arsenic, boron, cadmium, calcium, chromium, copper, iron, lead, mercury, selenium, tin and zinc. The feather samples were analysed for aluminium, arsenic, cadmium, copper, iron, lead, mercury, selenium and zinc, while fish and faecal samples were analysed for arsenic, cadmium, lead, mercury and selenium. The reduction in elements to be determined was due to limited funds available.

Prior to be run on an ICP, the material, or sampling matrix, needs to be prepared, so that all elements are available in their soluble form within the analyte. This is predominantly achieved by acid digestion at a temperature high enough to aid the solution process, but below the temperature where elements would become volatile, to avoid losses. For instance, mercury becomes volatile at temperatures above 95ºC. The specifics of acid digestion vary between studies and are, among other things, dependent on the sampling matrix. For instance, blood is much easier to digest than more solid material, such as muscle, feathers or bone. Most sample preparation methods involve nitric acid (e.g. Ciesielski *et al.*, 2006), some employ a mixture of nitric and hydrochloric acids (e.g. Debacker *et al.*, 2000), nitric and perchloric acids (e.g. Brothers & Brown, 1987), nitric and hydrogen peroxide acids (e.g.

Griesel *et al.*, 2008), or nitric, perchloric and sulphuric acids (e.g. Sakai *et al.*, 2000).

The method of sample preparation chosen in this study followed the standard procedure for preparation of biological samples for metal and metalloid determination at the National Measurement Institute (NMI), Melbourne, Australia. The detailed standard operating procedure is provided in Appendix 1. In brief, it involves the sample being dried, digested in nitric acid at 95ºC, filtered and made up to volume. A series of trial tests executed with chicken blood showed that nitric acid alone failed in extricating sufficient percentage recoveries of analytes (in particular, mercury). To address this issue, I made three adaptations: 1) the duration of acid digestion was increased from one hour to up to four hours - until there was little or no solid material visible in the analyte, 2) hydrochloric acid was added, and 3) a step of cooling the solution was added at the end of the digestion process, to avoid losses due to high pressure when opening the DigiTubes.

Victoria University has a strong tradition of research collaborating with the National Measurement Institute (NMI). The NMI is the peak Australian measurement body responsible for biological, chemical, legal, physical and trade measurement. I executed the preparation of blood and faecal samples (drying and acid digestion), feather (cleaning and drying) and fish prey samples (homogenising and dry-freezing) at the Victoria University laboratories at the Werribee campus. This was done to optimise available funds, most which was used to cover costs of ICP analysis at the NMI. Details of the analysis of samples at the NMI are given below and in each of the data chapters.

2.6.2. Blood sample digestion

A very detailed step-by-step standard operating procedure of the blood sample acid digestion is presented in Appendix 1. While some studies report wet weight blood metal concentrations (e.g. Ikemoto *et al.*, 2005; Thompson *&* Dowding, 1999), I chose to dry the blood prior to digestion to minimise bias. I observed that the water content in both blood and faecal samples varied considerably between individuals sampled. This is due to the varying hydration status of birds and likely highly depended on whether the penguin was fasting or feasting. To minimise this potentially large bias, all samples were oven-dried to constant weight and drying quotients are given in each of the data chapters in this thesis to facilitate conversion to wet weights and thus enable comparison with other studies.

The analytical precision of the digestion process was verified by using standard reference materials provided by the NMI. AGAL4 (bovine liver tissue) was chosen as the most appropriate reference material available. However, as AGAL4 does not contain the metal tin (Sn), so a second reference material, AGAL3 (prawn tissue), was also used. We followed the ICH guideline "Validation of Analytical Procedures: Text and Methodology Q2(R1)" (Guideline, 2005), which recommends a minimum range of 70 - 130% recovery of standard reference material to ensure the accuracy of the analytical procedure. Sample replicates with results outside this range were excluded from further analysis.

Samples were acid digested (Figure 2.8 top left), filtered (Figure 2.8 right), capped, sealed using Parafilm^{R} and delivered to NMI (Figure 2.8

bottom left), where they were stored at 5˚C until analysis, which generally occurred within four weeks of delivery.

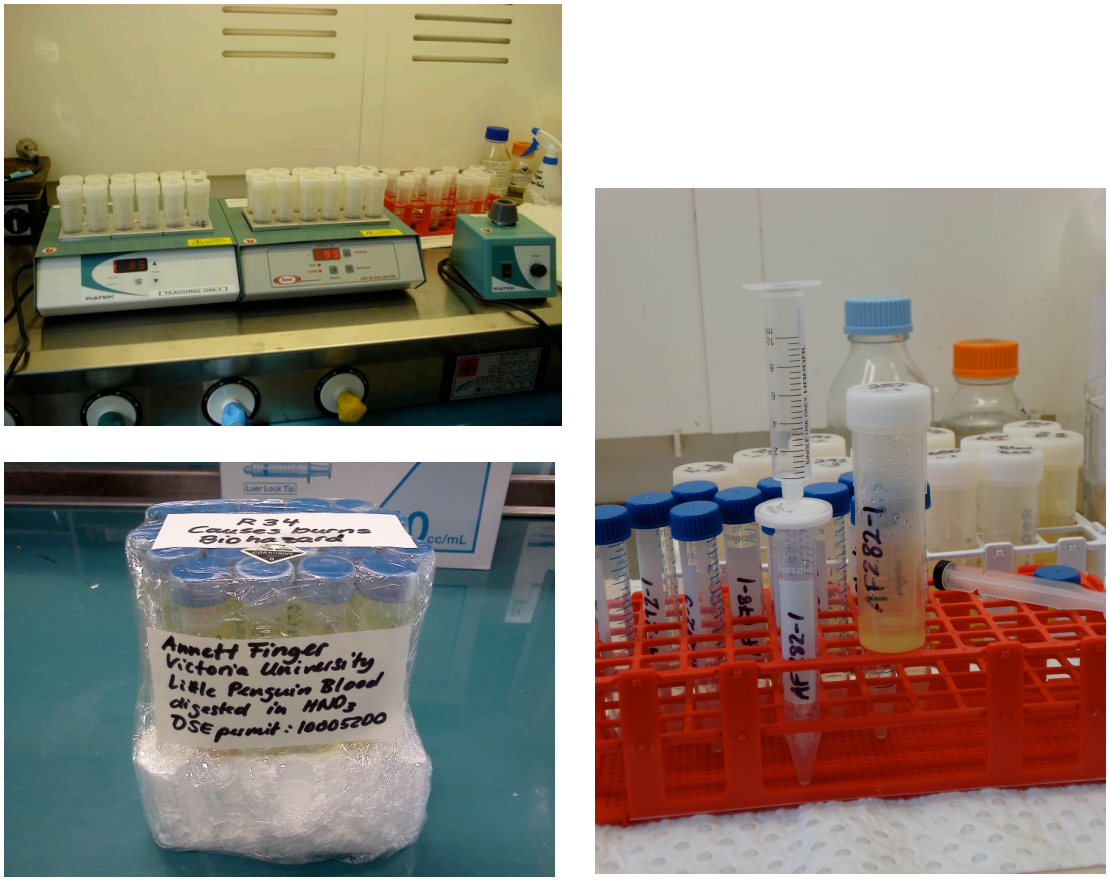

Figure 2.8: Sample preparation: replicates being heated in dry heater (top left), filtered into 14 mL Falcon tubes (right), and digested, sealed and packaged blood samples ready for delivery for elementary analysis to the NMI (bottom left).

2.6.3. Feather sample preparation

Feather samples were transferred into a clean 220 mL plastic container, filled about three quarters with Milli-Q water, tightly capped and vigorously shaken by hand for about 10 seconds. Using tweezers, the feathers were transferred to another clean 220 mL plastic container and the process of adding Milli-Q water and vigorously shaking the container was

repeated two more times (Figure 2.9 left). This process served to remove dust and dirt particles, which would sink to the bottom while the water-repelling feathers would float at the top. The feathers were transferred into a clean, labelled 70 mL yellow top specimen container. A clean sheet of Gladwrap^{R} was placed over the rim, fixed with a rubber band and pierced several times to allow water vapour to escape (Figure 2.9 right). The containers were placed into a fan-operated oven at 45˚C for ≥ 48 hours or until two consecutive weight measurements, two hours apart, were identical or within ± 0.5 mg. Dry weight of feather samples was recorded, samples were placed into labelled zip-locked bags and delivered to the NMI for acid digestion and metal analysis.

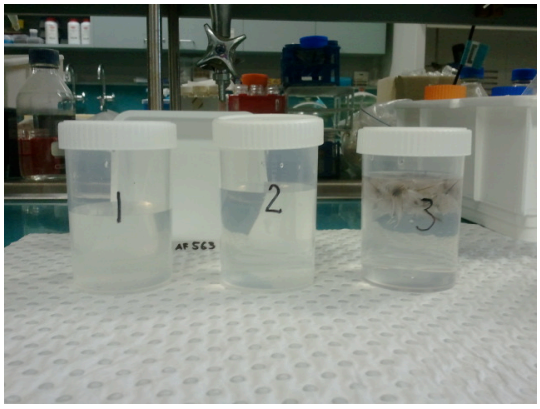

Figure 2.9: Feather sample preparation: cleaning process of Little Penguin feathers (left) and Little Penguin feather sample prior to drying process (right).

The samples were digested at the NMI at Port Melbourne, Australia, using NMI method VL 247. This method is similar to the digestion of blood samples described in chapter 2.6.2. In short, the entire sample of feathers was digested (with procedural blanks) in 3 mL of 65% trace analysis grade nitric acid (SUPRAPUR, trace metal grade, by Merck) and 0.5 mL of 37%

hydrochloric acid (EMSURE, trace metal grade, by Merck) in a heating block at 95˚C for one hour, followed by microwave digestion. Samples were not run in duplicates; instead the analytical precision of the digestion process was verified with three standard reference materials provided by the NMI: AGAL3 (shrimp tissue), AGAL4 (bovine liver tissue) and INCT-TL1 (tea leaves).

Aluminium, iron and zinc were analysed on ICP-AES with a limit of reporting of 0.5 mg/kg, 2 mg/kg and 0.01 mg/kg, respectively. Arsenic, cadmium, copper, mercury, lead and selenium were analysed on ICP-MS with a limit of reporting of 0.01 mg/kg for all. All samples returned readings over the limit of reporting. Little Penguin feather analysis was executed in two batches. Batch 1 included the Phillip Island samples for 2012 and 2013, as well as samples collected at Notch Island in 2012. Batch 2 included samples collected at St Kilda in 2012 and 2013, as well as samples collected at Seal Island in 2013. No samples returned readings under the method detection limit and all percentage recoveries for standard reference materials were within 70 to 130% for all metals and metalloids in both batches (Table 2.2). Results are reported in mg/kg dry weight and procedural blank values were subtracted from original instrument results for all metals and metalloids.

2.6.4. Fish sample preparation

Fish samples were allowed to thaw at room temperature. Total wet weight (± 0.05 g) and standard length (± 1 mm, from the tip of the snout to the posterior end of the last vertebra, excluding the caudal fin) of each individual were recorded. Each fish was cut into ~1 cm sections with clean stainless steel scissors, transferred into a clean bottle of a George Foreman Mix&Go

blender™ (GFBL300) (Figure 2.10 top left). About 30 - 50 mL of Milli-Q water was added to the container, the blender part with stainless steel blades screwed on and each fish was homogenised for about 3 minutes or until fully homogenised (Figure 2.10 top right). The mixture was transferred into a clean 120 mL specimen container and frozen at -20˚C.

Table 2.2: Mean percent recoveries of standard reference materials for Little Penguin feather sample analysis at the NMI, Melbourne, Australia.

Analyte	AGAL3 (Prawn tissue)		AGAL4 (Bovine liver tissue)		INCT-TL1 (Tea leaves)	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Aluminium	67%	80%	70%	70%	88%	91%
Arsenic	82%	101%	107%	73%	-	-
Cadmium	130%	86%	110%	96%	92%	80%
Copper	71%	85%	93%	96%	86%	90%
Iron	81%	94%	101%	104%	92%	97%
Mercury	107%	98%	111%	89%	107%	N/A
Lead	88%	118%	88%	80%	98%	80%
Selenium	119%	87%	89%	90%	-	-
Zinc	83%	104%	97%	104%	92%	97%

The bottle and blades attachment were thoroughly cleaned with running hot water and twice rinsed with Milli-Q water between samples. Following homogenization, the samples were freeze-dried using a DYNAVAC FD 300 freeze dryer (Figure 2.10 bottom left). Dry weight of each sample was recorded (± 0.05 g) and drying quotients are presented in chapter 5. The

freeze-dried material was re-homogenised by being carefully broken up and mixed with a clean stainless steel utensil. A subsample of ~3 g was transferred into a labelled 14 mL falcon tube (Figure 2.10 bottom right), capped and sealed using Parafilm^{TM}. Dried fish samples were delivered to NMI, where they were stored refrigerated until analysis. Recovery rates for standard reference materials for fish samples are given in Table 2.3.

Table 2.3: Mean percent recoveries of standard reference materials for fish sample analysis at the NMI, Melbourne, Australia.

% Recovery	**Arsenic**	**Cadmium**	**Mercury**	**Lead**	**Selenium**
Agal3 Batch 80&81	96%	88%	82%	76%	107%
Agal4 Batch 80&81	110%	99%	85%	84%	104%
Agal3 Batch 82&83	84%	117%	77%	77%	99%
Agal4 Batch 82&83	84%	96%	86%	84%	78%
Agal3 Batch 84&85	93%	100%	90%	84%	104%
Agal4 Batch 84&85	91%	101%	86%	83%	88%

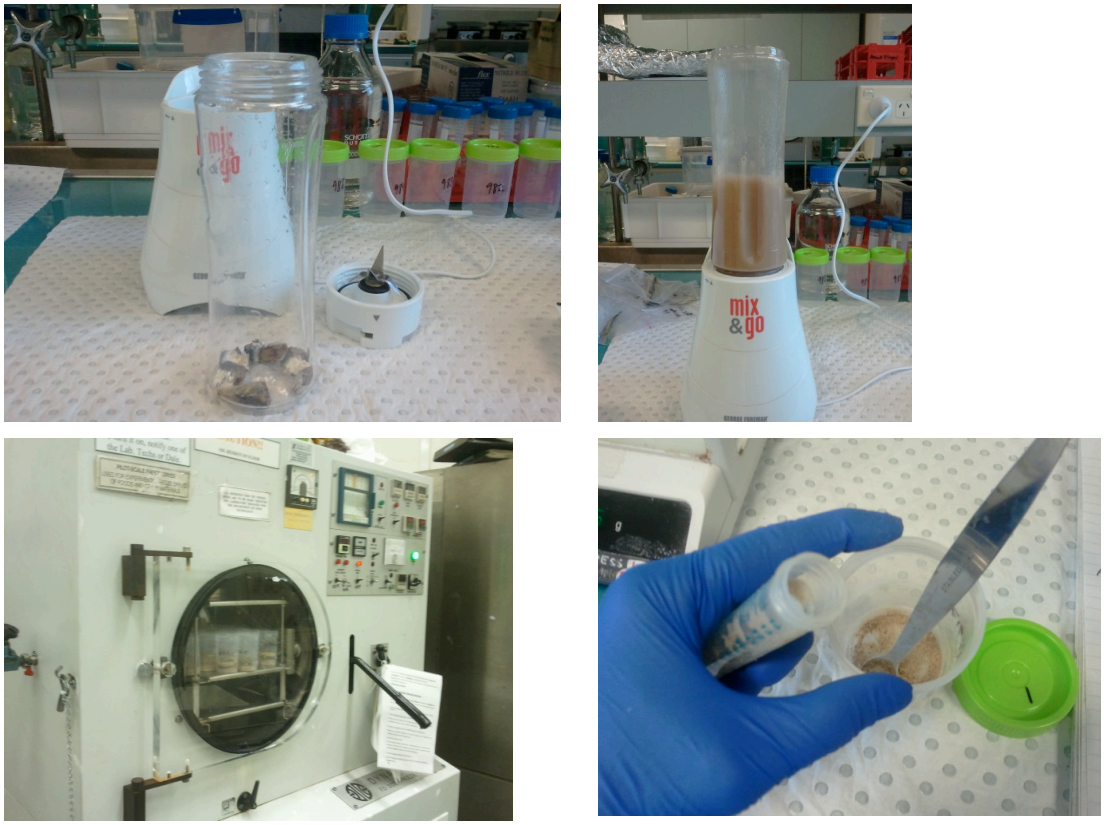

Figure 2.10: Fish sample preparation: fish cut in smaller pieces (top left), being homogenised (top right), freeze dried (bottom left), and re-homogenised and transferred into 14 mL falcon tubes (bottom right).

2.6.5. Faeces sample preparation

Faecal samples were prepared using the exact same procedure as for the blood samples (Chapter 2.6.2). In brief, samples were dried, acid-digested, filtered and delivered to the NMI, where five elements (arsenic, cadmium, mercury, lead and selenium) were determined using ICP-MS and ICP-AES. Details of recoveries are given in Table 2.4.

2.7. **Quality assurance**

As contamination and loss of analyte are of great concern in trace element analysis (Prichard *et al.*, 1996), special care was taken in the design

of this study to minimise both sources of bias. This was done in several ways. Great care was taken to store samples in clean containers. All samples were handled with gloves, in clean laboratories, with thoroughly cleaned utensils and equipments (acid-washed in 5% NO_3 where possible). Milli-Q water was carried to all field sessions and field blanks for both blood and faecal sampling were collected and treated as 'real' sample throughout the process. None of these field blanks returned results above the limit of reporting, so no corrections of any sample results were necessary. For each batch of nine subsamples (sample replicates), one procedural blank (Milli-Q water) was carried through the sample digestion process. Procedural blanks were analysed and replicate results were corrected for elements accordingly. This was done at the NMI prior to reporting of sample results.

Table 2.4: Mean percent recoveries of standard reference materials for Little Penguin faecal sample analysis at the NMI, Melbourne, Australia.

% Recovery	Arsenic	Cadmium	Mercury	Lead	Selenium
Agal3 Batch 61	81%	99%	80%	88%	64%
Agal4 Batch 61	68%	90%	87%	77%	35%
Agal3 Batch 62	81%	100%	75%	72%	63%
Agal4 Batch 62	83%	99%	94%	81%	37%
Agal3 Batch 63	81%	99%	87%	80%	92%
Agal4 Batch 63	81%	101%	98%	95%	84%
Agal3 Batch 64	84%	146%	103%	95%	101%
Agal4 Batch 64	83%	111%	97%	89%	88%
Agal3 Batch 65	73%	88%	99%	78%	91%
Agal4 Batch 65	64%	102%	98%	91%	80%
Agal3 Batch 66	83%	89%	96%	80%	95%
Agal4 Batch 66	73%	96%	93%	90%	88%
Agal3 Batch 67	82%	103%	96%	79%	97%
Agal4 Batch 67	71%	96%	92%	85%	87%
Agal3 Batch 68	74%	97%	94%	86%	82%
Agal4 Batch 68	75%	104%	92%	108%	79%
Agal3 Batch 69	80%	85%	101%	104%	89%
Agal4 Batch 69	75%	96%	96%	87%	81%
Agal3 Batch 70	81%	149%	93%	74%	104%
Agal4 Batch 70	80%	101%	93%	130%	87%
Agal3 Batch 71	80%	94%	91%	80%	100%
Agal4 Batch 71	83%	102%	92%	88%	86%
Agal3 Batch 72	81%	90%	91%	76%	94%
Agal4 Batch 72	78%	109%	94%	120%	84%
Agal3 Batch 73	86%	90%	94%	93%	99%
Agal4 Batch 73	78%	103%	98%	92%	88%
Agal3 Batch 74	78%	99%	82%	82%	94%
Agal4 Batch 74	75%	96%	80%	78%	79%
Agal3 Batch 75	83%	90%	87%	91%	97%
Agal4 Batch 75	83%	95%	82%	72%	88%
Agal3 Batch 76	77%	120%	84%	118%	94%
Agal4 Batch 76	74%	88%	80%	73%	79%
Agal3 Batch 77	84%	91%	98%	78%	99%
Agal4 Batch 77	82%	102%	96%	88%	85%

2.8. **Data treatment and analysis**

2.8.1. Study design

The study design dictates the types of statistical methods that can be applied during the analysis (Box *et al.*, 1978; Clarke & Green, 1988). All

penguins sampled in this study were selected at random and are considered to represent a valid cross-section of the population. Birds were caught where they were encountered, either in their burrows or on the way to and from their burrows. Birds under a certain weight or those appearing sick or injured were excluded from sampling (see details in Chapter 2.5.1). While this criteria skews the selection slightly towards the healthier, fitter part of the population, it was applied equally at all colonies, could not have been avoided and happened relatively seldom to be considered to not have a large impact on the results of this study. Only adult penguins were sampled, older than one year, as identified by their plumage. Even though I took notes of which penguins were found to be mated pairs (shared a burrow) wherever possible, the effect of pairing details was not considered during the statistical analysis, as it was not known for all samples.

2.8.2. Data cleaning

Data obtained for each metal or metalloid in Little Penguin blood, feather, faecal and fish prey samples underwent several data 'cleaning' algorithms: (1) scratching of data that was determined to be under the limit of reporting, (2) scratching of metal results in batches that returned unsatisfactory percentage recovery for standard reference materials, (3) analytical outlier detection, and (4) statistical outlier detection. All four algorithms are described in detail in the data chapters. Table 2.5 lists the number of blood and feather results retained for each metal at the conclusion of this process.

Table 2.5: Number (n) and percentage (%) of sample results retained for each sampling matrix and analyte following data cleaning algorithms ("-" = not analysed).

Analyte	Blood		Feather		Faeces		Fish	
	n	%	n	%	n	%	n	%
Aluminium	141	83%	35	100%	-	-	-	-
Arsenic	156	92%	35	100%	19	90%	60	100%
Boron	166	98%	-	-	-	-	-	-
Cadmium	22	13%	35	100%	18	86%	57	95%
Calcium	169	99%	-	-	-	-	-	-
Chromium	84	49%	-	-	-	-	-	-
Copper	168	99%	35	100%	-	-	-	-
Iron	170	100%	35	100%	-	-	-	-
Mercury	164	96%	35	100%	19	90%	60	100%
Lead	164	96%	35	100%	18	86%	52	87%
Selenium	166	98%	35	100%	19	90%	60	100%
Tin	62	46%	-	-	-	-	-	-
Zinc	170	100%	35	100%	-	-	-	-

2.8.3. Statistical strategy

The statistical strategy followed in this study is presented in Figure 2.11. Predictor variables were, for instance, location, sex, season (breeding, moult and non-breeding), year, body weight, flipper length, total head length and type of sampling matrix. Response variables in most instances were the metal or metalloid concentrations measured. The exact statistical method

employed depended on the particular research question posed, details of which are given in each of the data chapters.

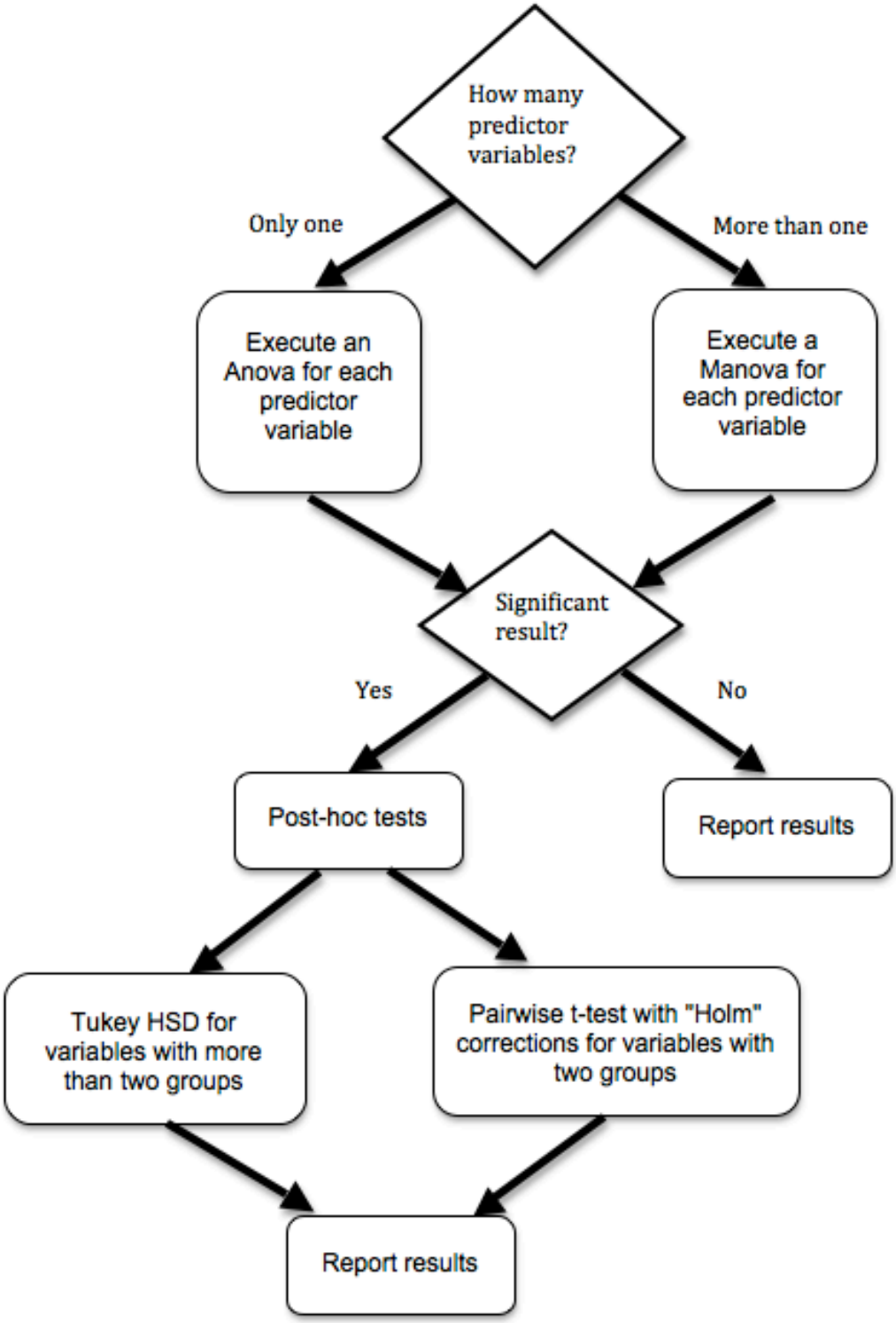

Figure 2.11: Flowchart diagram of the statistical strategy

As an example, one of the most fundamental questions was whether metal concentrations in Little Penguins differed between the locations sampled. After the data cleaning step nine metals / metalloids remained, which equals nine response variables. Following the flowchart in Figure 2.11, I executed a One-way Manova for the combination of metals / metalloids (transformed as needed to fulfil the assumptions for the test) by location. There was a significant difference between locations. However, the Manova result does not specify which of the nine metals / metalloids are significantly different, nor for which locations. To get those details, one must perform post-hoc tests. In Chapter 3, I compared three locations (groups): St Kilda, Phillip Island and Notch Island - hence I would follow up the significant Manova result with a Tukey HSD test. In Chapter four, I compared two locations (groups): St Kilda and Phillip Island. Hence, I performed pairwise *t*-tests with "Holm" correction for each metal / metalloid. The results were reported for each metal / metalloid, specifying exactly which locations differed for which metal / metalloid.

Statistical analyses were executed using R version 3.2.3 (R Core Team 2014) and SPSS (version 21, SPSS Inc., Chicago, IL). Significance was taken to be $p < 0.05$ for all statistical analyses, unless specified otherwise. All statistical tests were performed once the data passed the appropriate assumption tests. This included statistical extreme outlier detection, transformations, followed by tests for normality and homogeneity of variance (Logan, 2011). The details of transformations and extreme outliers removed are given in the Supplementary Materials for each data chapter, presented in the Appendices. Results were presented in text, as boxplot figures or in

tables. Correlations were conducted for data either using the Kendall test ($n > 30$) or Spearman's rank test ($n < 30$) (Logan, 2011).

Often in ecological research, we are interested in how the composition of a community changes between different sites. One very powerful statistical tool used to analyse and visually present such multivariate data is non-metrical multidimensional scaling (NMDS) (Kenkel & Orlóci, 1986). NMDS collapses information from complex datasets with multiple response variables into a reduced number of dimensions, so they can be visualised and interpreted (Dixon, 2003). I have chosen this technique over alternative ordination tools, such as Principle Component Analysis (PCA) (Jolliffe, 2002), because PCA uses absolute distance to ordinate between groups. Applying the PCA technique would therefore cause essential elements, such as iron, calcium and zinc, which are present in large concentrations in my data, to have a disproportionate effect on the result of the analysis. Contrary to PCA, NMDS uses rank orders, which are rankings of distances between points. This means NMDS can be applied to a large variety of data and is not sensitive to data transformations (Kenkel & Orlóci, 1986). The graphical results of the analyses and their interpretations are presented in each of the data chapters.

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Chapter 3

The Little Penguin (Eudyptula minor) as an indicator of coastal trace metal pollution

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Little Penguin (*Eudyptula minor*) nesting between rocks of the St Kilda breakwater (Photo courtesy: Earthcare St Kilda)

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GRADUATE RESEARCH CENTRE

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ABSTRACT

Monitoring trace metal and metalloid concentrations in marine animals is important for their conservation and could also reliably reflect pollution levels in their marine ecosystems. Concentrations vary across tissue types, with implications for reliable monitoring. We sampled blood and moulted feathers of the Little Penguin (*Eudyptula minor*) from three distinct colonies, which are subject to varying levels of anthropogenic impact. Non-essential trace metal and metalloid concentrations in Little Penguins were clearly linked to the level of industrialisation adjacent to the respective foraging zones. This trend was more distinct in blood than in moulted feathers, although we found a clear correlation between blood and feathers for mercury, lead and iron. This study represents the first reported examination of trace metals and metalloids in the blood of any penguin species and demonstrates that this high trophic feeder is an effective bioindicator of coastal pollution.

3. The Little Penguin (*Eudyptula minor*) as an indicator of coastal trace metal pollution

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Abstract

Monitoring trace metal and metalloid concentrations in marine animals is important for their conservation and could also reliably reflect pollution levels in their marine ecosystems. Concentrations vary across tissue types, with implications for reliable monitoring. We sampled blood and moulted feathers of the Little Penguin (*Eudyptula minor*) from three distinct colonies, which are subject to varying levels of anthropogenic impact. Non-essential trace metal and metalloid concentrations in Little Penguins were clearly linked to the level of industrialisation adjacent to the respective foraging zones. This trend was more distinct in blood than in moulted feathers, although we found a clear

correlation between blood and feathers for mercury, lead and iron. This study represents the first reported examination of trace metals and metalloids in the blood of any penguin species and demonstrates that this high trophic feeder is an effective bioindicator of coastal pollution.

Capsule Abstract: This study confirms the suitability of the Little Penguin as a bioindicator of coastal metal pollution in coastal areas using non-destructive sampling methods.

Keywords

Trace element, Blood, Seabird, Australia, Bioindicator

3.1. Introduction

Toxicants are present in our environment, particularly in the marine environment, because the ocean acts as a sink (Neff, 2002). Influx from rivers, agricultural and urban land runoff, sewage outfalls, long-range atmospheric transport and deposition all contribute to elevated levels of contaminants in marine ecosystems (Lamborg *et al.*, 2014). Monitoring this pollution and assessing its ecological and human health effects has become a global concern (Islam & Tanaka, 2004). Metals in particular, have been shown to have a negative impact on coastal ecosystems in highly industrialised areas (Geens *et al.*, 2010). Metals are released by both natural (erosion, volcanism, upwelling) and anthropogenic sources (mining, smelting, metallurgy), often accumulate in organisms at higher trophic levels and have been studied in a range of seabirds to investigate temporal and spatial variation (Furness, 1993; Becker, 2003; Vo *et al.*, 2011).

Many researchers report on trace metal concentrations in internal tissues and samples are often collected destructively, i.e. by sacrificing randomly selected members of a population (Bacher, 1985; Smichowski *et al.*, 2006), or opportunistically, i.e. starved or killed 'by misadventure' (Lock *et al.*, 1992; Gibbs, 1995; Choong *et al.*, 2007). While the former has ethical implications, and cannot be applied to species of conservation concern, the latter carries potentially unquantified biases including (1) the sample not being representative of a random cross-section of the population, (2) unknown provenance, (3) unknown cause of death, and (4) altered lipid content in a starved organ which can lead to falsely elevated elemental concentrations (Bryan *et al.*, 2007). Feather collection is the most common non-destructive sampling protocol (Burger, 1993). Moulted feathers can be obtained non-invasively and transported and stored with minimal cost. Shunting non-essential trace metals to the feathers is a main method of detoxification in seabirds (Furness *et al.*, 1986), but feather metal concentrations are often a poor indicator for internal tissue concentrations (Eagles-Smith *et al.*, 2008; Lavers & Bond, 2013) and results can be highly biased by external contamination (Jaspers *et al.*, 2004).

Recent advances in trace analysis such as atomic absorption spectrometry (AAS) and inductively-coupled plasma spectrometry (ICP) promote non-destructive cross-section sampling, e.g. the taking of a small sample of blood (< 2 mL) from a free-ranging bird without sacrificing it (Becker, 2003). The success of non-lethal blood sampling techniques is evident in the literature (Eagles-Smith *et al.*, 2008; Carvalho *et al.*, 2013; Fort *et al*., 2014). Trace element concentrations in avian blood reflect current

dietary exposure and often correlate strongly with those in internal tissues, presenting a potentially more suitable non-lethal matrix than feathers for investigating a biologically relevant contaminant load (Monteiro & Furness, 2001).

Penguins are ideal sentinels for marine pollution monitoring (Boersma, 2008) due to their high trophic position, long lifespan, philopatry, conspicuous nature and our extensive knowledge of their life history (Trathan *et al.*, 2014). Compared to other seabird bioindicators, they have the advantage of being relatively sedentary, therefore reflecting local conditions (Ropert-Coudert *et al.*, 2004). While penguins have been used in ecotoxicological studies using internal tissues and feathers (Gibbs, 1995; Smichowski *et al.*, 2006; Jerez *et al.*, 2011), there are to date no trace metal concentration data published from the blood of any penguin species.

Little Penguins (*Eudyptula minor*) are the smallest of the penguins, breeding on offshore islands and small mainland colonies in southern Australia and New Zealand (Marchant & Higgins, 1990). Extensive research has been conducted on their breeding and foraging ecology (Reilly & Cullen, 1981; Fortescue, 1999; Dann & Chambers, 2013). Little Penguins are generalist feeders whose diet can vary greatly among colonies and even years at the same colony (Chiaradia *et al.*, 2010; Kowalczyk *et al.*, 2013). They inhabit a wide range of coastal habitats - from relatively pristine offshore islands in the Bass Strait (Hoskins *et al.*, 2008) to human-made structures in highly urbanised bays (Preston *et al.*, 2008). Little Penguins from the St Kilda colony (Figure 3.1) forage exclusively within Port Phillip Bay (Preston *et al.*, 2008) a semi-enclosed body of water bordering the highly industrialised

metropolis of Melbourne, Australia. This relatively shallow bay (mean depth 13.6 m, area 1930 km^2) is joined to Bass Strait through a 3 km-wide channel and contains areas with highly contaminated sediments due to historical and current industrial discharges through storm water run-off (Harris *et al.*, 1996). Semi-enclosed coastal areas are contamination hotspots; with sediments remaining polluted, sometimes for decades after the primary source is removed due to restricted currents and wave action (Aly *et al.*, 2012). Notably, little is known about the contaminant load in Little Penguins breeding at St Kilda and how it compares to that of conspecifics at less urban environments, such as the nearby Phillip Island colony and more remote Bass Strait island colonies.

Our emphasis in this study was to evaluate high-level trophic feeding Little Penguins as indicators of the presence of bioavailable metals and metalloids within their relatively small foraging areas. The specific objectives were to: (1) investigate the spatial variation of trace metals and metalloids in the blood and feathers of Little Penguins; and (2) investigate correlations between the two sampling matrices in order to (3) assess the usefulness of blood and feathers as non-destructive sampling matrices for assessing exposure of Little Penguins to trace metal and metalloid pollution.

3.2. Materials and methods

3.2.1. Study sites

We collected blood (n = 31) and moulted feathers (n = 35) from wild Little Penguins during February and March of 2012 at three locations: St Kilda, Phillip Island and Notch Island (Figure 3.1). The St Kilda colony is

located 5 km from the central business district of Melbourne, Australia (37˚51'S, 144˚57'E), where approximately 1,000 Little Penguins nest on a 650 m long man-made breakwater structure (Z. Hogg, unpublished data). St Kilda Little Penguins remain in Port Phillip Bay throughout the year and feed predominantly on Australian anchovy (*Engraulis australis*) (Preston *et al.*, 2008), but their diet varies depending on prey availability (Kowalczyk *et al.*, 2013). Phillip Island (38˚30'S, 145˚10'E) is located 140 km southeast of Melbourne, Australia, and has approximately 32,000 penguins nesting on the Summerland Peninsula (Sutherland & Dann, 2012). Phillip Island Little Penguins feed on a range of clupeoid fish (Australian anchovy, Pilchard *Sardinops sagax,* Barracouta *Thyrsites atun*, Red Cod *Pseudophysis bachus*) and Arrow squid (*Nototodarus gouldi*) in Bass Strait (Chiaradia *et al*., 2010), but during the winter months often forage within Port Phillip Bay, partially overlapping with the foraging area of penguins from the St Kilda colony (Chiaradia *et al.*, 2012). Notch Island (38˚94'S, 146˚68'E) lies 19 km east of Wilsons Promontory, Victoria, Australia, and home to approximately 660 penguins in summer (Schumann *et al.*, 2014). The foraging ecology of Notch Island's penguins is not well-studied, but satellite tracking in 2007-08 showed they fed in open Bass Strait waters, away from terrestrial point sources of anthropogenic pollution (P. Dann, unpublished data). Schumann (2012) reported that penguins at Notch Island fed predominantly on unidentified post-larval fish, Australian pilchard, Barracouta and Arrow squid.

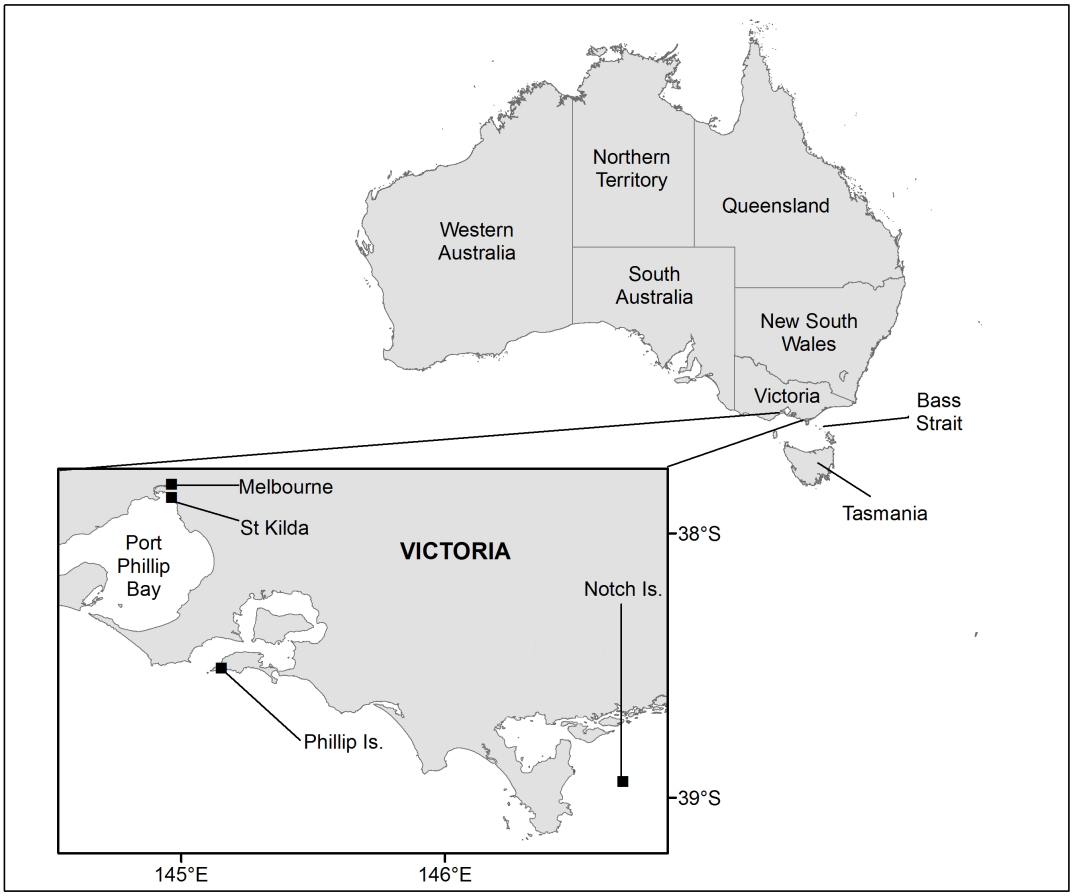

Figure 3.1: Little Penguin breeding colonies sampled in February and March 2012: St Kilda, Phillip Island and Notch Island.

3.2.2. Sample collection

All samples collected in this study were from moulting penguins. Penguins undergo a complete (catastrophic) moult over a period of 15 to 20 days, during which time they cannot return to sea to forage (Brasso *et al.*, 2013). Adult Little Penguins forage extensively for a few weeks at the end of each breeding season, increasing their weight considerably (Reilly & Cullen, 1983). While moulting, penguins fast and become increasingly anorexic. Trace element concentrations circulating in the blood at the time of moult are thought to be a combination of what has been consumed in the weeks of pre-

moult feeding and a remobilisation of especially non-essential metals sequestered from internal tissues (Furness *et al.*, 1986; Bearhop *et al.*, 2000). The blood supply to feathers is closed off after formation of the new feathers. Therefore, the data presented here are an indication of the contaminant body burden in 2011 (via feathers moulted in 2012) and 2012 (via 2012 blood).

We caught Little Penguins individually by hand from their burrows and plucked a handful of moulting feathers from the dorsal and another from the ventral region of the penguin (~30 feathers in total). We kept the feathers in labelled sterile, laboratory-grade press and seal bags stored at room temperature. We aspirated up to 2 mL of blood from the medial metatarsal (caudal tibial) vein using a 25-gauge butterfly needle with a 3 mL syringe (Sergent *et al.*, 2004) and transferred it into 6 mL Vacutainers® (BD Diagnostics, trace element tube plus K_2EDTA, product number 368381). We placed blood samples in a cooler with ice packs, transferred them to a freezer within 12 hours of sampling and kept them frozen at -20˚C until analysis.

3.2.3. Trace element analysis

Blood samples were thawed at room temperature before being homogenised. We divided each blood sample into two or three replicate samples of 0.5 g aliquots, depending on available blood volume. We dried replicate samples to constant weight at 60ºC in 50 mL digestion tubes (DigiTubes by SCP Science, product number 010-500-261) for ≥ 48 hours (mean drying quotient: 5.87 ± 0.88 SD). A sublimation test found no significant losses during the drying process (Tables S1 and S2, Supplementary Material). The dried blood samples were then digested in 3 mL 65% nitric acid

(SUPRAPUR, trace metal grade, Merck) and 0.5 mL 37% hydrochloric acid (EMSURE, trace metal grade, Merck) for 3 hours at 95ºC with one intermittent vortex. Then we cooled, filtered (syringe filter, 28 mm diameter, 0.45 µm pore size) and diluted the solutions with Milli-Q^{TM} water to a final volume of 14 mL. For quality assurance, we ran each batch of nine replicate samples with one procedural blank (Milli-Q^{TM} ultrapure water) and two Standard Reference Materials (SRM) provided by the National Measurement Institute (NMI), Melbourne, Australia: AGAL3 (prawn tissue) and AGAL4 (bovine liver tissue). Aluminium (Al), calcium (Ca), iron (Fe) and zinc (Zn) were analysed at the NMI using a Perkin Elmer Optima 8300 Dual View Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) with a limit of reporting of 0.5, 10, 2 and 0.01 mg/kg, respectively; while arsenic (As), boron (B), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and tin (Sn) were analysed on an Agilent 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) with a limit of reporting of 0.01 mg/kg. All results were corrected for procedural blanks. Mean percentage recoveries of SRM in blood samples ranged from 71% to 143% for trace elements analysed. We excluded replicate results of samples run concurrently with SRM, where both SRM for that element returned recoveries outside 70% - 130% from statistical analysis. If only one replicate result remained for the sample, we excluded that sample result for that element; else we took the mean of the (remaining) replicate results.

We cleaned feather samples by vigorously washing them in Milli-Q water three times. We oven-dried feathers at 45˚C for ≥ 48 hours until constant weight and delivered them to NMI for acid digestion and analysis of

Al, As, Cd, Cu, Fe, Hg, Pb, Se and Zn, using the same instrumentation and limits of reporting as for blood analysis. Mean percentage recoveries of SRM in feathers ranged from 78% to 102%. All feather samples returned results over the limit of reporting and had percentage recovery rates within the range of 70% to 130%.

We detected analytical outliers (i.e. outliers within replicate samples due to instrumental and laboratory variations) in both blood and feather samples following the NMI's outlier detection protocol (NMI, 2014), i.e. calculating the percentage relative standard deviation (%RSD) for each replicate sample and trace metal:

$$\%RSD = (\text{standard deviation} / \text{mean}) * 100$$

We then executed an acceptance test: replicate results that were lower than ten times the limit of reporting were accepted if the calculated %RSD was ≤ 38 for duplicates and ≤ 45 for triplicates. Replicate results that were higher than ten times the limit of reporting were accepted if the calculated %RSD was ≤ 24 for duplicates and ≤ 29 for triplicates. We excluded all replicate results failing the acceptance test from further analysis. In triplicate samples, if one replicate result failed the analytical outlier detection test, we removed this replicate result. We then recalculated %RSD for the remaining two replicate results and applied the acceptance test for duplicate samples stated above. We report final results as mg/kg dry weight (dw).

3.2.4. Statistical analyses

Data were statistically analysed using R version 3.1.1 (R Core Team, 2014) and SPSS (version 20, SPSS Inc., Chicago, IL). We took significance to

be $p < 0.05$ for all statistical analyses. Results under the limit of reporting were excluded from further analysis. Statistical population outliers (defined by > 3 * Cook's D), normality of distribution and residual normality for each element were tested using the Shapiro Wilk test and exploratory graphics. We used Levene's test to investigate homogeneity of variances and applied transformations to data as follows: blood concentrations of Al, As, Ca, Hg and Se were log- and B concentrations were arcsin-transformed; while feather concentrations of Al, As, Cd, Fe and Pb were log-transformed to meet normality assumptions of our statistical analysis. We executed non-metric multidimensional scaling (NMDS) using the R package 'vegan' (Oksanen *et al.*, 2015) to analyse dissimilarity among colonies. We conducted a one-way multivariate analysis of variance (MANOVA) for each tissue type to test whether the effect of the linear combination of all trace metal concentrations was significantly different among locations sampled. Where this was significant, we ran post-hoc tests (Tukey HSD) to investigate interactions among locations. We did not compare differences in trace metal concentrations between genders since sample sizes were too small for multivariate analysis. We executed Spearman correlations to measure the strength of the association between tissue types using a subset of penguins, where both blood and feathers were collected (paired data, n = 19).

3.3. Results

3.3.1. Trace elements in blood of Little Penguins

Cadmium (Cd), chromium (Cr) and tin (Sn) concentrations for most samples were either below the limit of reporting (0.01 mg/kg for all), returned

SRM percentage recovery results outside the acceptable range (70% - 130%) or failed the analytical outlier detection test, therefore we excluded these elements from statistical analysis. Concentrations of the remaining ten elements in the blood sampled at the three locations in February and March 2012 are given in Table 3.1. We found the greatest differences in mean concentrations among locations for the non-essential elements As, Hg and Pb (Figure 3.2, left panels). This was supported by NMDS analysis, which identified As, Hg and Pb as the largest vectors for dissimilarity among colonies (Figure 3.6 A). We found a statistically significant difference among the three locations sampled for mean trace element concentrations in the blood of Little Penguins, $F_{20,30} = 5.869$, $p < 0.001$; Wilk's $\lambda = 0.041$; partial $\eta^2 = 0.796$. Tukey HSD post-hoc tests showed that mean As, Hg and Pb blood concentrations were significantly different between St Kilda and Phillip Island ($p < 0.001$ for As and Hg, $p = 0.001$ for Pb), as well as between St Kilda and Notch Island ($p < 0.001$ for As and Hg, $p = 0.001$ for Pb), but not between Phillip Island and Notch Island. For the essential elements (Figure 3.2, right panels), mean Fe concentrations at Notch Island were significantly different when compared to St Kilda ($p = 0.018$) or Phillip Island ($p = 0.004$) but not between St Kilda and Phillip Island. Mean Se concentrations were marginally different between St Kilda and Phillip Island ($p = 0.06$), similar at Phillip Island and Notch Island, but significantly different between St Kilda and Notch Island ($p = 0.001$). Mean Zn concentrations differed significantly between St Kilda and Phillip Island ($p = 0.036$) as well as between Phillip Island and Notch Island ($p = 0.015$), but not between St Kilda and Notch Island.

3.3.2. Trace elements in feathers of Little Penguins

Concentrations of nine trace elements analysed in feathers of Little Penguins sampled at three locations in February and March 2012 are given in Table 3.2. Mean Pb concentrations in feathers showed the greatest differences among locations, with St Kilda penguin feathers containing on average 4.2 times and 5.2 times more Pb than penguin feathers from Phillip Island and Notch Island, respectively. The distribution of element concentrations in feathers was distinctly different at each of the three colonies sampled and their relatedness did not overlap when presented in an NMDS plot (Figure 3.6 B). One-way MANOVA found a significant effect for the distribution of mean trace element concentrations in the feathers of Little Penguins at the three locations sampled, $F_{18, 48} = 13.445$, $p < 0.001$; Wilk's $\lambda = 0.027$; partial $\eta^2 = 0.834$. Tukey HSD post-hoc tests found significant differences among locations in non-essential elements As, Cd, Hg and Pb (Figure 3.3) and essential elements Al, Fe and Se (Figure 3.4). Specifically, mean As and Cd feather concentrations were similar at St Kilda and Phillip Island, but significantly different between St Kilda and Notch Island ($p = 0.029$ for As, $p = 0.026$ for Cd), and Phillip Island and Notch Island ($p = 0.013$ for As, $p = 0.028$ for Cd). Aluminium, Fe, Hg and Se were significantly different among each of the locations sampled ($p < 0.05$ for all), while Pb at St Kilda was significantly different from both, Phillip Island ($p < 0.001$) and Notch Island ($p < 0.001$), but not between Phillip Island and Notch Island.

Table 3.1: Mean trace metal concentrations (mg/kg dry weight) ± standard deviation (sample number for that element, if different from the value given in first column) in whole blood of Little Penguins and other comparable species of birds, sorted by geographical distance from Australia. Blood analysed from adults unless otherwise specified. Range (where available) is given in square brackets. Absence of data is shown as '-'. <DL represents a value determined that was below detection limit. The levels of pollution given here do not reflect absolute values, but were chosen based on proximity to metropolitan centre as identified by author or background knowledge (NA = not available). * mean ± standard error ** geometric mean

Species	Year	Location	Level of Pollution	Al	As	B	Ca	Cu	Fe	Pb	Hg	Se	Zn	Source
Little Penguin (n = 10)	2012	St Kilda, Victoria, Australia	High	3.89 ± 1.26 [2.50 - 6.90]	3.72 ± 1.76 [1.40 - 6.97]	0.70 ± 0.21 [0.45 - 1.20]	408.5 ± 89.8 [315.0 - 626.7]	2.48 ± 0.44 [1.77 - 3.10]	2147 ± 228 [1833 - 2433]	0.07 ± 0.02 [0.05 - 0.12]	2.75 ± 0.85 [1.60 - 4.47]	12.46 ± 4.41 [6.1 - 18.7]	37.97 ± 5.28 [31.7 - 47.0]	This study
Little Penguin (n = 11)	2012	Phillip Is, Victoria, Australia	Medium	3.19 ± 0.84 (10) [1.87 - 4.30]	1.07 ± 1.22 [0.20 - 4.57]	0.68 ± 0.16 [0.52 - 1.13]	348.9 ± 49.7 [266.7 - 430.0]	2.14 ± 0.42 [1.27 - 2.90]	2162 ± 159 [1950 - 2433]	0.04 ± 0.01 [0.02 - 0.06]	0.86 ± 0.23 (10) [0.49 - 1.17]	19.20 ± 5.22 [10.0 - 25.0]	33.47 ± 3.27 [29.0 - 41.0]	This study
Little Penguin (n = 10)	2012	Notch Is, Victoria, Australia	Low	4.22 ± 1.67 (9) [2.4 - 6.7]	0.67 ± 0.43 [0.16 - 1.43]	0.81 ± 0.58 [0.59 - 2.47]	372.5 ± 59.3 [313 - 515]	2.32 ± 0.40 [1.70 - 2.93]	1877 ± 153 [1600 - 2033]	0.04 ± 0.01 (9) [0.02 - 0.08]	0.84 ± 0.37 [0.44 - 1.50]	26.85 ± 13.75 [12.3 - 50.0]	38.77 ± 6.76 [30.0 - 51.7]	This study
Pied Oystercatcher (n = 10)	1998	Mangere Inlet, New Zealand	High	-	-	-	-	-	-	0.45±0.14 [0.25 - 0.76]	0.16 ± 0.05 [0.10 - 0.24]	-	-	Thompson & Dowding, 1999
Pied Oystercatcher (n = 17)	1998	Sth Kaipara, New Zealand	Low	-	-	-	-	-	-	0.22 ± 0.04 [0.25 - 0.76]	0.16 ± 0.05 [0.07 - 0.26]	-	-	Thompson & Dowding, 1999
Spectacled Petrel (n = 38)	2006, 2007	Brazil	NA	-	-	-	-	4.77 ± 4.46 [0.79 - 20.77]	-	9.30 ± 4.33 [5.02 - 26.03]	3.41 ± 2.14 [0.84 - 9.86]	-	14.11 ± 3.03 [10.95 - 28.02]	Carvalho *et al*. 2013
White-chinned Petrel (n = 30)	2006, 2007	Brazil	NA	-	-	-	-	3.49 ± 1.82 [0.62 - 10.40]	-	8.21 ± 3.53 [5.72 - 24.03]	3.20 ± 3.67 [0.20 - 15.82]	-	13.64 ± 2.76 [10.73 - 24.69]	Carvalho *et al*. 2013
Black-footed albatross chick (n = 30)	2002	Torishima Is, Japan	Low	-	-	-	-	2.33 ± 0.17	-	0.05 ± 0.02	3.12 ± 1.32	8.8 ± 2.4	17.4 ± 1.5	Ikemoto *et al*., 2005
Little Auk (n = 14)	2011	Newfound-land, Canada	Medium	-	-	-	-	-	-	-	2.86±0.78 [1.72-4.25]	-	-	Fort *et al*. 2014
Little Auk (n = 63)	2009, 2010	East Greenland, Canada	Low	-	-	-	-	-	-	-	0.84±0.20 [0.44-1.25]	-	-	Fort *et al*. 2014
Osprey chick (n = 10)	2000	Baltimore Harbor, Maryland, USA	High	<DL	1.18** [0.83 - 1.68]	2.01** [<ML - 2.41]	-	1.38** [1.17 - 2.02]	1926** [1780 - 2110]	<DL	0.17** [0.11 - 0.28]	5.75** [4.44 - 7.86]	24.9** [21.4 - 29.0]	Rattner *et al*., 2008
Osprey chick (n = 12)	2000	South River, Maryland, USA	Low	<DL	0.55** [<ML - 1.05]	2.12** [<ML - 2.56]	-	1.30** [1.10 - 1.67]	1916** [1780 - 2170]	<DL	0.18** [0.11 - 0.24]	6.81** [5.00 - 7.89]	22.8** [19.2 - 27.7]	Rattner *et al*., 2008
Great tit (n = 16)	2006	Antwerp, Belgium	High	-	-	-	-	0.52 ± 0.08*	-	1.12 ± 0.08*	-	-	22 ± 2*	Geens *et al*., 2010
Great tit (n = 10)	2006	Antwerp, Belgium	Low	-	-	-	-	0.52 ± 0.12*	-	0.08 ± 0.08*	-	-	27.6 ± 2.8*	Geens *et al*., 2010

Note: Species are as follows, Little Penguin *E. minor*, Pied Oystercatcher *H. longirostris*, Spectacled Petrel *P. conspicillata*, White-chinned Petrel *P. aequinoctialis*, Little Auk *A. alle*, Osprey *P. haliaetus*, Great Tit *P. major*.

Table 3.2: Mean trace metal concentrations (mg/kg) ± standard deviation (sample number for that element, if different from the value given in first column) in feathers of Little Penguins and other comparable species of birds, sorted by geographical distance from Australia. Feathers analysed from adults unless otherwise specified. Range values (where available) are given in square brackets. Absence of data is shown as '-'. <DL represents a value determined that was below detection limit. The levels of pollution given here do not reflect absolute values, but were chosen based on proximity to metropolitan centre as identified by author or background knowledge (NA = not available).

Species	Year	Location	Level of Pollution	Al	As	Cd	Cu	Fe	Pb	Hg	Se	Zn	Source
Little Penguin (n = 13)	2012	St Kilda, Victoria, Australia	High	40.38 ± 22.96 [17 - 91]	0.16 ± 0.05 [0.07 - 0.24]	0.04 ± 0.02 [0.012 - 0.07]	11.42 ± 2.19 [7.7 - 15.0]	71.31 ± 43.99 [29 - 170]	0.42 ± 0.20 [0.16 - 0.80]	4.13 ± 0.98 [1.7 - 5.9]	2.31 ± 0.59 [1.4 - 3.5]	84.77 ± 11.28 [64 - 100]	This study
Little Penguin (n = 12)	2012	Phillip Is, Victoria, Australia	Medium	16.78 ± 16.11 [6.3 - 67]	0.18 ± 0.10 [0.06 - 0.40]	0.04 ± 0.03 [0.01 - 0.13]	10.77 ± 1.50 [8.6 - 13.0]	28.0 ± 16.2 [13 - 77]	0.08 ± 0.03 [0.03 - 0.12]	2.7 ± 0.37 [2.1 - 3.2]	4.21 ± 0.94 [2.7 - 5.6]	80.58 ± 8.06 [65 - 89]	This study
Little Penguin (n = 10)	2012	Notch Is, Victoria, Australia	Low	6.25 ± 2.79 [2.8 - 9.6]	0.09 ± 0.03 [0.03 - 0.13]	0.06 ± 0.02 [0.03 - 0.10]	10.54 ± 2.07 [8.5 - 16.0]	13.03 ± 3.64 [8.2 - 19.0]	0.10 ± 0.05 [0.05 - 0.18]	1.50 ± 0.82 [0.87 - 3.50]	3.11 ± 0.65 [2.4 - 4.2]	76.80 ± 7.15 [69 - 91]	This study
Little Penguin (n = 10)	2008 - 2012	Phillip Island, Victoria, Australia	Medium	-	-	-	-	-	-	2.92 ± 2.88	-	-	Brasso *et al*. 2013
Little Penguin (n = 12)	2012	Garden Is, South Western Australia	High	-	-	0.031 ± 0.01 [0.015 - 0.055]	-	-	0.45 ± 0.28 [0.15 - 0.75]	2.84 ± 0.74 [1.6 - 3.8]	3.41 ± 0.48 [2.5 - 4.2]	-	Dunlop *et al*. 2013
Little Penguin (n = 9)	2009	Penguin Is, South Western Australia	Medium	-	-	0.043 ± 0.02 [0.025 - 0.073]	-	-	0.93 ± 0.9 [0.32 - 3.10]	1.67 ± 0.62 [0.78 - 2.6]	2.73 ± 0.99 [1.6 - 4.8]	-	Dunlop *et al*. 2013
Little Penguin (n = 10)	2010	Mistaken Is, South Western Australia	Medium	-	-	0.093 ± 0.054 [0.03 - 0.20]	-	-	0.82 ± 0.98 [0.28 - 3.4]	1.32 ± 0.66 [0.82 - 3]	5.9 ± 1.41 [3.9 - 8.6]	-	Dunlop *et al*. 2013
Little Penguin (n = 10)	2009	Woody Is, South Western Australia	Low	-	-	0.302 ± 0.445 [0.049 - 1.50]	-	-	0.38 ± 0.13 [0.16 - 0.58]	0.61 ± 0.17 [0.44 - 0.89]	3.07 ± 0.78 [2.2 - 4.4]	-	Dunlop *et al*. 2013
Little Penguin (n = 5)	<1983	New Zealand	NA	-	-	0.4 ± 0.5	29.4 ± 11.8	-	1.7 ± 1.7	3.4 ± 3.7	-	99.2 ± 16.0	Lock *et al*. 1992
Silver Gull (n = 45)	1996	Sydney, Australia	High	-	-	0.38 ± 0.34	-	-	10.10 ± 3.78	0.82 ± 1.31	0.51 ± 0.25	-	Burger & Gochfeld, 1999
Silver Gull (n = 15)	1996	Blue Mountains NP, Australia	Low	-	-	0.01 ± 0.05	-	-	3.81 ± 1.42	0.96 ± 1.06	0.62 ± 0.35	-	Burger & Gochfeld, 1999
Flesh-footed Shearwater (n = 33)	2008	Woody Is, South Western Australia	NA	222.45 ± 195.80	3.09 ± 8.57	0.29 ± 0.82	18.38 ± 3.05	-	0.52 ± 0.37	6.04 ± 4.0	-	92.24 ± 33.94	Bond & Lavers, 2011
Flesh-footed Shearwater (n = 18)	2008	Lord Howe Is, Eastern Australia	NA	63.26 ± 34.52	0.24 ± 0.16	0.09 ± 0.1	9.63 ± 1.41	-	0.47 ± 0.29	11.22 ± 5.61	-	8.59 ± 24.06	Bond & Lavers, 2011

Flesh-footed Shearwater (chicks, n = 37)	2011	Lord Howe Is, Eastern Australia	NA	112.53 ± 72.79	0.22 ± 0.13 (n = 29)	0.49 ± 0.17 (n = 6)	14.64 ± 16.99	-	0.30 ± 0.29	2.40 ± 1.70	-	91.70 ± 11.23	Lavers *et al.* 2014
Adelie Penguin (n = 10)	1981	Antarctica	Low	-	-	0.20 ± 0.09 [0.07 - 0.45]	12.9 ± 1.45 [9.42 - 15.9]	23 ± 15.3 [5.71 - 66.2]	0.28 ± 0.07 [0.12 - 0.54]	0.172 ±0.045 [0.008 - 0.304]	-	78.2 ± 5.3 [64 - 100]	Honda *et a*l. 1986
Gentoo Penguin (n = 20)	2005 - 2012	King George Is, Antarctica	Medium	39.76 ± 24.74[1]	0.05 ± 0.04[1]	0.03 ± 0.03[1] (n = 7)	16.44 ± 3.16[1]	77.60 ± 134.55[1]	0.51 ± 0.46[1]	0.28 ± 0.05[2] (n = 10)	2.46 ± 0.70[1]	85.12 ± 14.84[1]	[1]Jerez *et a*l. 2011, [2]Brasso *et al*. 2013
Gentoo Penguin (n = 17)	2005 - 2007	Ronge Is, Antarctica	Low	22.92 ± 27.87	0.04 ± 0.02 (n = 8)	0.03 ± 0.01 (n = 8)	16.02 ± 2.09	69.45 ± 109.94	0.25 ± 0.44	-	2.15 ± 0.80	72.89 ± 7.46	Jerez *et al*. 2011
Chinstrap Penguin (n = 25)	2005 - 2012	King George Is, Antarctica	Medium	132.4 ± 198.1[1]	0.10[1] (n = 1)	< DL	20.29 ± 8.30[1]	126 ± 103.63[1]	1.76 ± 1.74[1]	0.69 ± 0.18[2] (n = 10)	4.44 ± 1.71[1]	77.12 ± 45.15[1]	[1]Jerez *et a*l. 2011, [2]Brasso *et al*. 2013
Chinstrap Penguin (n = 20)	2005 - 2007	Ronge Is, Antarctica	Low	14.26 ± 9.72	0.05 ± 0.03 (n = 7)	0.10 ± 0.05	19.23 ± 3.65	22.47 ± 11.63	0.14 ± 0.09	-	6.77 ± 3.23	97.27 ± 21.35	Jerez *et al*. 2011
Adelie Penguin (n = 25)	2005 - 2012	King George Island, Antarctica	Medium	43.36 ± 69.03[1]	< DL	< DL	12.68 ± 7.09[1]	59.74 ± 45.26[1]	0.64 ± 1.09[1] (n = 5)	0.32 ± 0.08[2] (n = 10)	6.37 ± 2.52[1]	50.84 ± 17.38[1]	[1]Jerez *et a*l. 2011, [2]Brasso *et al*. 2013
Adelie Penguin (n = 22)	2005 - 2007	Avian Island, Antarctica	Low	5.08 ± 3.03	0.07 ± 0.03 (n = 3)	0.04 ± 0.02	13.16 ± 3.04	27.98 ± 41.20	0.14 ± 0.21	-	6.06 ± 3.05	77.69 ± 15.17	Jerez *et al*. 2011
White-chinned Petrel (n = 30)	2006, 2007	Brazil	NA	-	-	7.34 ± 1.70 [5.72 - 24.03]	10.74 ± 5.56 [2.68 - 23.92]	-	33.05 ± 8.48 [18.62 - 55.51]	1.84 ± 2.48 [0.19 - 8.91]	-	67.48 ± 11.64 [48.96 - 93.54]	Carvalho *et al.* (2013)
Spectacled Petrel (n = 38)	2006, 2007	Brazil	NA	-	-	7.33 ± 1.57 [3.76 - 10.44]	7.91 ± 5.05 [1.05 - 21.57]	-	32.26 ± 8.71 [16.53 - 59.00]	11.17 ± 3.78 [4.24 - 24.03]	-	62.05 ± 7.58 [45.30 - 81.49]	Carvalho *et al.* (2013)
Laysan Albatross (Juv, n=40)	2012	Midway Atoll	NA	-	0.06 ± 0.07	-	-	48.34 ± 85.32	1.61 ± 3.39	1.74 ± 0.66	1.70 ± 0.18	66.16 ± 19.01	Lavers & Bond (in press)
Bonin Petrel (Juv, n = 7)	2012	Midway Atoll	NA	-	0.14 ± 0.13	-	-	230.26 ± 185.77	1.19 ± 0.9	2.04 ± 0.91	2.11 ± 0.13	26.88 ± 10.06	Lavers & Bond (in press)
Little Auk (n = 81)	2011	Newfoundland, Canada	Medium	-	-	-	-	-	-	3.17 ± 0.83 [1.53 - 5.73]	-	-	Fort *et al.,* 2014
Little Auk (n = 78)	2009, 2010	East Greenland, Canada	Low	-	-	-	-	-	-	1.53 ± 0.84 [0.71 - 4.33]	-	-	Fort *et al*., 2014

Note: Species are as follows, Little Penguin *E. minor*, Silver Gull *C. novaehollandiae*, Flesh-footed Shearwater *P. carneipes*, Adelie Penguin *P. adeliae*, Gentoo Penguin *P. papua*, White-chinned Petrel *P. aequinoctialis*, Spectacled Petrel *P. conspicillata*, Laysan Albatross *P. immutabilis*, Little Auk *A. alle*.

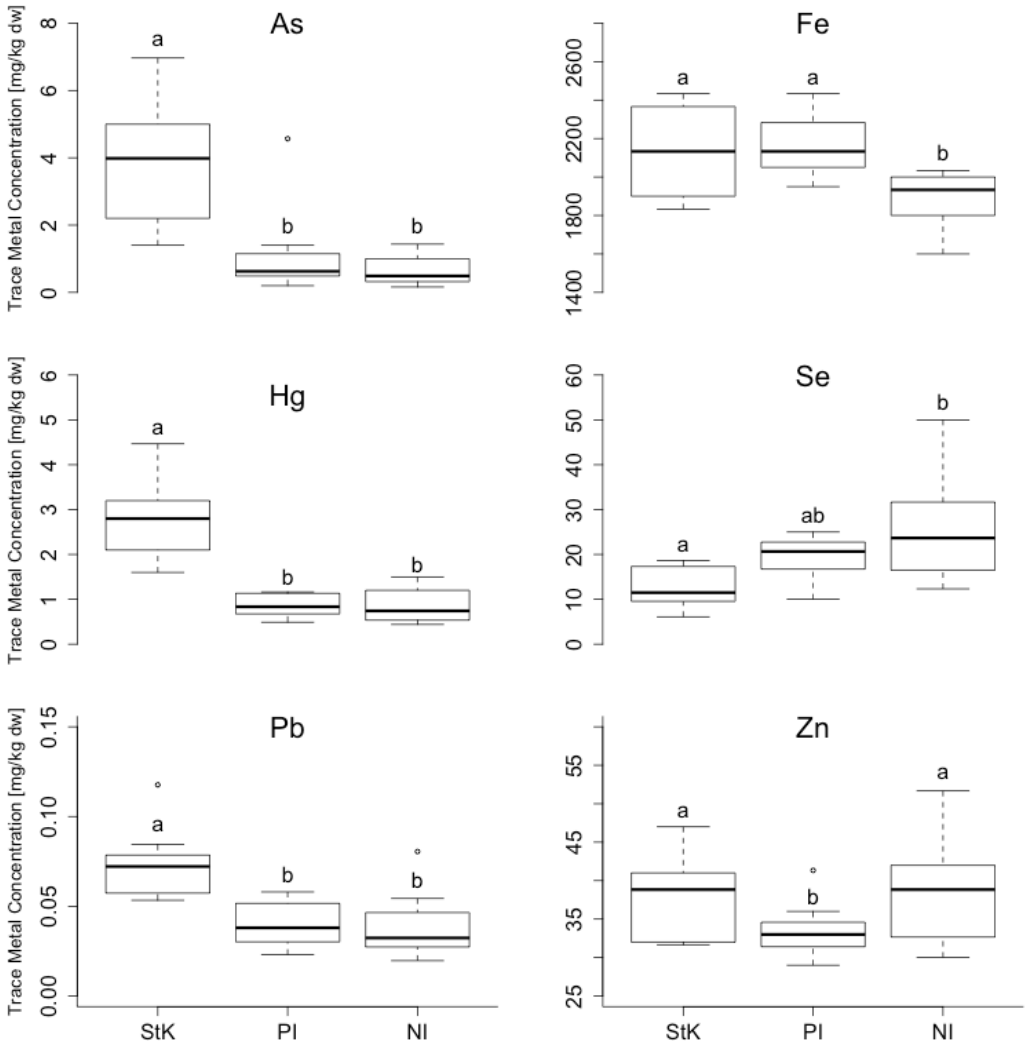

Figure 3.2: Concentrations of non-essential (left) and essential (right) trace metals and metalloids (mg/kg dry weight) in whole blood of Little Penguins sampled at three locations (StK = St Kilda, PI = Phillip Island, NI = Notch Island) in February and March of 2012. Different lower case letters indicate significant differences among locations (Tukey HSD, $p < 0.05$). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (°) are defined as between 1.5 and 3*IQR.

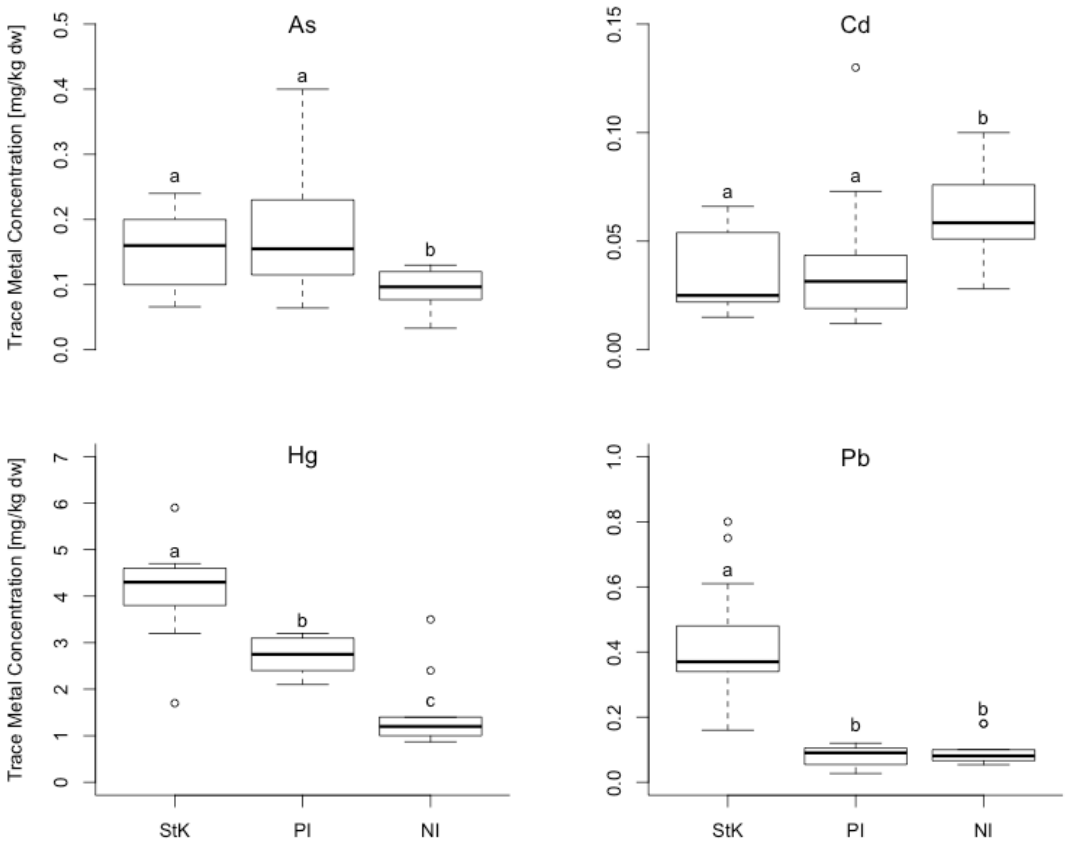

Figure 3.3: Concentrations of non-essential trace metals and metalloids As, Cd, Hg and Pb (mg/kg dry weight) in feathers of Little Penguins sampled at three locations (StK = St Kilda, PI = Phillip Island, NI = Notch Island) in February and March of 2012. Different lower case letters indicate significant differences among locations (Tukey HSD, $p < 0.05$). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

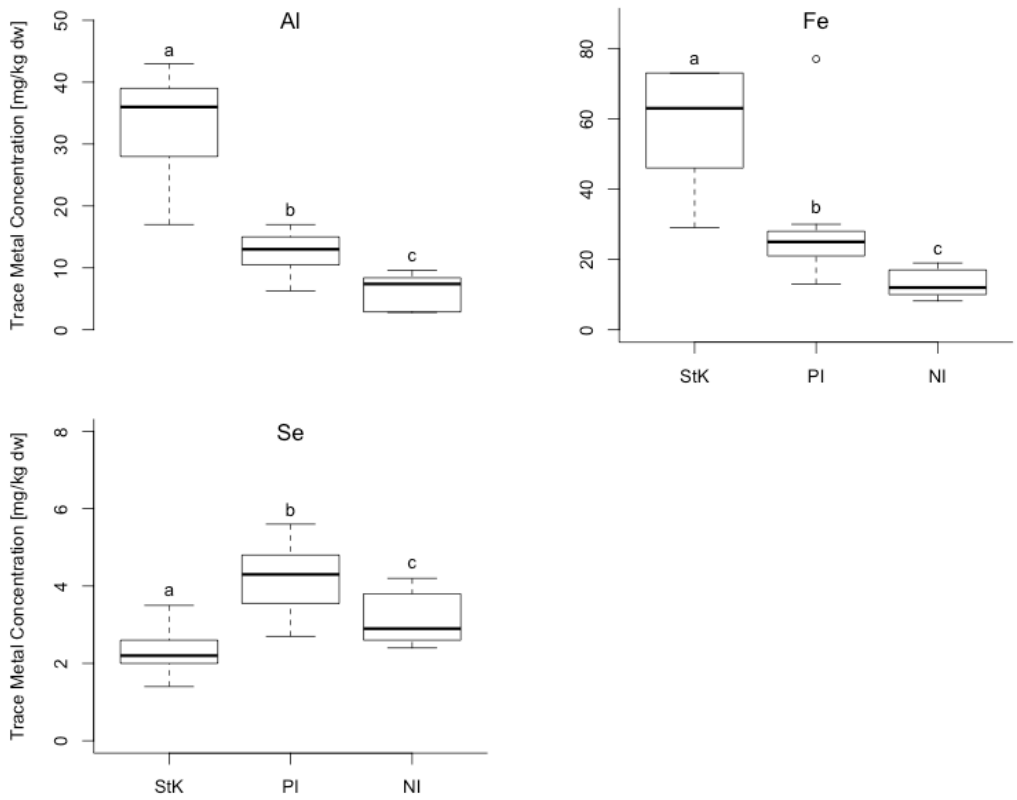

Figure 3.4: Concentrations of essential trace metals Al, Fe and Se (mg/kg dry weight) in feathers of Little Penguins sampled at three locations (StK = St Kilda, PI = Phillip Island, NI = Notch Island) in February and March of 2012. Different lower case letters indicate significant differences among locations (Tukey HSD, $p < 0.05$). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

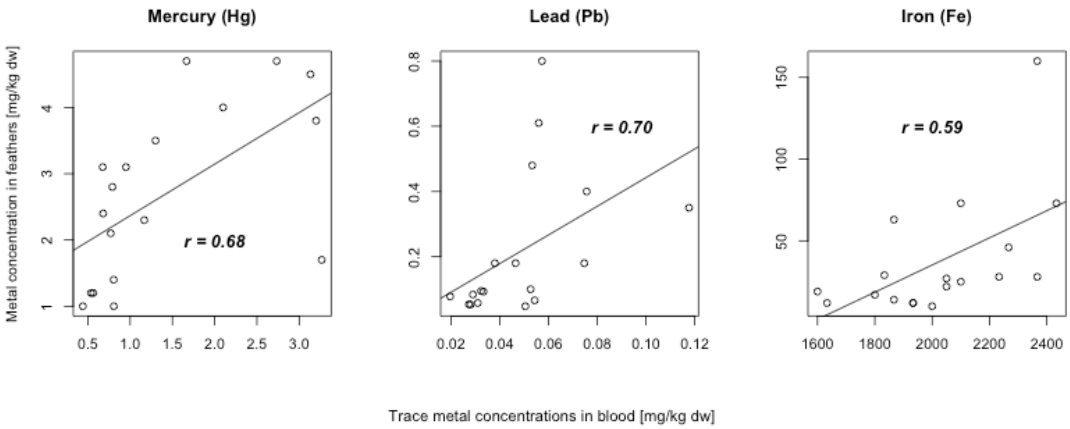

Figure 3.5: Spearman correlations between trace metal concentrations in blood and feathers of Little Penguins (paired data from all locations sampled, n = 19).

3.3.3. Comparison of element concentrations measured in blood and feathers of Little Penguins

We executed Spearman correlations for eight elements measured in both blood and feathers of 19 Little Penguins and found significant positive correlations between tissue types for Hg (r_S (363) = 0.68, p = 0.001), Pb (r_S (289) = 0.70, p = 0.001) and Fe (r_S (463) = 0.59, p = 0.007) (Figure 3.5). Most of the eight trace elements, namely Al, Cu, Pb, Hg and Zn, were present in larger concentrations in feathers compared to blood concentrations. Additionally, concentrations of Al, Cu, Fe and Pb were more variable (as indicated by standard deviations) in feathers of Little Penguins, while As, Se and Zn showed greater variation in the blood.

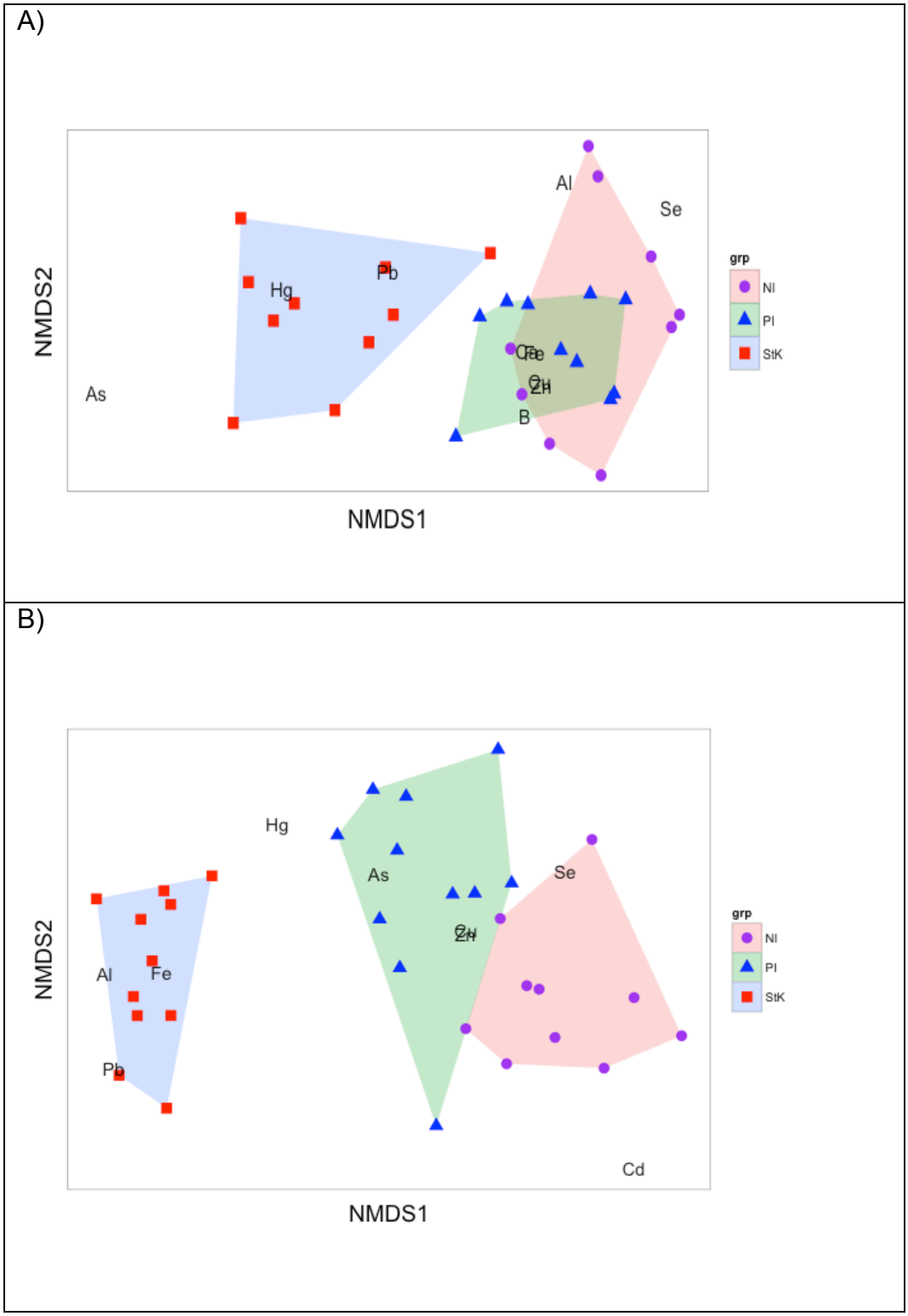

Figure 3.6: Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin A) blood (stress = 0.12) and B) feather samples (stress = 0.11). Polygon ellipse lines are drawn for each sampling location: St Kilda (StK, squares), Phillip Island (PI, triangles) and Notch Island (NI, full circles). Trace elements are displayed by their periodic symbols.

3.4. Discussion

3.4.1. Non-essential trace elements

Arsenic (As) in the blood of Little Penguins showed the greatest geographical differences and the values observed in St Kilda penguins (mean 3.72 mg/kg, max 6.97 mg/kg) are well above levels reported in seabirds elsewhere (Table 3.1). Correspondingly, high As concentrations have been measured in Port Phillip Bay sediments (max 150 mg/kg; EPA, 2013), water (mean As in water 2.8 µg/L; Fabris *et al.*, 1999) and fish (snapper *Pagruss auratus,* edible tissue*,* mean 12.1 mg/kg wet weight; Fabris *et al.*, 2006). The source of these elevated concentrations is thought to be due to natural sediment mineralogy (Harris *et al.*, 1996), although Pettigrove & Hoffmann (2003) suggest past gold mining activities in the 19th century released large quantities of As into the Bay through catchment inputs. In comparison, snapper caught around Phillip Island and Notch Island contained four to five times less As in edible tissues (mean 2.8 - 4.2 mg/kg; Fabris *et al.*, 2006). Feather As concentrations exhibited less variation among locations and showed little distinction between St Kilda and Phillip Island (Table 3.2). Arsenic in feathers can be highly variable, likely due to external contamination, and can vary greatly among locations for the same seabird species (Bond & Lavers, 2011). Elevated As concentrations are common in marine species (Eisler, 1988) and while some studies observed detrimental effects in birds (e.g. Fairbrother *et al*., 1994), chronic toxicity has not yet been adequately verified in experimental or wild animals (Huff *et al*., 2000). To establish whether high As concentrations found in the blood of St Kilda penguins are a potential concern, we propose that future studies measure the

relative proportions of inorganic As, which is highly toxic (Eisler, 1988) and has been implicated as an endocrine disruptor (see review by Kunito *et al*., 2008).

Cadmium (Cd) concentrations in the blood of Little Penguins were below the limit of reporting for all samples from St Kilda and half the samples from Phillip Island and Notch Island. All measurements were < 0.3 mg/kg. This is likely a result of Cd in birds being predominantly stored in the kidneys and liver (Scheuhammer, 1987). Feather Cd concentrations were highest at Notch Island (Figure 3.3), which could be due to penguins at that location having a larger dietary proportion of cephalopods (17 - 25%; Schumann, 2012), which contain higher concentrations of Cd (Szefer *et al.*, 1993; Bustamante *et al*., 1998). However, all samples returned results well below the hypothesised adverse effect level in feathers (2 mg/kg; Burger, 1993).

Mercury (Hg) is one of the most important environmental contaminants monitored in seabirds (Scheuhammer *et al.*, 2014). Most Hg in the marine environment is anthropogenic (UNEP, 2013), has increased over the last decades (Lamborg *et al.*, 2014) and bioaccumulates up the food chain (Neff, 2002). Chronic and acute Hg toxicity in birds has been associated with reduced hatchability, egg weight, chick survival and increased mortality (Burger & Gochfeld, 1997). Mercury in Port Phillip Bay has in the past been largely anthropogenic in origin (Walker, 1982), but has decreased since the implementation of stricter effluent controls in the late 1970s and now compares to other uncontaminated coastal waters elsewhere in the world (mean Hg in water 1.7 ng/L; Fabris *et al.*, 1999). Fabris *et al.*, (2006) found that edible tissue of sand flathead (*Platycephalus bassensis*) within Port

Phillip Bay contained a mean Hg concentration of 0.21 mg/kg wet weight, while the closely related tiger flathead (*Neoplatycephalus richardsoni*) in areas surrounding Phillip and Notch Islands measured only 0.06 mg/kg and 0.07 mg/kg wet weight, respectively. Blood Hg concentrations of Phillip and Notch Island penguins measured in this study are similar to what Fort *et al.* (2014) reported for Little Auks (*Alle alle*) in their Arctic breeding grounds, while the St Kilda penguins matched the blood Hg concentrations of Little Auks wintering in more polluted northwest Atlantic areas. Feather Hg concentrations reported here are similar to those reported in other Little Penguin studies and higher than in Antarctic penguins (Table 3.2). One individual from St Kilda exceeded the lowest hypothesised adverse effect level for Hg in feathers (5 mg/kg; Eisler, 1987), but all samples were well below 20 mg/kg; a threshold concentration for significant reproductive concern often applied to piscivorous birds (Evers *et al.*, 2014; Bond *et al*., 2015). However, interspecies comparisons are difficult, often exacerbated by differences in methodology and Shore *et al.* (2011) found evidence for substantial differences in susceptibility toward trace metal burdens among bird species. Diet composition often affects Hg body burdens, but does not explain the pattern observed in this study. Krill is the prey item most associated with high Hg concentrations (Minganti *et al.*, 1996; Bond & Diamond, 2009), and while krill has been found in the diet of penguins from Bass Strait island colonies, it has not been reported for the St Kilda colony (Chiaradia *et al.*, 2012). Hence, local differences in prey composition are unlikely to be a contributing factor in the pattern observed. To interpret the strong correlation found between Hg concentrations in blood and feathers of Little Penguins (Figure 3.5), we need

to consider that Hg contained in the blood during moult is a combination of recent dietary intake and fast-induced remobilised Hg from body tissues (Furness *et al.*, 1986; Bearhop *et al.*, 2000). This aids Hg sequestration into new feathers and provides an effective means of detoxification (Furness *et al.*, 1986). Moult blood may therefore be a proxy for Hg sequestered into the new feathers. Since the Little Penguin feathers used in our study were grown in the year prior, this correlation may also suggest a relative stability of Hg in the prey of these penguins between 2011 and 2012.

While lead (Pb) measured in fish and crustacea showed little variation among Port Phillip Bay and fishing zones overlapping with foraging areas of Bass Strait penguin colonies (Fabris *et al.*, 2006), both matrices measured in this study clearly reflected the following Pb pollution gradient: St Kilda > Phillip Island > Notch Island. Lead concentrations in Little Penguins were correlated between matrices, but geographical differences were more pronounced in feathers than in blood. This may be explained by the affinity of Pb to attach itself to the outside of feathers (Jaspers *et al.*, 2004). As such, Pb concentrations in feathers reflect the exogenous contamination of their environment as well as the endogenous contamination. Port Phillip Bay has an annual Pb input of 31 t (Harris *et al.*, 1996), which is reflected in surface waters containing Pb concentrations of 0.19 µL/L and up to 197 mg/kg in sediments (Fabris *et al.*, 1999). According to the guidelines established by Long *et al.* (1995), the Pb sediment value in Port Phillip Bay is within the "possible effects" range. However, at present, Pb concentrations in St Kilda penguin feathers and blood are well below the hypothesised deleterious effect level (4 mg/kg; Burger, 1993).

Essential trace elements

Essential trace elements are required for biochemical processes in the organism, but can become toxic at high concentrations. Copper and Zn, in particular, are anthropogenically added to the marine environment and are known to bioaccumulate, therefore it is important to understand the limits of 'healthy' concentrations in organisms (Neff, 2002). For essential elements, penguins would be expected to exert homeostatic control and metabolically regulate blood concentrations, which homogenises variability among sites. In concurrence, there was no clear geographical pattern detected for essential trace element concentrations in the blood (Figure 3.2, right panels), while Al and Fe concentrations in feathers (Figure 3.4) followed a pollution gradient similar to that expressed in Hg and Pb blood and feather concentrations. The reasons for this are unknown, but Jaspers *et al.* (2004) identified both Al and Fe as sources of external contamination in feathers.

Selenium is an important trace element that has been included here because of its important role in reducing Hg assimilation and aiding Hg detoxification in marine animals (Cuvin-Aralar & Furness, 1991). There are no data on Se concentrations in the penguin prey items at any of the locations sampled, but Dunlop *et al.* (2013) executed a controlled feeding study on captive Little Penguins and found Se prey concentrations correlated with that in feathers. Hence, it would appear that the penguin diet at Notch Island is higher in Se than at St Kilda and Phillip Island (Figure 3.4). Dunlop *et al*. (2013) also found higher Hg feather concentrations in the captive group (mean 2.61 mg/kg) compared to samples collected from Mistaken Island, Western Australia (mean 1.32 mg/kg, Table 3.2), an area historically

contaminated with Hg (Francesconi *et al*., 1997). Dunlop *et al.* (2013) suggested that Se rich food, evidenced as high Se concentrations in the feathers at that colony, might help decrease Hg bioaccumulation. Conversely, the low Se fish fed to the captive group might have led to their disproportionally high Hg feather concentrations (Dunlop *et al.*, 2013). Both blood and feather Se concentrations of St Kilda penguins were relatively low, indicating low dietary intake. Therefore, any long-term high Hg food intake by this urban colony could result in exacerbated deleterious effects.

3.4.2. Suitability of the Little Penguin as a bioindicator for local trace element contamination

Penguins, in comparison with other high trophic indicators (other seabirds, cetaceans, sharks), have the distinction of being fairly easily and reliably sampled and their foraging areas are relatively small, meaning, their body burdens indicate local pollution. Analysis of moderate sample numbers showed pronounced spatial differences in both tissue types (Figure 3.6). Non-essential trace element concentrations in blood, in particular, provided strong evidence of a pollution gradient with increased concentrations observed in the metropolitan St Kilda colony. Our findings correspond well with previous data on elevated trace element concentrations in seawater, sediment and vertebrates in Port Phillip Bay (Fabris, 1995; Harris *et al*., 1996; Fabris *et al*., 1999). With only limited data available on trace element concentrations in biota of Bass Strait, this study furthers our understanding of background levels at a relatively uncontaminated site, such as Notch Island. While Phillip Island penguins exhibited larger body burdens of non-essential trace

elements than penguins at Notch Island, they differed significantly from those at St Kilda and might provide a rare opportunity to investigate demographic or behavioural effects of contamination. Demographic effects of chronic exposure to contaminants in seabirds can be measured by population growth rates, survival rates and long-term breeding performance (Tartu *et al.*, 2013; Fort *et al.*, 2014; Goutte *et al.*, 2014a; Goutte *et al.*, 2014b). While the St Kilda and Phillip Island colonies are regularly surveyed and many penguins have passive induction transponders (Dann *et al.*, 2014), therefore enabling mark-recapture data collection, the differences in nesting (rock crevasses at St Kilda, burrows at Phillip Island) confound a direct comparison of these measures. Nevertheless, further coordinated research at both colonies, coupled with continued feather and/or blood collection might uncover indicators that can be connected to the difference in contaminant exposure of this high trophic feeder.

3.4.3. Utility of blood and feathers as sampling matrix

Both blood and feathers of Little Penguins provided suitable matrices to investigate spatial differences of contamination with moderate sample sizes (Figure 3.6). Moulted feather collection is non-invasive and inexpensive, but can suffer from increased bias due to external contamination (Jaspers *et al.*, 2004). Little Penguins moult on land over a relatively brief period (Reilly & Cullen, 1983), providing a reliable, time window to collect feathers. While Hg, Se, and to some degree Pb, are adequately reflected in feathers, some trace elements that are of biological and ecological interest in environmental monitoring are not, or are heavily biased by exogenous contamination. The

collection of blood samples is more invasive and has more specific skill, storage and transportation requirements. However, the analysis of blood allows for finer scale temporal investigations than feathers, and metals in blood are often a better representation of the biologically relevant contaminant load (Carvalho *et al.*, 2013).

3.5. Conclusions

This study reports on trace element concentrations in the blood and feathers of Little Penguins at three locations with varying degrees of anthropogenic contamination. The results provide the first data on metal and metalloid concentrations in blood of any penguin species and indicate a strong link between non-essential trace element concentrations in the blood of Little Penguins and the level of industrialisation of their foraging zone. While feathers provided a less precise indicator of penguin body burden for most metals and metalloids, they provide a simplified, non-invasive means of monitoring pollution levels if collected over extended time periods. We advocate that the Little Penguin be utilised as a suitable proxy of coastal ecosystem health and, in light of the pollution patterns detected during this study, recommend more detailed coordinated monitoring of the St Kilda and Phillip Island populations. The design of this study did not include any measures to determine the effect of increased toxicant load, and therefore, an assessment of any potential health impacts on Little Penguins was not made. However, there is to date no evidence of any colony-level deleterious effects of the increased contaminant load at St Kilda when compared to Phillip Island,

with relatively secure population status and breeding success being reported for both colonies (Z. Hogg, unpublished data; Sutherland & Dann, 2014).

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Chapter 4

Seasonal variation and annual trends of metals and metalloids in the blood of the Little Penguin (Eudyptula minor)

Little Penguin flipper measurement
(Photo courtesy: Nikki Filby)

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GRADUATE RESEARCH CENTRE

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ABSTRACT

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4. Seasonal variation and annual trends of metals and metalloids in the blood of the Little Penguin (*Eudyptula minor*)

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Abstract

Little Penguins (*Eudyptula minor*) are high-trophic coastal feeders and are effective indicators of bioavailable pollutants in their foraging zones. Here, we present concentrations of metals and metalloids in blood of 157 Little Penguins, collected over three years and during three distinct seasons (breeding, moulting and non-breeding) at two locations: the urban St Kilda colony and the semi-rural colony at Phillip Island, Victoria, Australia. Penguin metal concentrations were foremostly influenced by location (St Kilda > Phillip Island for non-essential elements) and differed among years and seasons at both locations, reflecting differences in seasonal metal bioaccumulation or seasonal exposure through prey. Mean blood mercury concentrations showed

an increasing annual trend and a negative correlation with flipper length at St Kilda. Notably, this study is the first to report on blood metal concentrations during the different stages of moult, showing the mechanism of non-essential metal mobilisation and detoxification.

Keywords

Seabird, Bioindicator, Mercury, Port Phillip Bay, Bass Strait

4.1. Introduction

Studying annual and seasonal trends of contaminants in coastal waters is essential to understand impacts of anthropogenic activities on sensitive species and inshore marine ecosystems, which are under increasing pressure due to continuing human population growth. In Australia, the proportion of the country's population living near the coast in 2001 was 85% and is predicted to increase (ABS, 2011). Higher concentrated coastal populations are likely to increase coastal pollution levels. Another factor adding pressure to coastal ecosystems is climate change, which will raise sea surface temperatures and sea levels, change rainfall run-off patterns into marine areas, increase the frequency and intensity of severe weather events and increase the acidity of sea-water, all of which affect contaminant exposure and toxic effects (Sokolova & Lannig, 2008; Millero *et al*., 2009; Noyes *et al*., 2009). While point-source industrial output of contaminants has been more tightly regulated and has in some locations decreased over time (Fabris *et al*., 1999), historical discharges persist for decades in the sediments of bays and inlets near industrialised areas (Aly *et al*., 2013). Highly toxic pollutants can become bioavailable, if sediments are physically disturbed and re-suspended in the

water column, e.g. due to dredging (Hedge *et al*., 2009; Edge *et al*., 2015; Fetters *et al*., 2015). Taken up by plankton, these elements can enter the food web and potentially accumulate with each increase in trophic position. Hence, high-level predators are often used to indicate the ecological risks of bioavailable coastal pollution (Becker, 2003).

An increasing number of studies use blood of seabirds to elucidate issues relating to marine contamination (Eagles-Smith *et al*., 2008; Carvalho *et al*., 2013; Tartu *et al*., 2015). Taking a small blood sample non-destructively from a randomly selected individual has the advantage of being more ethical than destructive (sacrificial) sampling, but also more representative than opportunistic collection of specimens that died of unknown causes (Becker, 2003). As blood concentrations reflect recent dietary exposure, these data can give insight into seasonal variations of exposure through prey, but also highlight differences in bioaccumulation due to varying seasonal needs in the species' life stages. It may even be possible to observe sampled individuals over time to gauge an effect of the contaminant load on their behaviour (Tartu *et al*., 2013). However, studies that document annual variations often report on metal content in feathers (e.g. Carravieri *et al*., 2014; Bond *et al*., 2015), while studies investigating variations in pollution load of resident high-trophic feeders between seasons within a given year are rare. The benefits of such studies include getting a more accurate gauge on small-scale temporal changes and providing insight into the physiological mechanisms of the study species. This line of research has the potential to influence scientific investigation more broadly and to provide more comprehensive information as to what factors, other than diet, affect metal load in top feeders.

Port Phillip Bay is adjacent to the City of Melbourne, Australia - a metropolitan city, which currently hosts a human population of 4 million (ABS, 2011). Port Phillip Bay is Australia's largest shipping port, is 1930 km^2 in area, 13.6 m deep on average and joined to the Bass Strait through a narrow 3 km-wide channel (Figure 4.1). Semi-enclosed bays like Port Phillip Bay may contain contamination hotspots as reduced wave action hinders the transport of polluted particles into open waters (Fukushima *et al*., 1992; Aly *et al*., 2013). Not surprisingly, sediments in Hobsons Bay (Figure 4.1) and the shipping channels historically contain high concentrations of arsenic, mercury and lead (Phillips *et al*., 1992; Fabris *et al*., 1999). These locations were recently directly impacted by dredging in 2008/09 when 23 million m^3 of rock, silt, clay and sand were removed from the mouth of the Yarra River and the shipping channels to increase vessel accessibility to the port (PoMC, 2010). Notably, the same areas have been identified as foraging hot spots for the all-year resident Little Penguin population, nesting at the breakwater in St Kilda (Preston *et al*., 2008; Kowalczyk *et al*., 2013). Currently, records of metal and metalloid contaminants in the biota of Port Phillip Bay are only sporadic and not consistent in their choice of study species (Walker, 1988; EPA, 2009, 2013; Finger *et al*., 2015). Given the recent scale of developments and disturbances, as well as general pressures on the area, the lack of long-term data on contamination levels in biota is of concern.

Our previous study of metals and metalloids in Little Penguins, sampled at one point in time at three different locations, each with varying degrees of industrialisation, established the Little Penguin as a reliable bioindicator for local metal and metalloid contamination and showed Phillip

Island to be a viable reference site for the more industrialised St Kilda (Finger *et al*., 2015). In this study, we extend this work and present the analysis of a comprehensive multi-year data set to investigate annual, seasonal and within-moult variation of blood metal and metalloid concentrations in this high trophic feeder.

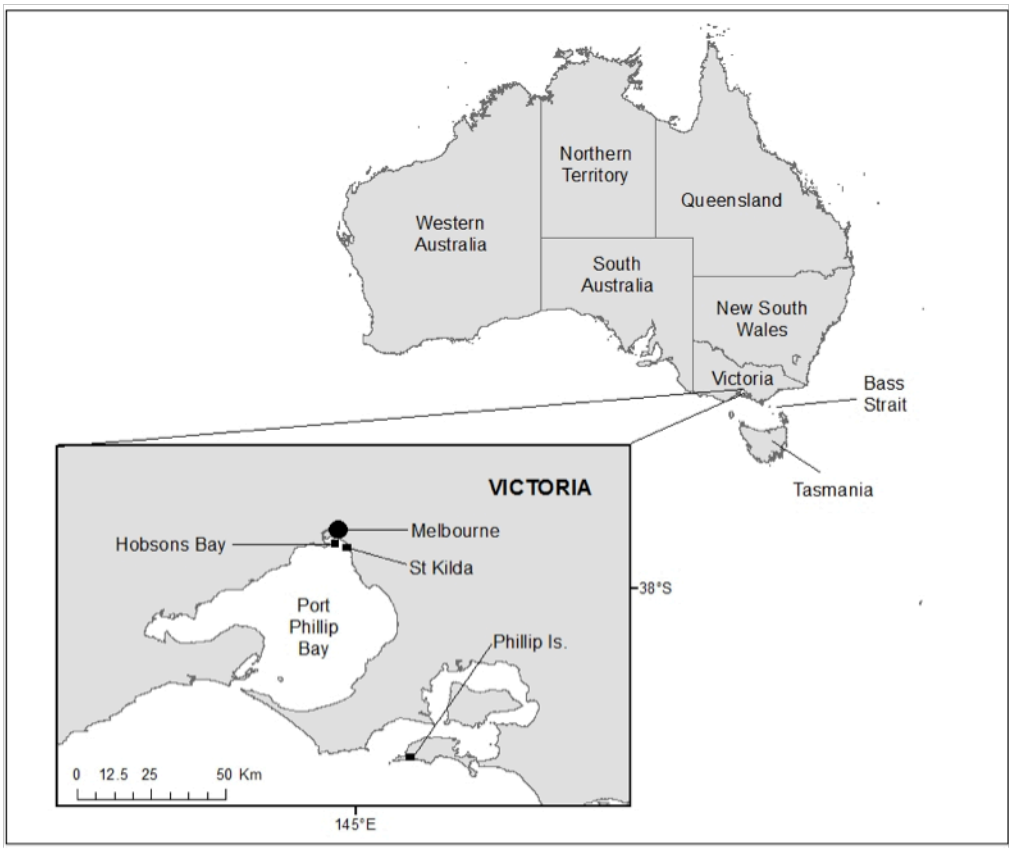

Figure 4.1: Little Penguins were sampled at St Kilda and Phillip Island from 2011 to 2013.

4.2. Materials and methods

4.2.1. Study sites

Blood was collected from adult Little Penguins at two locations: St Kilda (n = 101) from March 2011 to December 2014 and Phillip Island (n = 56) from November 2011 to May 2013 (Figure 4.1, Table 4.1). The St Kilda colony

(37˚51'S, 144˚57'E) is located 5 km from the central business district of Melbourne, Australia. Approximately 1,000 Little Penguins nest between the rocks of a 650 m long man-made breakwater structure (Z. Hogg, unpublished data). St Kilda's Little Penguins adjust their diet depending on prey availability (Kowalczyk *et al*., 2015c), but mostly feed on clupeoids, such as Australian anchovy (*Engraulis australis*), southern garfish (*Hyporhamphus melanochir*), and luminous bay squid (*Loliolus noctiluca)* (Preston *et al*., 2008). The Phillip Island colony (38˚30'S, 145˚10'E) is located 140 km southeast of Melbourne, Australia, and has approximately 32,000 penguins nesting on the Summerland Peninsula (Sutherland & Dann, 2012). These penguins feed on Australian anchovy, Pilchard (*Sardinops sagax*), Barracouta (*Thyrsites atun*), Red Cod (*Pseudophysis bachus*), various other juvenile fish and Arrow squid (*Nototodarus gouldi*) (Chiaradia *et al*., 2010).

4.2.2. Sample collection

We collected all samples (n = 157) during three distinct life history stages, namely ‘breeding’, ‘moult’ and ‘non-breeding’; henceforth called 'seasons'. Breeding starts with egg-laying and concludes with the fledging of the chicks, lasting between three and five months (Reilly & Cullen, 1981). During moult, which follows the breeding season, penguins stay inside or near their burrows and fast while the entire plumage is replaced (~ 22 days, Reilly & Cullen, 1983). Non-breeding is defined as the time between moult and breeding, where penguins forage without the restraints of caring for young. We captured penguins by hand on their way to or from their burrows. We restrained the animal in a light cotton bag and aspirated up to 2 mL of blood

from the medial metatarsal (caudal tibial) vein using a 25-gauge butterfly needle with a 3 mL syringe. This was transferred into 6 mL Vacutainers® (BD Diagnostics, trace element tube plus K_2EDTA, product number 368381), which we placed in a cooler with ice packs, transferred to a freezer within 12 hours of sampling and kept frozen at -20˚C until analysis. We weighed penguins to the nearest 10 g using a hand-held spring balance. Animals that weighed less than 900 g were released without sampling for ethical and logistical reasons. Different weight considerations applied for sampling during moult. This is because penguins nearly double their weight in preparation for the 22 day fasting associated with moult (Reilly & Cullen, 1983). Following consultation with research staff from the Phillip Island Nature Parks (PINP), we adjusted the minimal weight criterion for a sampled bird for each moult stage as follows: M1 - 1400 g, M2 - 1300 g, M3 - 1100 g, M4 - 1000 g, M5 - 900 g. Moult stages are defined as follows: M0 - penguin not undergoing moult, M1 - penguin at moult start, flippers swollen, old feathers stand upright but are still firmly attached, M2 - old feathers begin to fall out, M3 - 1/3 to 2/3 new feathers grown, M4 - more than 3/4 new feathers grown, M5 - all new feathers grown and all old feathers lost (Dann, unpublished data). We also took standard morphological measurements using digital callipers (± 0.1 mm) of the total head length (THL, length from back of the head to the tip of the beak), beak length (BL, length of the beak from the posterior section to the tip of the beak), beak depth (BD, taken just anterior to the nasal cavities and used to determine sex following Arnould *et al.*, 2004). We measured the length of the right flipper, extended at a 90˚ angle to the body using a stopped ruler (± 1 mm).

Table 4.1: Mean metal and metalloid concentrations (mg/kg dry weight) ± standard deviation (number of samples) in whole blood of adult Little Penguins by location and season. Range is given in square brackets. "<LOR" = results under the limit of reporting. "- " = no samples collected.

Year	Trace element: Season	Location	Aluminium	Arsenic	Boron	Calcium	Copper	Iron	Mercury	Lead	Selenium	Zinc
2011	Moulting	St Kilda	3.28 ± 0.77 (8) [2.50 - 4.55]	2.01 ± 0.52 (10) [1.2 - 2.7]	0.62 ± 0.21 (8) [0.43 - 1.10]	337 ± 61.9 (8) [261 - 455]	2.41 ± 0.53 (10) [1.58 - 3.30]	1948 ± 144.3 (10) [1800 - 2294]	1.75 ± 0.44 (9) [1.10 - 2.45]	0.07 ± 0.01 (8) [0.06 - 0.08]	6.21 ± 1.24 (10) [4.20 - 7.79]	34.35 ± 5.419 (10) [27.50 - 43.50]
		Phillip Island	-	-	-	-	-	-	-	-	-	-
	Non-breeding	St Kilda	3.70 ± 0.76 (13) [2.53 - 5.35]	2.89 ± 1.46 (14) [0.58 - 5.33]	0.68 ± 0.34 (14) [0.37 - 1.37]	315 ± 35.2 (13) [273 - 370]	2.24 ± 0.18 (12) [1.93 - 2.50]	2070 ± 85.7 (14) [1933 - 2233]	2.08 ± 0.56 (13) [1.43 - 3.00]	0.08 ± 0.02 (14) [0.05 - 0.13]	5.57 ± 1.49 (14) [3.57 - 8.30]	32.04 ± 5.33 (14) [22.50 - 40.50]
		Phillip Island	-	-	-	-	-	-	-	-	-	-
	Breeding	St Kilda	1.84 ± 0.73 (9) [0.65 - 2.80]	2.01 ± 0.72 (11) [0.66 - 3.27]	1.01 ± 0.64 (13) [0.36 - 1.90]	354 ± 51.4 (14) [290 - 485]	2.40 ± 0.50 (14) [1.83 - 3.45]	2173 ± 325.4 (10) [1650 - 2650]	2.71 ± 0.56 (13) [1.60 - 3.60]	0.06 ± 0.02 (12) [0.04 - 0.10]	9.05 ± 3.78 (14) [3.45 - 16.00]	32.65 ± 6.81 (12) [25.30 - 47.30]
		Phillip Island	3.91 ± 1.67 (14) [1.03 - 6.17]	0.56 ± 0.38 (17) [0.17 - 1.73]	1.18 ± 0.74 (14) [0.08 - 2.13]	310 ± 61.0 (13) [220 - 473]	2.14 ± 0.34 (17) [1.23 - 2.63]	2282 ± 482.4 (17) [1750 - 3250]	1.39 ± 0.45 (16) [0.72 - 2.27]	0.04 ± 0.02 (16) [0.02 - 0.07]	37.52 ± 16.74 (17) [12.00 - 70.50]	35.21 ± 4.59 (17) [27.00 - 42.67]
2012	Moulting	St Kilda	3.89 ± 1.26 (10) [2.5 - 6.9]	3.72 ± 1.76 (10) [1.40 - 6.97]	0.70 ± 0.21 (10) [0.45 - 1.20]	384 ± 49.7 (9) [315 - 453]	2.48 ± 0.44 (10) [1.77 - 3.10]	2147 ± 228.4 (10) [1833 - 2433]	2.75 ± 0.85 (10) [1.60 - 4.47]	0.07 ± 0.02 (10) [0.05 - 0.12]	12.46 ± 4.41 (10) [6.10 - 18.67]	37.97 ± 5.28 (10) [31.7 - 47.0]
		Phillip Island	3.19 ± 0.84 (10) [1.87 - 4.30]	0.72 ± 0.39 (10) [0.20 - 1.40]	0.68 ± 0.16 (11) [0.52 - 1.13]	349 ± 49.7 (11) [267 - 430]	2.14 ± 0.42 (11) [1.27 - 2.90]	2162 ± 158.7 (11) [1950 - 2433]	0.86 ± 0.23 (10) [0.49 - 1.17]	0.04 ± 0.01 (11) [0.02 - 0.06]	19.20 ± 5.22 (11) [10.00 - 25.00]	33.47 ± 3.27 (11) [29.00 - 41.33]
	Non-breeding	St Kilda	3.16 ± 0.86 (8) [2.15 - 4.60]	1.74 ± 0.86 (8) [0.82 - 3.20]	0.67 ± 0.21 (8) [0.43 - 0.98]	321 ± 32.8 (8) [277 - 375]	2.31 ± 0.28 (8) [1.83 - 2.65]	2008 ± 101.6 (8) [1850 - 2167]	2.74 ± 0.59 (7) [1.90 - 3.37]	0.06 ± 0.01 (8) [0.05 - 0.07]	19.27 ± 8.23 (8) [10.67 - 34.50]	33.62 ± 5.47 (8) [26.00 - 42.00]
		Phillip Island	2.85 ± 1.55 (3) [1.87 - 4.63]	1.83 (1)	1.45 ± 0.74 (4) [1.00 - 2.57]	316 ± 52.9 (4) [240 - 363]	2.11 ± 0.34 (4) [1.63 - 2.43]	2125 ± 226.7 (4) [1900 - 2433]	1.21 ± 0.59 (3) [0.53 - 1.60]	0.05 ± 0.01 (4) [0.04 - 0.06]	21.00 ± 7.75 (4) [13.00 - 29.33]	29.58 ± 2.25 (4) [26.67 - 31.67]
	Breeding	St Kilda	1.97 ± 0.49 (14) [1.20 - 2.70]	2.81 ± 1.11 (18) [1.40 - 5.20]	0.80 ± 0.29 (18) [0.21 - 1.25]	340 ± 78.6 (16) [267 - 510]	2.44 ± 0.30 (16) [2.00 - 3.00]	2047 ± 153.2 (18) [1800 - 2367]	3.30 ± 0.61 (17) [2.65 - 4.65]	0.06 ± 0.02 (15) [0.03 - 0.12]	34.92 ± 12.79 (18) [13.50 - 61.00]	31.16 ± 3.84 (18) [25.5 - 40.0]
		Phillip Island	2.66 ± 0.91 (12) [1.80 - 4.53]	1.09 ± 0.60 (8) [0.22 - 2.35]	0.84 ± 0.18 (13) [0.59 - 1.10]	321 ± 49.6 (13) [253 - 407]	2.15 ± 0.42 (13) [1.40 - 2.80]	2247 ± 153.9 (13) [1950 - 2500]	1.10 ± 0.32 (13) [0.65 - 1.60]	0.03 ± 0.01 (11) [0.02 - 0.05]	42.54 ± 12.68 (13) [20.00 - 62.33]	29.47 ± 3.39 (13) [23.50 - 36.00]
2013	Moulting	St Kilda	2.24 ± 1.05 (6) [1.20 - 4.10]	2.37 ± 1.17 (8) [0.84 - 4.40]	0.67 ± 0.37 (8) [0.15 - 1.25]	382 ± 37.9 (8) [347 - 443]	2.45 ± 0.47 (8) [1.87 - 3.25]	2156 ± 186.0 (8) [1850 - 2367]	2.90 ± 0.64 (8) [1.87 - 3.95]	0.07 ± 0.03 (8) [0.04 - 0.11]	27.02 ± 10.01 (7) [16.50 - 40.33]	30.62 ± 3.00 (8) [26.67 - 36.00]
		Phillip Island	2.15 ± 1.73 (3) [1.07 - 4.15]	0.61 ± 0.30 (3) [0.37 - 0.95]	0.17 ± 0.01 (3) [0.16 - 0.18]	367 ± 52.1 (3) [307 - 400]	1.89 ± 0.05 (3) [1.83 - 1.93]	2344 - 50.9 (3) [2300 - 2400]	0.89 ± 0.09 (3) [0.81 - 0.99]	0.04 ± 0.004 (3) [0.03 - 0.04]	28.22 ± 11.60 (3) [15.00 - 36.67]	34.56 ± 0.38 (3) [34.33 - 35.00]
	Non-breeding	St Kilda	< LOR	1.16 ± 0.39 (6) [0.52 - 1.65]	0.16 ± 0.03 (6) [0.12 - 0.19]	336 ± 36.6 (6) [285 - 390]	2.37 ± 0.26 (6) [2.00 - 2.80]	2231 ± 99.7 (6) [2100 - 2350]	1.94 ± 0.33 (6) [1.60 - 2.55]	0.06 ± 0.02 (5) [0.04 - 0.09]	30.42 ± 16.44 (6) [15.00 - 57.00]	33.58 ± 3.55 (6) [28.33 - 37.50]
		Phillip Island	2.49 ± 0.85 (6) [1.75 - 4.10]	1.49 ± 0.53 (4) [0.84 - 2.05]	0.79 ± 0.11 (8) [0.68 - 0.98]	376 ± 26.0 (7) [350 - 415]	1.93 ± 0.29 (8) [1.45 - 2.35]	1971 ± 120.1 (8) [1850 - 2200]	0.73 ± 0.26 (8) [0.43 - 1.25]	0.06 ± 0.03 (8) [0.02 - 0.10]	21.88 ± 6.65 (4) [14.50 - 27.50]	31.60 ± 2.51 (8) [26.50 - 34.33]

4.2.3. Trace element analysis

We prepared and analysed blood samples as described in Finger *et al.* (2015). In short, we dried the blood samples at 60ºC to constant weight (mean drying quotient 5.36, standard deviation 0.59) and digested them in 65% nitric acid (SUPRAPUR, trace metal grade, Merck) and 37% hydrochloric acid (EMSURE, trace metal grade, Merck) at 95ºC. The cooled, filtered and diluted solutions were delivered to the National Measurement Institute (NMI), Melbourne, Australia, for elemental analysis. Aluminium (Al), calcium (Ca), iron (Fe) and zinc (Zn) were analysed at the NMI using a Perkin Elmer Optima 8300 Dual View Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) with a limit of reporting of 0.5, 10, 2 and 0.01 mg/kg, respectively. Arsenic (As), boron (B), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), selenium (Se) and tin (Sn) were analysed on an Agilent 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) with a limit of reporting of 0.01 mg/kg. All results were corrected for procedural blanks. The mean percentage recoveries of standard reference materials (SRM) ranged from 72 to 111 % for AGAL3 (shrimp) and 80 to 134 % for AGAL4 (bovine liver) for elements analysed (Table S1, Supplementary Materials). We excluded replicate results of samples run concurrently with SRM where both SRM for that element returned recoveries outside 70 - 130 % from statistical analysis. If only one replicate result remained for the sample, we excluded that sample result for that element; else we took the mean of the (remaining) replicate results.

Analytical outliers were detected by calculating the percentage relative standard deviation (%RSD) for each replicate sample and trace element,

followed by an acceptance test (see Finger *et al*., 2015). All replicate results failing the acceptance test were excluded from further analysis. Final results are reported as mean mg/kg dry weight (dw), and standard deviations (SD) are given.

4.2.4. Statistical analyses

Data were statistically analysed using R version 3.2.3 (R Core Team, 2015) and SPSS (version 21, SPSS Inc., Chicago, IL). Significance was taken to be $p < 0.05$ for all statistical analyses. Cd, Cr and Sn concentrations for most samples were either below the limit of reporting (0.01 mg/kg for all), returned SRM percentage recovery results outside the acceptable range (70% - 130%), or failed the analytical outlier detection test. We therefore excluded these elements from statistical analysis. Aluminium (Al) was valid for 128 out of the 157 samples analysed and is presented in Table 4.1, but excluded from further statistical analyses to preserve the size of the data set.

Concentrations of the remaining nine elements (As, B, Ca, Cu, Fe, Hg, Pb, Se and Zn) were treated as response variables in the statistical analyses. Categorical factors were Little Penguin colony location (St Kilda and Phillip Island), sampling year (2011, 2012, 2013), sampling season (breeding, moulting, non-breeding), sex (female, male) and moult stage (M0 to M5). Continuous factors were total head length, beak length, flipper length and body mass. Extreme statistical population outliers were identified in individual box plots by being further away than three times the inter-quartile range from the median (Logan, 2011). Normality of distribution for each element was tested using the Shapiro Wilk test, while we used Bartlett's test to investigate

homogeneity of variances ($p < 0.01$ for both, Quinn & Keough, 2002). Details of outliers removed and transformations applied are given in Table S2 to S4, Supplementary Material. Where needed, the continuous body mass data were transformed into four equally populated body mass categories using the R command "cut2" from the package "Hmisc" (Harrell Jr & Dupont, 2013). Non-metric multi-dimensional scaling (NMDS) was carried out using the R package "vegan" (Oksanen *et al*., 2013) and "ggplot2" (Wickham, 2009) to visually investigate which of the factors resulted in the greatest dissimilarities within the data set. One-way multivariate analyses of variance (MANOVA) were conducted by location, moult stage, THL, BL, FL and body mass category. Data analysis was then performed separately for St Kilda and Phillip Island to investigate the effects of year, season and sex. Little Penguin breeding seasons start in July to November of one year and finish in February of the following year (Reilly & Cullen, 1981). As we started sampling at St Kilda in the moulting season, breeding at St Kilda was assigned to the year it started in, so we were able to compare three years, complete with three seasons each. At Phillip Island, however, we collected the first samples in the breeding season. We assigned breeding at Phillip Island the year it finished and were thus able to compare two complete years with three sampling seasons each. For each location, we executed separate NMDS analyses, followed by one-way MANOVAs. Where we found significant effects, we performed post-hoc tests (Tukey HSD and pairwise *t*-tests with “Holm” correction) to elucidate differences between groups for each trace element. We investigated interannual variation and trend of elements using linear regression and 2-way analysis of variance (ANOVA) with year and season as fixed factors. Where

appropriate, we give Cohen's *d* as an effect size measurement (R package "compute.es"; Del Re, 2013), with small effect size = 0.2, moderate effect size = 0.5 and large effect size ≥ 0.8 (Cohen, 1988).

4.3. Results

4.3.1. Colony location, morphometrics and moult stage

The mean and SD of trace element concentrations in the blood of Little Penguins at two locations, over three years and three seasons are presented in Table 4.1. Location had the greatest effect on the combination of metals and metalloids in the blood of Little Penguins, as presented by the NMDS plot (Figure 4.2). The non-essential elements As, Hg and Pb were the largest vectors defining St Kilda penguins' dissimilarity, while Se was identified as Phillip Island penguins' most defining dissimilarity vector. The essential elements B, Ca, Cu, Fe and Zn were in or near the overlap area of both locations (Figure 4.2). A one-way MANOVA found a statistically significant difference in mean blood metal concentrations of Little Penguins between the two locations, $F_{1,110} = 32.11$, $p < 0.001$; Wilk's $\lambda = 0.26$; partial $\eta^2 = 0.75$. Pairwise *t*-tests with “Holm” correction were significant for As, Cu, Hg, Pb, Se ($p < 0.001$ for all, Cohen's d = -1.67, -0.76, -2.36, -1.41 and 0.94, respectively, Figure 4.3). Mean blood As concentrations were almost 3 times higher at St Kilda compared to Phillip Island. For Hg and Pb, that factor was 2.5 and 1.6, respectively. Mean Se blood concentrations were 1.6 times higher at Phillip Island compared to St Kilda penguins. Penguin body mass data (n = 157) was transformed into 4 categories using "cut2" from the R package "Hmisc": BM1: 900 g - 1080 g (n = 44), BM2: 1090 g - 1170 g (n = 35), BM3: 1180 g - 1310 g

(n = 44) and BM4: 1320 g - 1870 g (n = 34). Overall, penguin body mass category had a significant effect on blood trace element concentrations, $F_{3,103}$ = 2.33, p < 0.001; Wilk's λ = 0.55; partial η^2 = 0.18. Tukey's HSD tests were significant for As, Hg and Zn (p < 0.05). Arsenic and Hg followed a general pattern of increasing concentration with increasing body mass, while Zn exhibited the opposite pattern (Figure 4.4).

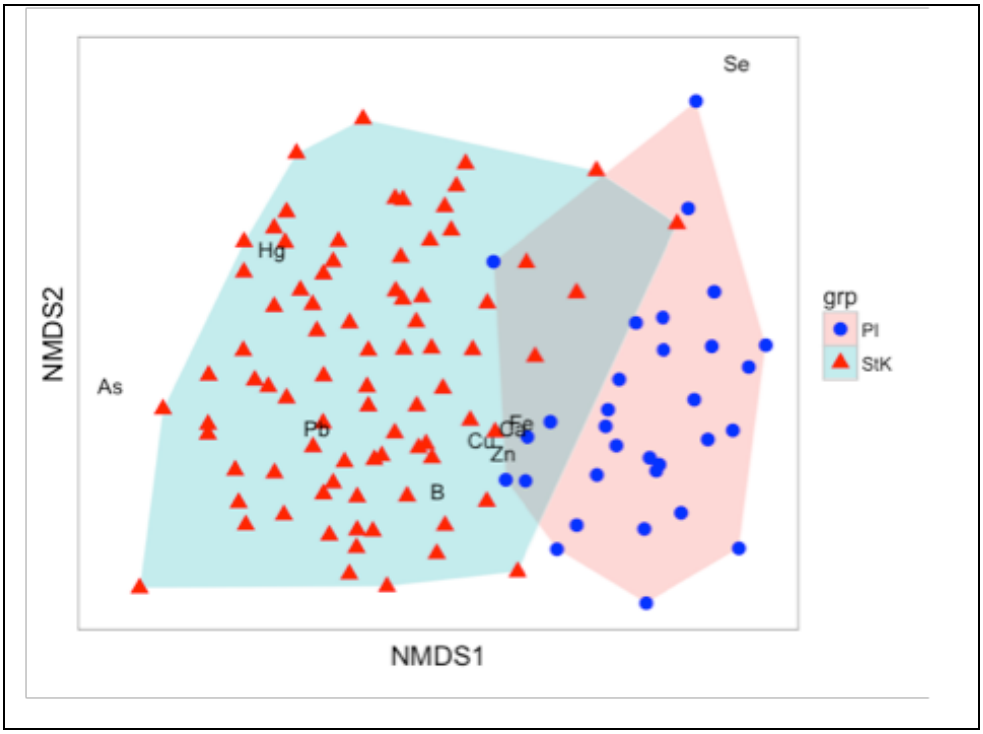

Figure 4.2: Two-dimensional NMDS plot with Bray-Curtis distance for Little Penguin blood samples by colony location (StK = St Kilda, PI = Phillip Island, stress = 0.20). Polygon ellipse lines are drawn for each sampling location. Metals and metalloids are displayed by their periodic symbols.

Total head length and beak length of Little Penguins were not significantly different between the sampled colonies. However, non-moulting Little Penguins were significantly heavier at St Kilda (mean weight 1199.7 g, SD 156.18 g) compared to non-moulting penguins at Phillip Island (mean weight 1118.8 g, SD 109.10 g, t_{113} = -2.96, p < 0.01, Cohen's d = -0.47).

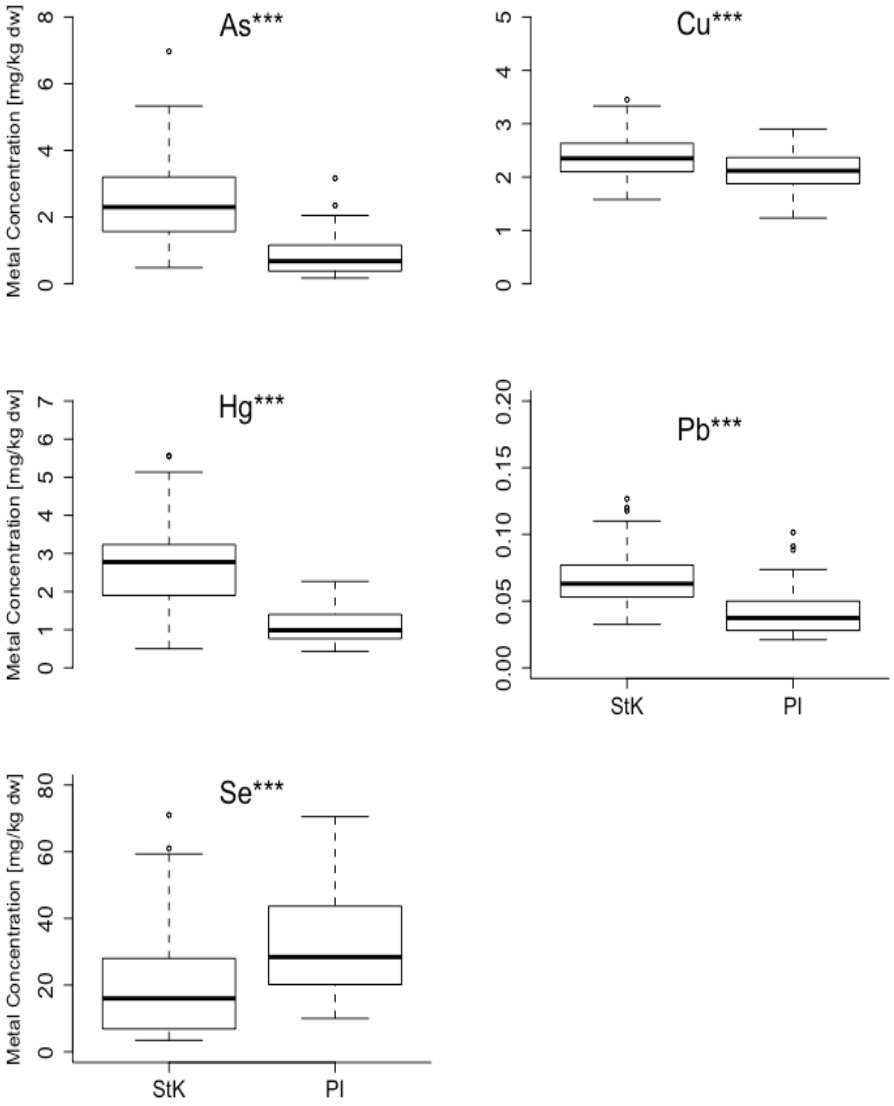

Figure 4.3: Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins that were significantly different (pairwise *t*-tests with "Holm" correction) by colony location (StK = St Kilda, Pl = Phillip Island). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

Penguins from St Kilda also had shorter flippers (mean 106.7 mm, SD 4.27 mm) than penguins at Phillip Island (mean 109.8 mm, SD 4.60 mm, t_{155} = -

4.21, $p < 0.001$, Cohen's $d = 0.70$). We found a significant negative correlation of blood Hg concentrations with flipper length at St Kilda (Pearson correlation, $r = -0.26$, $t_{94} = -2.59$, $p < 0.05$, r [95 %CI] = 0.44, Figure 4.5) but not Phillip Island. No other metals or metalloids showed any correlation with flipper length. Since flipper length overall varied significantly by sex ($t_{155} = -6.43$, $p < 0.001$, Cohen's $d = 1.06$), we executed a 2-way ANOVA of flipper length by sex (fixed) and Hg (random). At St Kilda, sex had a significant effect on flipper length ($F_{1,92} = 20.96$, $p < 0.001$, partial $\eta^2 = 0.19$), while Hg was just below significance levels ($F_{1,92} = 3.29$, $p = 0.07$, partial $\eta^2 = 0.03$), and there was no effect of sex:Hg ($p > 0.05$). At Phillip Island, sex ($F_{1,49} = 17.04$, $p < 0.001$, partial $\eta^2 = 0.26$) and sex:Hg ($F_{1,49} = 4.33$, $p < 0.05$, partial $\eta^2 = 0.08$) had a significant effect on penguin flipper length, but Hg did not ($p > 0.05$).

Moult stage of penguins sampled had a significant effect on blood metal concentrations, $F_{5,99} = 1.67$, $p < 0.01$; Wilk's $\lambda = 0.47$; partial $\eta^2 = 0.14$. However, Tukey's HSD tests were not significant for any metals or metalloids. Zn blood concentrations remained moderately constant throughout; while all other metals and metalloids followed a pattern of increase at the beginning of moult, decrease during the middle of moult, with a slight increase again at the end of moult (Figure 4.6).

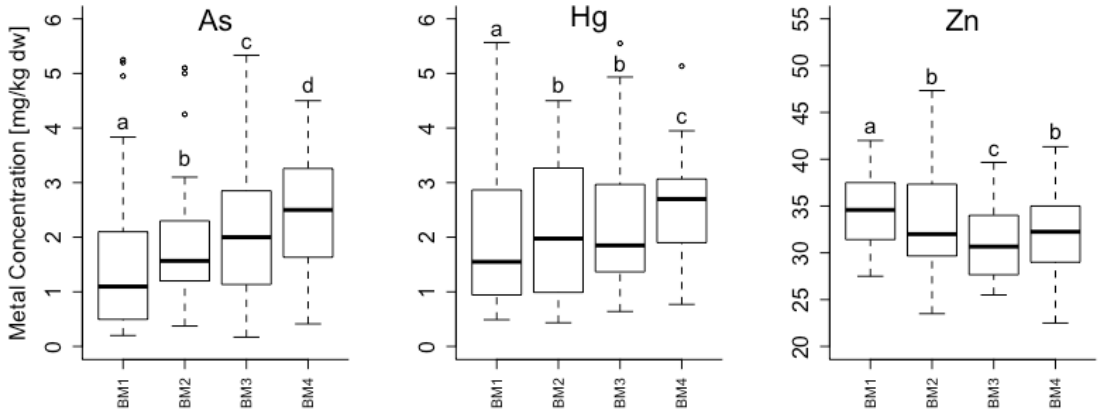

Figure 4.4: Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins that were significantly different (lower case letters, Tukey HSD, $p < 0.05$) by body mass category. BM1: 900 - 1080 g (n = 44), BM2: 1090 - 1170 g (n = 35), BM3: 1180 - 1309 g (n = 44), BM4: 1320 - 1870 g (n = 34). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

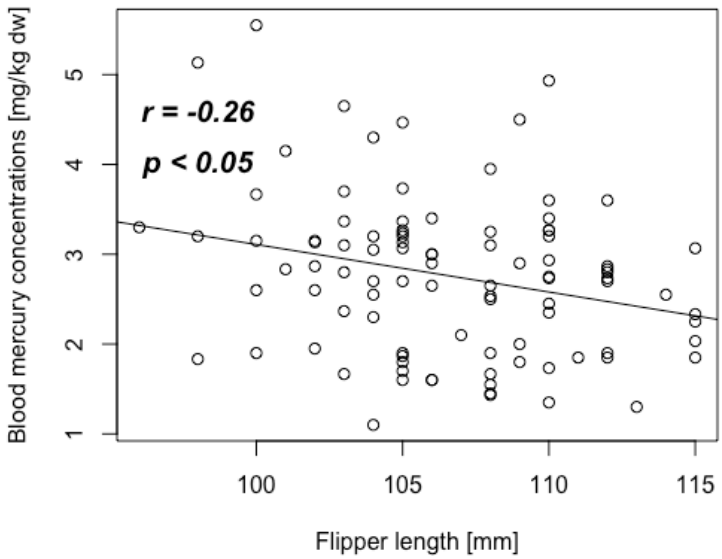

Figure 4.5: Pearson correlation between Little Penguin blood mercury concentrations and flipper length at St Kilda (n = 96).

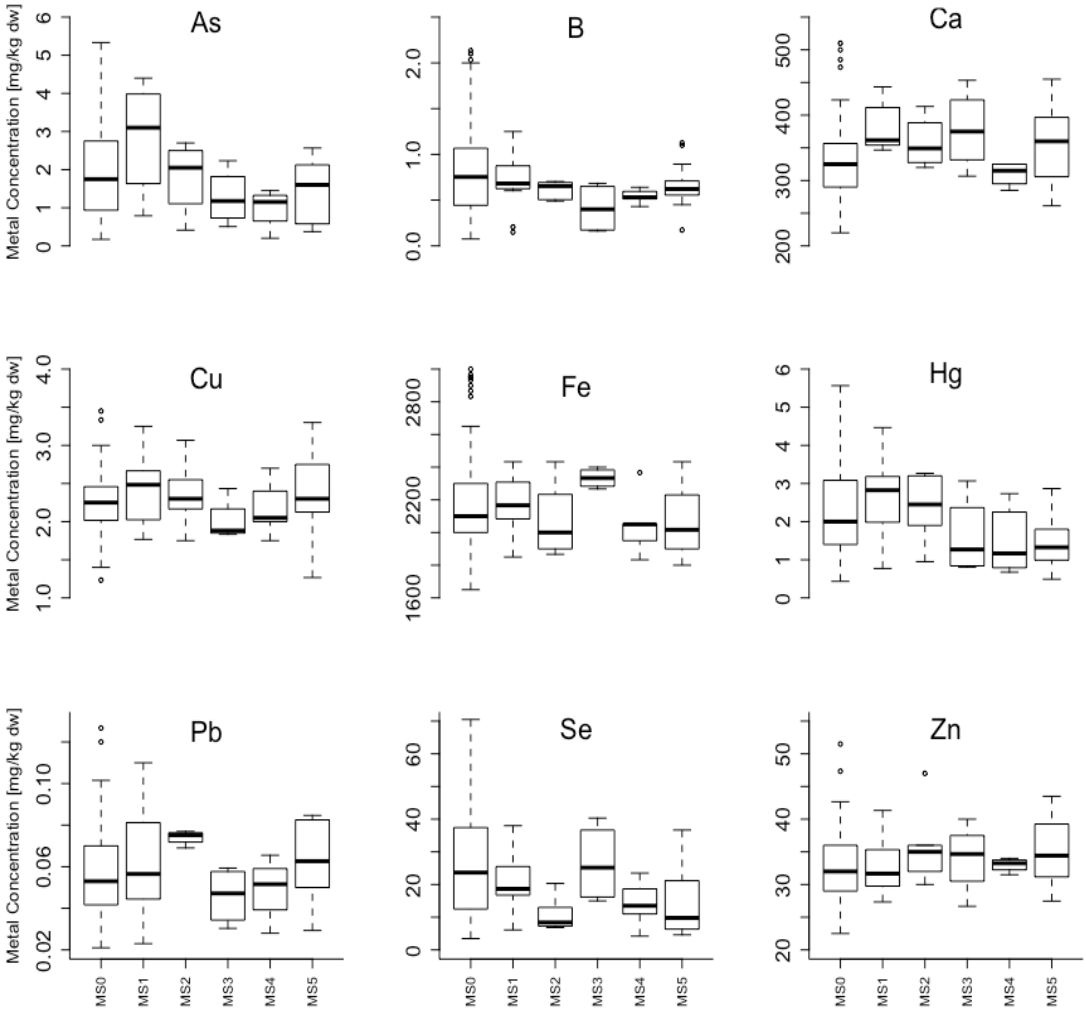

Figure 4.6: Metal and metalloid concentrations (mg/kg dry weight) in whole blood of Little Penguins by moult stage. MS0 means sample was taken outside of moult. M1 to MS5 are progressive moult stages, see methods for details. Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

4.3.2. Annual and seasonal variation - St Kilda

Metal and metalloid concentrations in the blood of Little Penguins at St Kilda varied significantly with year ($F_{2,67}$ = 10.21, p < 0.001; Wilk's λ = 0.15; partial η^2 = 0.61) and season ($F_{2,67}$ = 3.92, p < 0.001; Wilk's λ = 0.39; partial

η^2 = 0.37), but not sex (p > 0.05). NMDS analyses plots show Se, Hg and Pb as the most defining vectors by year and season at St Kilda (Figure 4.7, left panels). Hg and Se exhibited the same pattern between years (2011 < 2012 ~ 2013) and season (non-breeding ~ moulting < breeding), while Pb exhibited the opposite pattern (Figure 4.8 and 4.9). Post-hoc Tukey's HSD tests were significant for B, Fe, Hg, Pb and Se between years (Cohen's d = 0.04, 0.001, 0.24, -0.08 and 0.50, respectively) and for B, Ca, Hg, Pb, Se and Zn between seasons (Cohen's d = -0.54, -0.03, -0.10, -0.05, -0.02 and -0.07, respectively). In further analysis, blood Hg concentrations in St Kilda penguins presented an increasing trend over the three years measured (t = 5.53, p < 0.001, Cohen's d = 0.24, Figure 4.12). Two-way ANOVA of blood Hg concentrations, with year and season as fixed factors, found a significant effect for year ($F_{2,47}$ = 9.74, p < 0.001, partial η^2 = 0.19) and season ($F_{2,47}$ = 22.59, p < 0.001, partial η^2 = 0.31), but no combined effect year:season (p > 0.05, partial η^2 = 0.09) at St Kilda.

4.3.3. Annual and seasonal variation - Phillip Island

We found blood metal and metalloid concentrations of Phillip Island Little Penguins to be significantly affected by year ($F_{1,30}$ = 3.87, p < 0.01; Wilk's λ = 0.39; partial η^2 = 0.61) and season ($F_{2,29}$ = 4.36, p < 0.001; Wilk's λ = 0.12; partial η^2 = 0.65). One-way MANOVA found an overall significant effect for sex at Phillip Island ($F_{1,30}$ = 2.59, p < 0.05; Wilk's λ = 0.49; partial η^2 = 0.52), however, pairwise t-tests with "Holm" correction found no metals or metalloids were significantly different by sex. NMDS analyses plots are presented in Figure 4.7 (right panels).

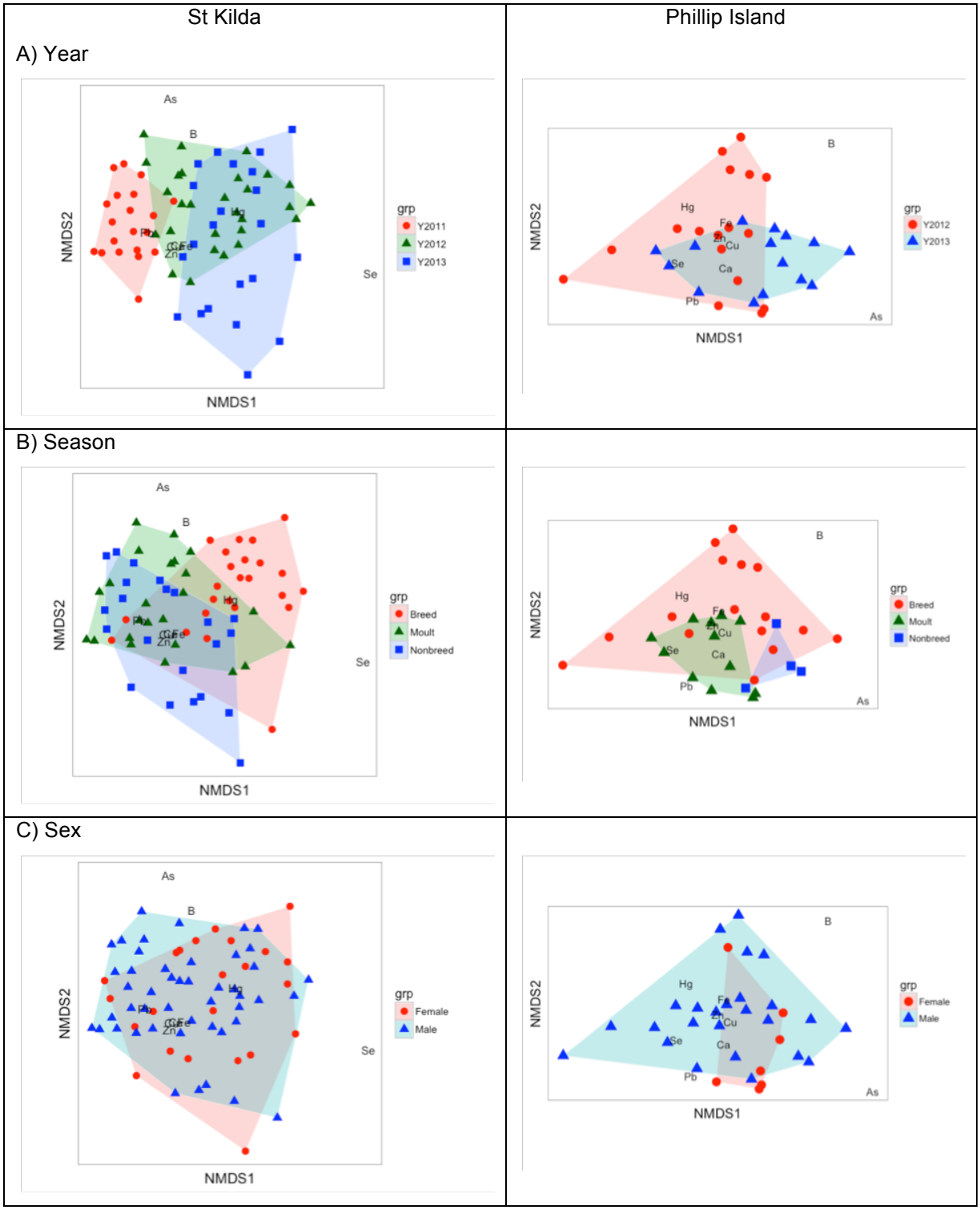

Figure 4.7: Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin blood samples from St Kilda (stress = 0.18, left panels) and Phillip Island samples (stress = 0.23, right panels) by factors A) year, B) season and C) sex. Polygon ellipse lines are drawn for each grouping; see legends for details for each. Metals and metalloids are displayed by their periodic symbols.

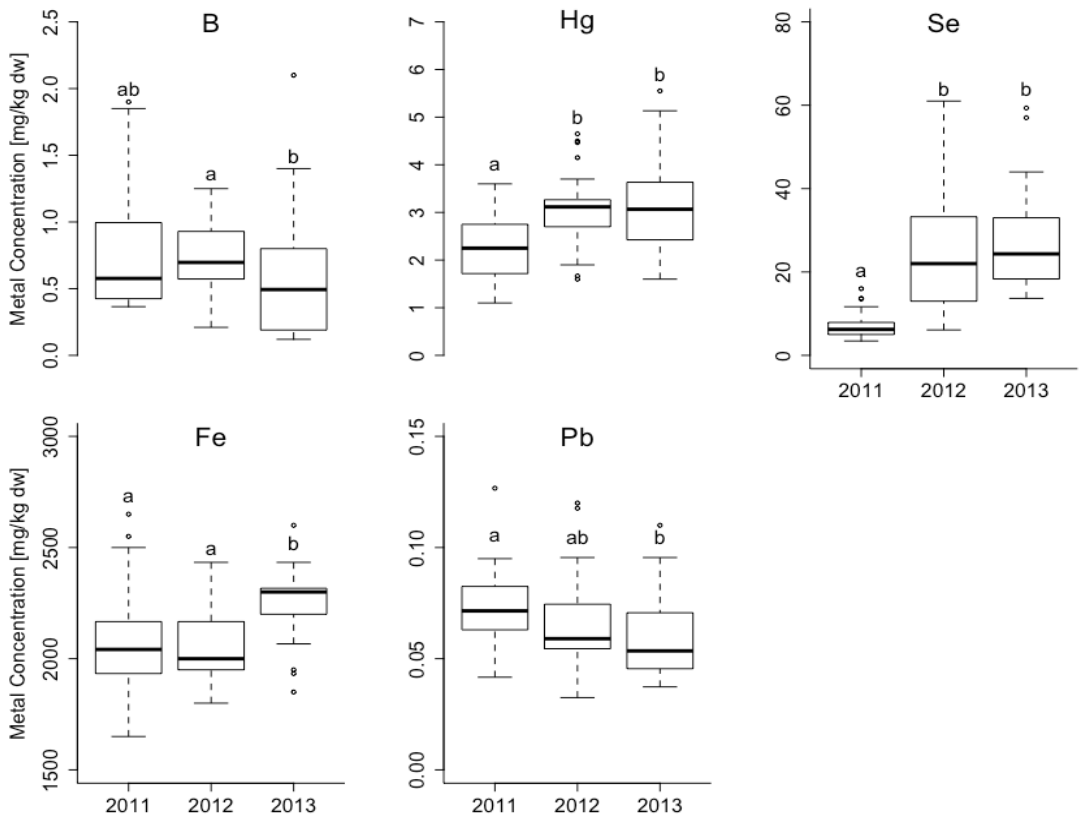

Figure 4.8: Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at St Kilda that were significantly different between years (lower case letters, Tukey HSD, $p < 0.05$). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

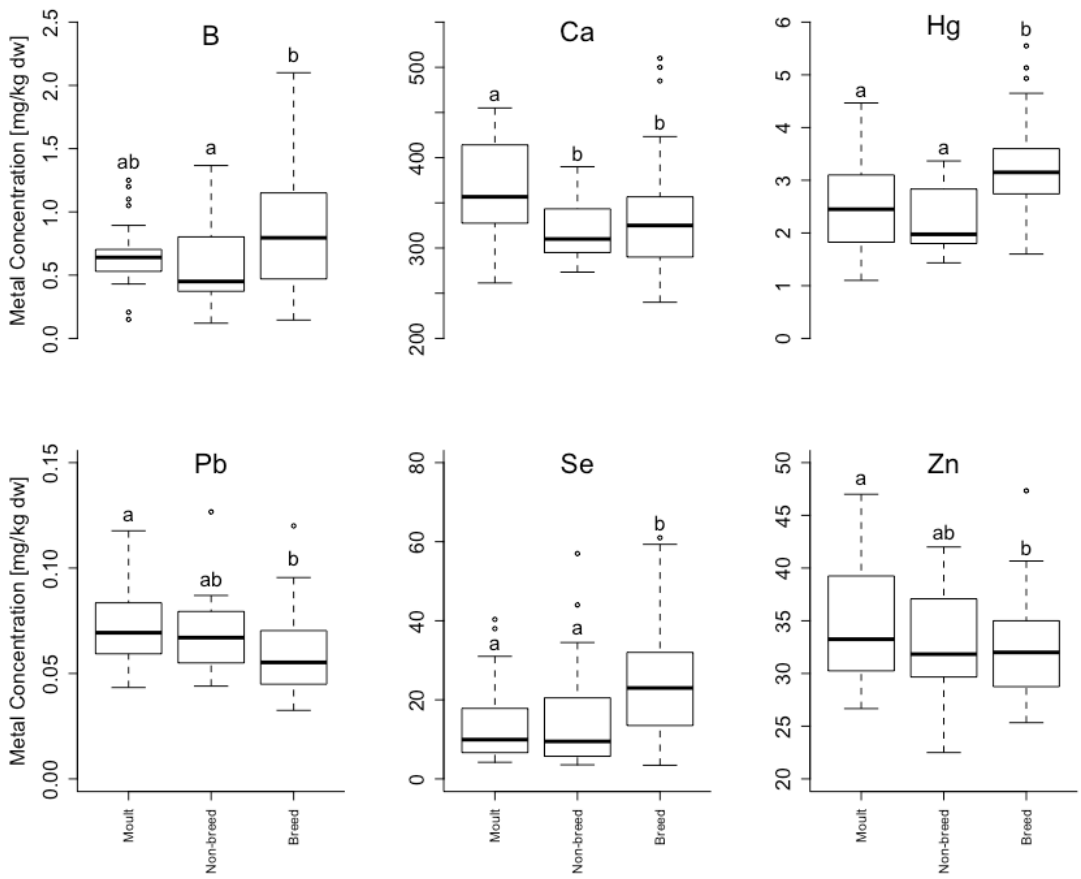

Figure 4.9: Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at St Kilda that were significantly different (lower case letters, Tukey HSD, $p < 0.05$) in three distinct seasons (moult, non-breeding and breeding). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

Post-hoc pairwise *t*-tests with "Holm" correction revealed significant annual differences between years for As, Hg and Zn (Cohen's d = 0.90, -0.60 and -0.79, respectively). Blood concentrations of As increased from 2012 to 2013, while Hg and Zn concentrations declined over the same time frame (Figure 4.10). Differences between seasons at Phillip Island were significant for As, Ca, Cu and Hg (Tukey's HSD, $p < 0.05$, Cohen's d = 0.71, -0.06, 0.01 and

0.01, respectively). Hg and Ca show a similar pattern between seasons (moult ~ non-breeding < breeding), while As showed an increase in the non-breeding season (Figure 4.11). Over the two years of sampling at Phillip Island, penguins carried decreasing concentrations of Hg (t = -3.44, p < 0.01, Cohen's d = -0.60) (Figure 4.12). Two-way ANOVA of blood Hg concentrations at Phillip Island, with year and season as fixed factors, found a significant effect for season ($F_{2,47}$ = 6.83, p < 0.05, partial η^2 = 0.26), but not for year or year:season interaction (both p > 0.05, partial η^2 = 0.12 and 0.05, respectively).

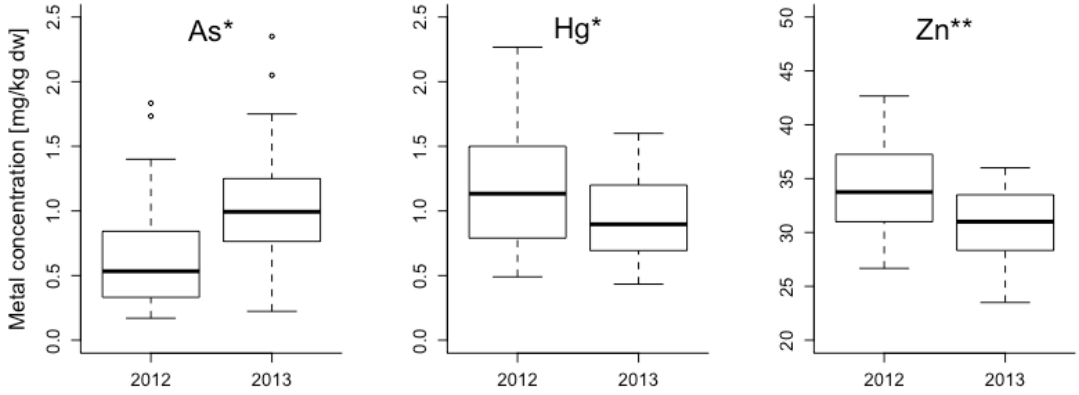

Figure 4.10: Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at Phillip Island that were significantly different (univariate ANOVA, p < 0.025) between the two years sampled (2012 to 2013). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

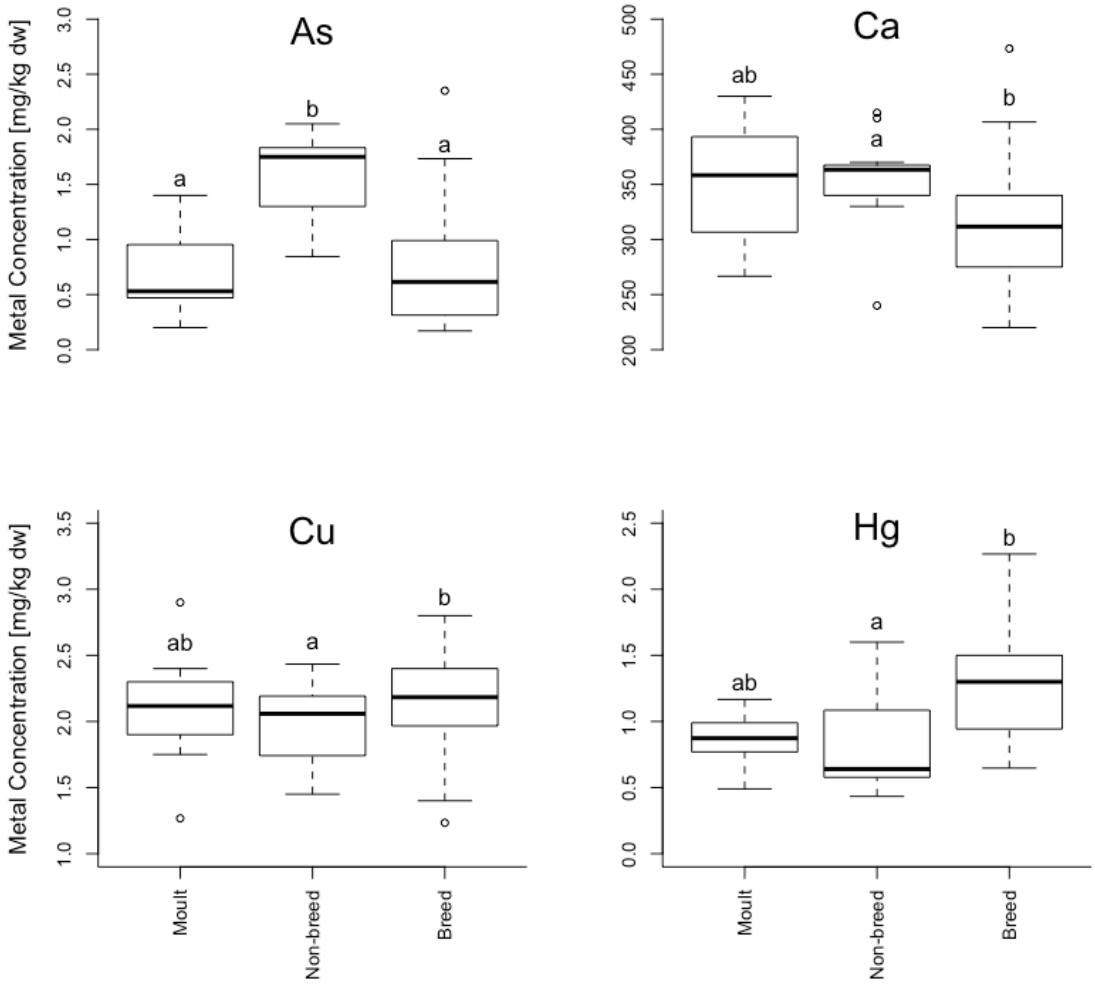

Figure 4.11: Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at Phillip Island that were significantly different (lower case letters, Tukey HSD, $p < 0.05$) in three distinct seasons (moult, non-breeding and breeding). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. The length of the box is the interquartile range (IQR). Lower and upper whiskers are minimum and maximum observation, or in case of outliers, calculated as Q1 - 1.5*IQR and Q3 + 1.5*IQR. Mild outliers (º) are defined as between 1.5 and 3*IQR.

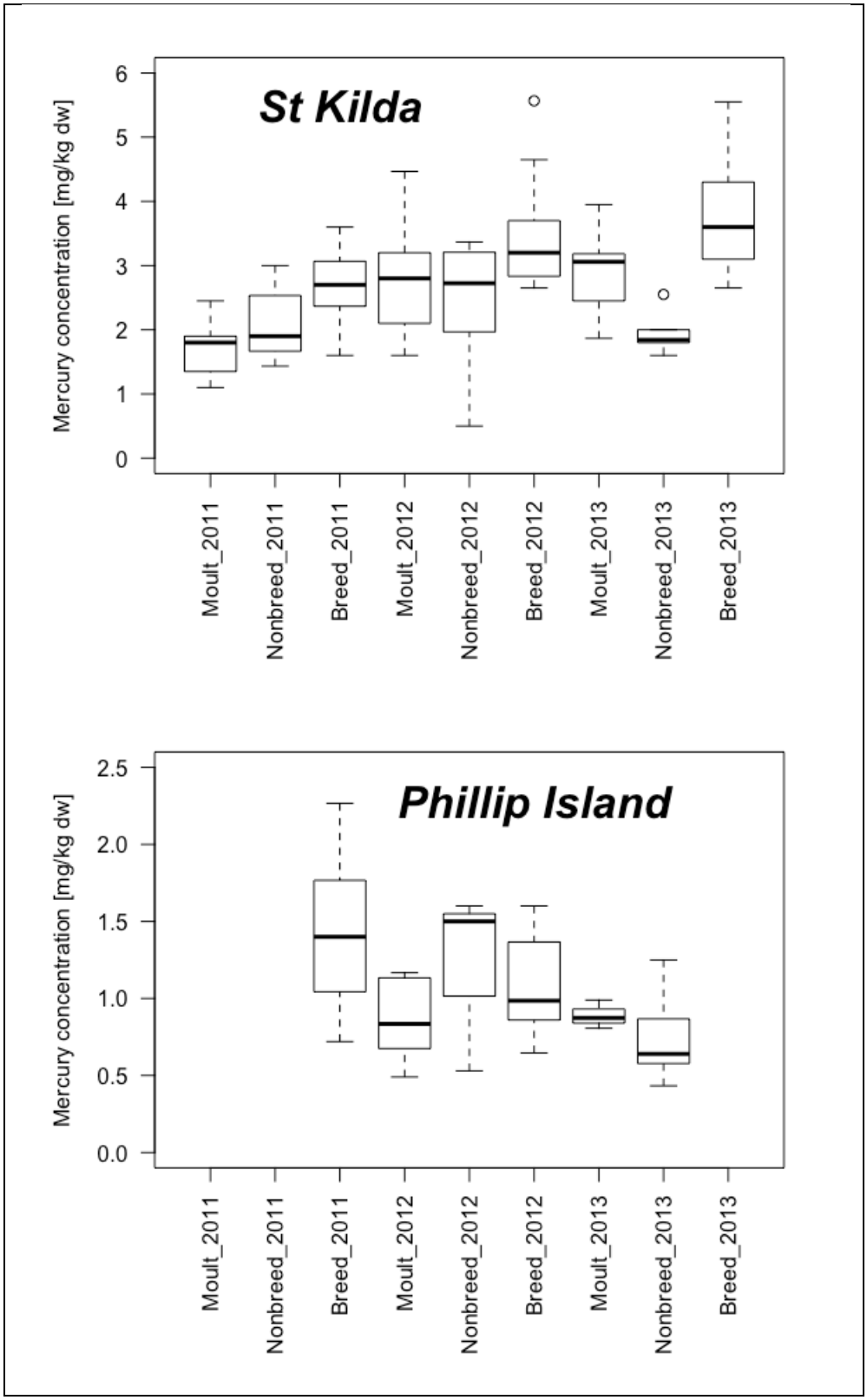

Figure 4.12: Mercury concentrations (mg/kg dw) in blood of Little Penguins, sampled from March 2011 to December 2013 at St Kilda and from November 2011 to May 2013 at Phillip Island.

4.4. Discussion

4.4.1. Differences between colonies

We found clear differences in blood metal and metalloid concentrations between the St Kilda and Philip Island penguin colonies, especially for As, Cu, Hg, Pb and Se (Figure 4.4). However, our analysis of the data also shows some overlap. It is possible that this could be a result of a partial overlap in the foraging range of the two sampled colonies during their non-breeding period. As central place foragers (Orians & Pearson, 1979), Little Penguins are restricted in their home range during the breeding season (Collins *et al*., 1999; Kowalczyk *et al*., 2015a). Due to the consistent and reliable presence of clupeoid fish in suitable size classes (6-10 cm) within Port Phillip Bay (Hirst *et al*., 2010; Hirst *et al*., 2011), adult St Kilda Little Penguins feed exclusively within Port Phillip Bay throughout the year (Preston *et al*., 2008; Kowalczyk *et al*., 2015a). Phillip Island penguins forage near their colony in the open waters of Bass Strait during the breeding season, but enter Port Phillip Bay during the winter months for the benefits of more reliable prey and calmer waters (McCutcheon *et al*., 2011; Chiaradia *et al*., 2012). Despite this foraging overlap, the multi-year results presented here confirm our earlier findings from Little Penguin blood metal concentrations during moult 2012 (Finger *et al*. 2015) and indicate that the spatial pattern of metal contamination observed, St Kilda > Phillip Island, is consistent across years.

The increased blood concentrations of As in St Kilda penguins are likely due to high bioavailability of this metalloid within Port Phillip Bay owing to natural sediment mineralogy (Harris *et al*., 1996) while higher Hg and Pb concentrations are likely the result of historic and current anthropogenic

deposits into the Bay (Walker, 1982; Harris *et al*., 1996). The significantly higher blood Se concentrations at Phillip Island are likely the result of those penguins feeding on more Se-rich prey items compared to their conspecifics at St Kilda. No local data exist on Se concentrations in the penguins' prey items, but Dunlop *et al*. (2013) found a strong correlation between Se in prey and Little Penguin feathers in Western Australia, and Se is generally lower in anchovies than in pilchards (Yamashita *et al*., 2011; Olmedo *et al*., 2013), the latter of which are more frequently found in the diet of Phillip Island penguins (Chiaradia *et al*., 2012).

While it is not uncommon for metal concentrations in birds to differ between sexes, particularly for Hg (see review in Robinson *et al*., 2012), we only found an effect of sex on metal concentrations at Phillip Island, but not St Kilda. This could be due to slight sexual dimorphism in foraging being present in one colony, but not the other (Shaw, 2008). The slight weight differences between penguin sexes may have a larger impact on diet variation in the open Bass Strait waters than they do in shallow bay waters, as heavier male penguins are able to dive deeper and catch different prey to females. However, a post-hoc test failed to identify any metals or metalloids that were significantly different between sexes. Also, the NMDS graphs for sex (Figure 4.7, C panels) show a large overlap between trace elements in females and males at both locations. The post-hoc analyses' effect sizes were 'moderate' only for three out of the nine elements measured (Cohen's d = -0.46, 0.54 and 0.50 for Fe, Pb and Zn, respectively, else Cohen's d < 0.29). A more even distribution of male and female samples, and a larger overall sample size is required to confirm whether there is an effect of sex on penguin blood metal /

metalloid blood concentrations and if so, which metals or metalloids are driving this effect at Phillip Island.

Non-moulting St Kilda penguins (moulting penguins were excluded to prevent bias due to uneven sample effort between locations and moult stages) were on average heavier than Phillip Island penguins. This is likely due to more consistent availability of food throughout the year within Port Phillip Bay (Kowalczyk *et al*., 2015b) and St Kilda penguins' shorter foraging trips (~20 km, Preston *et al*., 2008). However, it is unclear why St Kilda penguins have shorter flippers. This is the first time this has been reported. Notably, all measurements were taken by the same person (AF), using the same technique and equipment. While it is possible that epigenetic selection might have occurred (Overeem et al., 2008), we cannot dismiss a link with pollution, as increased metal loads have been associated with reduced wing length in fledgling Flesh-footed Shearwaters (*Ardenna carneipes*) (Lavers *et al*., 2014) and with reduced growth rates in Little Blue Heron chicks (*Egretta caerulea*) (Spahn & Sherry, 1999). The negative correlation of flipper length with blood Hg concentrations at St Kilda found in this study suggests a potential pollution link at the urban colony. The results of our analysis of sex and Hg effects on flipper length did not show a conclusive Hg effect, however, probability was close to significance levels (p = 0.07) and the effect size was low (partial η^2 = 0.03). We strongly encourage continued data collection at both colonies to find out whether the shorter flippers of this urban dweller are in fact an indicator of a population-wide deleterious physiological effect of pollution in their foraging area.

4.4.2. Seasonal variation

Overall, the seasonal variation of blood metal concentrations was different at St Kilda and Phillip Island (Figure 4.7, B panels). Blood Hg concentrations, however, followed a distinct seasonal pattern at both locations: Non-breeding < Moulting < Breeding (Figures 4.9 and 4.11). This pattern is also visible during each individual sampling year at both locations (Figure 4.12). This suggests a stronger effect of physiological mechanisms associated with this persistent pollutant, such as accumulation, mobilisation and detoxification of Hg, rather than dietary differences between seasons. The only time our data did not conform to this pattern was in the first year at St Kilda (Figure 4.10). However, moult data in 2011 were collected about two weeks later than in the following years and most of the penguins sampled were in the last moult stages. Mean blood Hg concentrations are likely to have been higher if we had sampled more penguins in the early stages of moult (see graph for Hg in Figure 4.6). All other times, at both locations, non-breeding Hg was lower than moulting Hg. This is in accordance with the fact that in birds, Hg stored in internal organs gets mobilised during fasting and, as a prominent way of detoxification, is sequestered into the feathers (Braune & Gaskin, 1987; Moneiro & Furness, 2001a & 2001b; Bearhop *et al*., 2000). The low Hg during the non-breeding season might also be explained by the influx of juvenile pilchards into the Bay (Neira *et al*., 1999), i.e. this prey species does not mature in the bay and would have lower Hg concentrations than fish that were spawned and matured in the Bay. Blood Hg concentrations were highest during the breeding season, when penguins were accumulating more food, and hence more Hg, because they were providing for their chicks.

Blood concentrations at Phillip Island showed seasonal variation for As, Ca, Cu and Pb (Figure 4.11). Of those elements, As displayed the most pronounced variation between seasons. The data are influenced by low sample numbers (Table 4.1), but mean blood As concentrations were consistently highest during the non-breeding season. This might be a reflection of Phillip Island penguins feeding in Port Phillip Bay during a time when they are not restricted by needing to provide for offspring (Chiaradia *et al*., 2012). Ca metabolism in birds is closely regulated, particularly in females (Reynolds & Perrins, 2010). While inter-seasonal variation of Ca at St Kilda (Moult > Non-breeding ~ Breeding, Figure 4.9) was different to Phillip Island (Moult ~ Non-breeding > Breeding, Figure 4.11), it was consistent in low Ca levels during breeding, as females deposit large amounts of Ca into their eggs.

Notably, this study is the first to report on blood metal concentrations during the different stages of moult. Little Penguins fast during the approximately 22 days of moult each year (Reilly & Cullen, 1983). During this time, in particular non-essential metals get mobilised from internal stores (liver, fat) and sequestered into the new feathers (Burger, 1993). Monteiro & Furness (2001a) executed one of very few dose-response field studies and found that for Cory's Shearwaters (*Calonectris diomedea*), blood Hg levels correlated with feather Hg, independent of dose. Moult is the most effective and important means of detoxification in birds, as 70 to 93 % of the Hg body burden gets excreted through moult (Evers *et al*., 1998). Most of the nine elements presented in this study, but in particular the non-essential elements As, Hg and Pb, followed a distinct 'wave' pattern during moult: increasing at

the start, were lowest mid-way through and increasing again at the end of moult (Figure 4.6). Interestingly, Spalding *et al*. (2000), in a rare captive feeding study of fledging Great Egrets (*Ardea albus*) also reported that Hg blood concentrations increased once the new feathers were grown. We have thus been able to describe this complex mechanism of non-essential metal mobilisation, depuration / detoxification and end of fast upswing in Little Penguins.

4.4.3. Inter-annual variation

We found a trend of increasing blood mercury concentrations in Little Penguins nesting at St Kilda from 2011 to 2013 (Figure 4.12). The source of the increased bioavailable mercury observed in this study for Port Phillip Bay is unknown. Input of mercury into Port Philip Bay has been stable or decreasing since new regulations for industrial point-sources were implemented in the 1990s (Harris *et al*., 1996). One possibility for an increase in bioavailable Hg is that penguins at St Kilda over the three years 'gradually' switched to more Hg-rich prey. Unfortunately, no multi-year data exist on metals in the main prey species of the Little Penguin in Port Phillip Bay. Another possibility could be climate change driven increased sea temperatures and acidity increasing the absorption of metals (Sokolova & Lannig, 2008; Millero *et al*., 2009), but it is unlikely that this alone could have a major effect over such a short time-frame. Yet another possibility is the global trend of increasing oceanic Hg, but this applies to a lesser degree to the Southern hemisphere (Lamborg *et al*., 2014). Only two of seven penguin species in the southern Indian Ocean exhibited an increasing Hg pattern in

their feathers (Carravieri *et al*., 2016) and it is certainly questionable why St Kilda penguins would reflect this global trend when penguins from Phillip Island did not.

Alternatively, it is possible that the Hg annual increase might be linked to the Port Phillip Bay Channel Deepening Project, which was executed in 2008 and 2009 (PoMC, 2010). Metals and other pollutants stored in marine sediments can become bioavailable when these sediments are physically disturbed and toxicant particles are re-suspended in the water column (Hedge *et al*., 2009; Edge *et al.,* 2015; Fetters *et al*., 2015). EPA Victoria, the environmental governing body of the state, have executed a monthly monitoring protocol of water quality measures since 2000 for several stations within the bay (EPA, 2015), including Hobsons Bay, which is within the known foraging range of St Kilda penguins (Preston *et al*., 2008; Kowalczyk *et al*., 2015a). These measurements recorded a number of spikes in total suspended solids (TSS), Cr, Cu, Pb and Zn for Hobsons Bay in 2008 and 2009 (Figure S1, Supplementary Materials), exceeding the State Environment Protection Policy guidance limits for TSS and Cu, and coinciding with the timing of dredge works (PoMC, 2010). We are not aware of any other activity or climatic event that could have caused these spikes. While Hg was measured, all but one measurement in November 2009 (0.1 µg/L, EPA, 2015) returned results under the limit of reporting (0.1 µg/L, EPA, 2015). However, it is safe to assume that the same sediments that were dredged contained high levels of Hg (Fabris *et al*., 1999) and that Hg-rich particles were re-suspended in the water column and thus entered the lower trophic levels of Port Phillip Bay. As part of the environmental impact obligations, a bay-wide contaminant

study measured trace metals and organic pollutants in black bream (*Acanthopagrus butcheri*) in January 2009 (EPA, 2009) and found no elevated concentrations of any pollutants compared to an investigation on the same fish in 2006 (EPA, 2007). However, dredge works were only completed in September 2009 (PoMC, 2010), and furthermore, the black bream of the size sampled (> 26 cm, EPA, 2009) would not have been impacted by any potential trophic transfer of increased contaminants until much later. Unfortunately, the physiochemical mechanisms of mobilisation / uptake of Hg into aquatic food chains are non-trivial, dependent on many factors (Lavoie *et al*., 2013), and there is scant experimental data on the time it takes for contaminants to travel up trophic levels. But a timeline from the best-known case of mercury poisoning, at Minamata Bay, suggests years rather than months for Hg to travel from the food web base to the level of piscivores (Harada, 1995). With the current data available, we are unable to confidently state whether inter-annual variations of Hg concentrations in Little Penguins reflect changes in food-chain contamination, or are the result of a reorganization/modification of the Port Phillip Bay food web. Perhaps it is a combination of all these mechanisms? While the concentrations reported in this study are below effect levels recorded for other bird species (Evers *et al*., 2008), it is wise to caution against species to species comparisons. In light of the trend observed, long-term surveying of mercury levels in this resident seabird in Port Phillip Bay is warranted. This will ensure the penguins' continued health, conservation and management.

4.5. Conclusion

The physiology of trace elements during the different moult stages, between seasons and years is important in the interpretation of future Little Penguin blood data. How do we know that the pattern observed is due to variations in contaminant exposure and not the result of naturally occurring seasonal variation? Only by having knowledge of a 'baseline' pattern, gleaned from large data sets, can that distinction be made. The details of seasonal, annual and within-moult variations provided here do not exist for any resident high-trophic feeder anywhere in the world. It is hoped that this information can be used to elucidate long-term changes in contaminant exposure in this iconic species as well as provide insight into changes in the bioavailability of metal pollution within Port Phillip Bay, Melbourne, Australia. We recommend this bioindicator be used to inform future environmental impact statements.

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Chapter 5

Metals and metalloids in Little Penguin (Eudyptula minor) prey, blood and faeces

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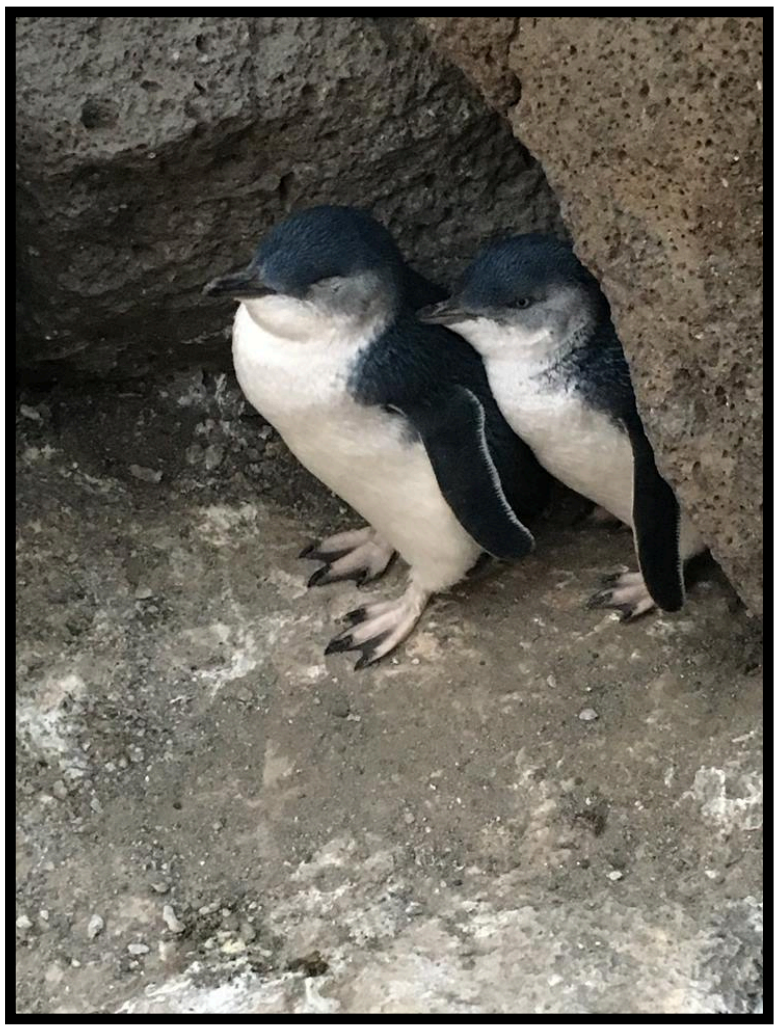

Little Penguin breeding pair at St Kilda
(Photo source: @DELWPPortPhillip)

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1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Annett Finger	76	Co-designed study, field sampling, sample analysis, data analysis, preparation of mauscript	[redacted]	21/10/16
Jenn Lavers	5	Revision of manuscript		21/10/16
Peter Dann	5	Co-designed study, revision of manuscript		21/10/16
Dayanthi Nugegoda	5	Co-designed study, revision of manuscript		21/10/16
John Orbell	3	Revision of manuscript		21/10/16
Carol Scarpaci	3	Co-designed study		21/10/16
Nicole Kowalczyk	3	Contributed stable isotope data set		21/10/16

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5. Metals and metalloids in Little Penguin (*Eudyptula minor*) prey, blood and faeces

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Abstract

Piscivorous species like the Little Penguin (*Eudyptula minor*) are particularly at risk of being negatively impacted by pollution due to their heightened exposure through aquatic food chains. Therefore, determining the concentration of heavy metals in the fish prey of seabirds is an essential component of assessing such risk. In this study, we report on arsenic, cadmium, mercury, lead and selenium concentrations in three fish species, which are known to comprise a substantial part of the diet of Little Penguins at the urban colony of St Kilda, Melbourne, Australia. Metal concentrations in the fish sampled were generally within the expected limits, however, arsenic and

mercury were higher than reported elsewhere. Anchovy (*Engraulis australis*) and sandy sprat (*Hyperlophus vittatus*) contained higher Hg concentrations than pilchard (*Sardinops sagax*), while sandy sprat and pilchard contained more selenium. We present these findings together with metal concentrations in Little Penguin blood and faeces, sampled within weeks of the fish collection. Mercury concentrations were highest in the blood, while faeces and fish prey species contained similar concentrations of arsenic and lead, suggesting faeces as a primary route of detoxification for these elements. We also investigated paired blood - faecal samples and found a correlation for selenium only. Preliminary data from stable isotope ratios in penguin blood indicate that changes in penguin blood mercury concentrations cannot be explained by trophic changes in their diet alone, suggesting a variation of bioavailable Hg within this semi-enclosed bay.

Capsule: Metals and metalloids in prey, blood and faeces of a resident urban high trophic feeder are presented together with paired blood - faecal sample correlations and stable isotope ratios.

Keywords

Coastal pollution, blood, guano, fish trace metal, Port Phillip Bay

5.1. Introduction

Contamination loads in high trophic feeders are widely regarded as reliable indicators of bioavailable pollutants throughout their foraging range (Markert *et al.*, 2003a). Such information is especially useful for managing a large semi-enclosed embayment like Port Phillip Bay, where pollutants accumulate as a result of activities undertaken by the adjacent 5.5 million

people metropolis of Melbourne, Australia (ABS, 2011). Contaminants released into the marine environment are often stored in the sediments, but can be taken up into the food chain by microbial action (Hedge *et al.*, 2009; Edge *et al.*, 2015; Fetters *et al.*, 2015). The concentration of these elements is usually higher in predators than in their prey, as the rate of dietary intake exceeds the rate of loss in the organism (Rand *et al.*, 1995). This is called biomagnification, or trophic magnification (Markert *et al.*, 2003b). The higher the trophic position of an animal, the greater the rate of transfer of pollutants; and the highest rate of increase in concentration is often from water-breathing prey to air-breathing predators (Neff, 2002). Biomagnification, particularly of mercury (Hg), occurs in seabirds and marine mammals (O'Shea, 1999; Ciesielski *et al.*, 2006; Bond and Diamond, 2009). Temporal variations in contaminant concentrations in resident high trophic feeders can be either (1) due to the animals switching to prey from different trophic levels (Carvalho *et al.*, 2013), or (2) due to changes in the quantity of a bioavailable toxicant in their environment (e.g. spill or remediation), which has impacted the toxicant concentrations in the prey (Braune, 2007), or a combination of both. The trophic level of the ingested prey can be inferred from stable isotope ratios in the tissues of the predator (Hobson, 1999). Some recent studies have incorporated information on changes in the trophic level of prey items, as determined by stable nitrogen isotope ratios, to make assertions as to whether trophic or environmental change were the predominant contributor to an observed temporal variation in contaminant concentration of a high trophic feeder (Dehn *et al.*, 2006; Cui *et al.*, 2011; Burgess *et al.*, 2013).

The trophic structure of Port Phillip Bay is typical for shallow coastal ecosystems, and is based on phytoplankton, detritus, seagrass and macroalgae, which sustain zooplankton, filter- and deposit-feeders, and these in turn support a range of small clupeoid and juvenile fish, forming the food base for larger fish, seabirds, marine mammals and sharks (Crawford *et al.*, 1992; Officer and Parry, 1996; Fulton and Smith, 2004). Of the high trophic feeders within Port Phillip Bay, the Little Penguin (*Eudyptula minor*) is a good bioindicator of contamination effects, as they are long-lived, conspicuous, exhibit strong site-fidelity and are robust to being handled (Reilly and Cullen, 1981; Dann *et al.*, 2005). Seabirds are often used as contamination bioindicators, but interpretation of tissue concentrations is usually confounded spatially by their migration patterns (Wilson *et al.*, 2004; Ackerman *et al.*, 2008; Lavoie *et al.*, 2014). However, the 1,300 Little Penguins nesting at St Kilda (Z. Hogg, unpublished data) remain within Port Phillip Bay all year around (Preston *et al.*, 2008; Kowalczyk *et al.*, 2013) and their pollutant body burdens represent exposure through prey caught within 15 km of their nesting site (Kowalczyk *et al.*, 2015a). Contamination concentrations in faeces (guano) and its importance as a mode of depuration, has been investigated in laboratory studies (Lewis and Furness, 1991; Kenow *et al.*, 2007) and in the field (Morrissey *et al.*, 2005; Costa *et al.*, 2012; Celis *et al.*, 2015), but never in the Little Penguin. Also, investigations into blood and faecal correlations in seabirds are scant.

Little Penguin diet at St Kilda varies depending on availability, but predominantly consists of Australian anchovy (*Engraulis australis*), pilchard (*Sardinops sagax*), southern garfish (*Hyporhamphus melanochir*), and

luminous bay squid (*Loliolus noctiluca*) (Preston, 2010; Kowalczyk *et al.*, 2015b). Some fish in Port Phillip Bay have been found to contain elevated concentrations of mercury, arsenic and lead (Gagnon *et al.*, 2016; Harris *et al.*, 1996; Walker, 1988), but to date, no heavy metal data exist for Little Penguin prey items within Port Phillip Bay. Diet is the predominant source of pollution in seabirds (DesGranges *et al.*, 1998; Monteiro *et al.*, 1998; Carvalho *et al.*, 2013). Blood is as good a predictor for seabird pollutant body burdens as are internal organs (Eagles-Smith *et al.*, 2008). Blood can be collected non-destructively, without an adverse effect on individuals or populations, and allows for spatial and seasonal investigations (Finger *et al.*, 2016). The restricted foraging range of Little Penguins makes them particularly suitable bioindicators for local pollution and we have previously reported on significantly higher concentrations of heavy metals in the St Kilda population compared to more remote breeding sites (Finger *et al.*, 2015). This study aimed to 1) establish metal(loid) concentrations in Little Penguin fish prey items, 2) compare these to blood and faecal metal(loid) concentrations collected within weeks of fish sampling, 3) investigate blood - faeces metal(loid) correlations, and 4) using penguin blood stable nitrogen isotope ratios, calculate diet-corrected blood metal(loid) concentrations to assess bioavailability trends of metal(loid)s within Port Phillip Bay.

5.2. Materials and methods

5.2.1. Study site and sample collection

All samples were collected within Port Phillip Bay, south-eastern Australia (Figure 5.1) from February 2011 to December 2013. Port Phillip Bay

is a temperate, semi-enclosed, relatively shallow bay that encompasses 1930 km^2 and is bordered by the large city of Melbourne. Three species of fish, Australian anchovy, sandy sprat (*Hyperlophus vittatus*) and pilchard were obtained frozen in bulk from commercial catches in Port Phillip Bay at specific dates, matching four distinct Little Penguin field sessions (Table 5.1), henceforth called "events". The fish species selected are commonly caught by Little Penguins in Port Phillip Bay, as has been established by stomach flushing (Preston, 2010) and stable isotope analysis (Kowalczyk *et al.*, 2013; Kowalczyk *et al.*, 2015b), as well as anecdotal evidence by the commercial fisherman who provided the fish samples (P. Mc Adams, pers. comm.). Fish were caught by net in large cohorts and the individuals within a batch had similar standard lengths, which minimised variations in metal concentrations due to size of the organism. Pilchards were caught in two distinct age/size classes. The cohorts were classified as juvenile and young adult based on size data obtained from commercial catches in Port Phillip Bay (Neira *et al.*, 1999). The fish were allowed to partially thaw, until single fish could be safely (without breaking) separated from the bulk. Entire single fish were then individually stored in labelled sterile, lab-grade press and seal bags and kept frozen at -20˚C until analysis.

Blood and faecal samples were collected from adult Little Penguins at the St Kilda colony (Figure 5.1). Up to 2 mL of blood was aspirated from the medial metatarsal (caudal tibial) vein using a 25-gauge butterfly needle as described in Finger *et al.* (2015). For faecal collection, penguins were placed individually into clean plastic boxes for up to 30 minutes. Any faecal material deposited by the birds was transferred into 70 mL specimen containers using

clean wooden spatulas. The plastic boxes were cleaned after each penguin with hot water and paper towels. During each field sampling session, one faecal control sample was collected by emptying ~10 mL of Milli-Q ultrapure water (Merck Millipore) into a cleaned plastic box and collecting the control sample as described above. We labelled all faecal, blood and control samples and stored them in a cool box. All samples were transferred to a -20˚C freezer within 12 hrs of sampling.

5.2.2. Trace element analysis

The blood and faecal samples were prepared as described in Finger *et al.* (2015). Briefly, samples were measured into 50 mL digestion tubes (DigiTubes by SCP Science, product number 010-500-261), oven-dried at 60ºC and then digested in 65% nitric acid (SUPRAPUR, trace metal grade, Merck) and 37% hydrochloric acid (EMSURE, trace metal grade, Merck) at 95ºC. The fish samples were individually measured (standard length, ± 1 mm), weighed (± 0.5 mg) and then individually homogenised with 30 - 50 ml of Milli-Q ultrapure water (Merck Millipore) using a blender, freeze-dried and ground to powder. A subsample of ~3 g of this fish powder was transferred into individually labelled 14 mL falcon tubes, capped and sealed using Parafilm™. Fish samples were delivered to the National Measurement Institute (NMI) in Port Melbourne, Australia, where they were stored refrigerated until standard acid digestion and analysis (NMI method VL247, SRM recovery 77% - 117%).

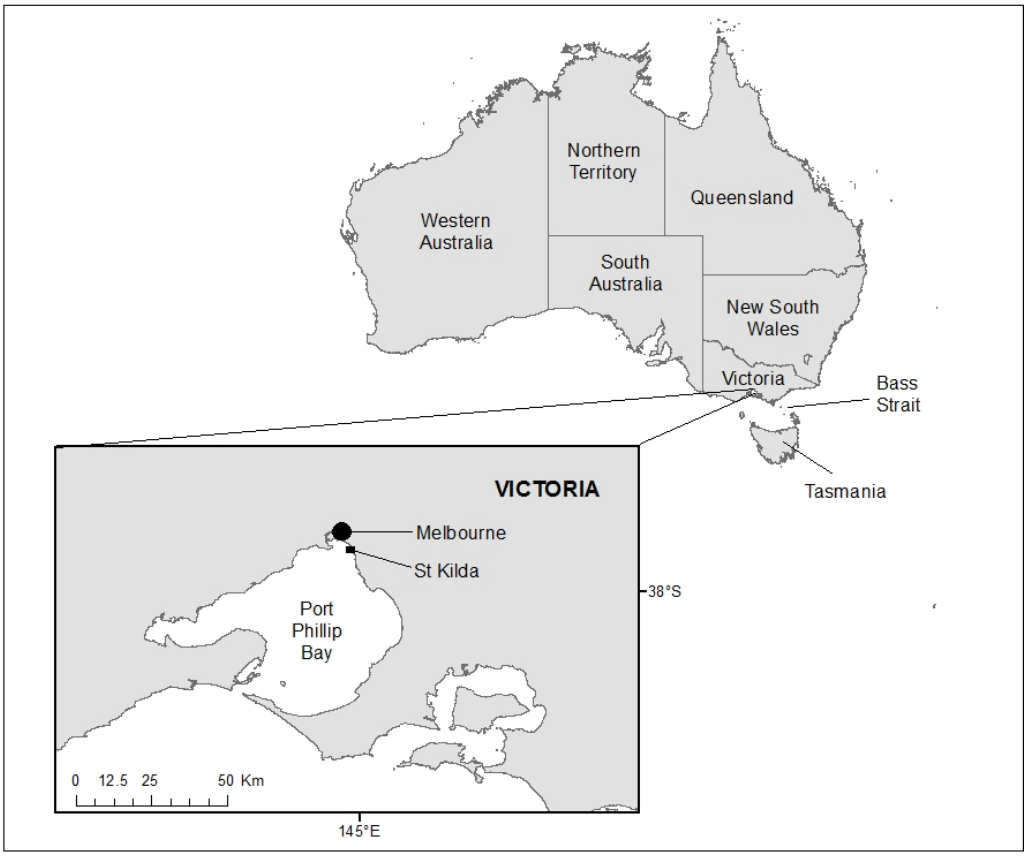

Figure 5.1: Little Penguins were sampled at St Kilda from 2011 to 2013.

The NMI carried out analyses on blood, faecal and fish samples for Arsenic (As), Cadmium (Cd), Mercury (Hg), Lead (Pb) and Selenium (Se) on an Agilent 7700x Inductively Coupled Plasma Mass Spectrometer (ICP-MS) with a limit of reporting of 0.01 mg/kg. All results were corrected for procedural blanks. All blood and faecal field blanks returned results under the limit of reporting. All results are reported as mg/kg dry weight (dw).

5.2.3. Stable Isotope data

To adjust blood Hg concentrations for dietary shifts, ∂^{15}N data analysed from blood samples taken from the same individual Little Penguins (n = 16) sampled during 2012 (for details on collection, processing and stable isotope

analysis, were used (see Kowalczyk *et al.*, 2014). Blood Hg concentrations were corrected for any dietary (trophic) influence (Braune, 2007):

$$Hg_{adj} = Hg_{measured} + A * (\partial^{15}N_{average} - \partial^{15}N_{measured}) \quad (1)$$

where A is the correlation coefficient for the blood Hg - blood $\partial^{15}N$ relationship at St Kilda, and $\partial^{15}N_{average}$ is the mean blood $\partial^{15}N$ value at St Kilda in 2012 (Table S1, Supplementary Material). This calculation was done to present the change in penguin blood metal(loid) values, independent of temporal changes in trophic status of penguin dietary items.

Table 5.1: Details of fish, Little Penguin blood and faeces samples collected in Port Philip Bay during 2011 to 2013.

Event	Penguin life stage	Fish species	# Fish samples	Date fish sampled	Date blood & faeces sampled	# Blood samples	# Faecal samples
#1	Moult	Sandy sprat *Hyperlophus vittatus*	10	15/02/2011	9/03/2011 - 30/03/2011	10	5
#2	Breed	Pilchard-1 (juvenile) *Sardinox sagax*	10	20/08/2012	10/09/2012	6	6
#3	Breed	Anchovy *Engraulis australis*	10	30/10/2012	7/11/2012	6	6
#4	Breed	1. Anchovy 2. Sandy sprat 3. Pilchard-2 (young adult)	10 10 10	31/12/2013	12/12/2013	6	4

5.2.4. Statistical analyses

Statistical analyses were executed using R version 3.2.3 (R Core Team 2015) and SPSS (version 20, SPSS Inc., Chicago, IL). Significance was taken to be $p < 0.05$ for all statistical analyses. Extreme statistical population outliers were identified in individual box plots as values further away than three times

the inter-quartile range from the median (Table S2, Supplementary Material, Logan, 2011). Normality of distribution for each element was tested using the Shapiro Wilk test, while Bartlett's test was used to investigate homogeneity of variances ($p < 0.01$ for both, Quinn and Keough, 2002). Non-metric multidimensional scaling (NMDS) was executed using the R package 'vegan' (Oksanen *et al.*, 2013) and 'ggplot2' (Wickham, 2009) to visually investigate dissimilarity among tissues for each event. Kendall (where $n > 30$) and Spearman's rank (where $n < 30$) correlations were executed between paired samples to establish relationships between blood and faecal metal(loid) concentrations (Table S3, Supplementary Material), and between blood Hg concentrations and blood $\partial^{15}N$ values.

5.3. Results

5.3.1. Metal(loid)s in fish prey of Little Penguins at Port Phillip Bay

Morphometric measurements and metal(loid) concentrations in whole fish prey samples are given in Table 5.2. Pilchards were caught in two distinct size classes (mean standard length 57.4 mm at event 2 and 108.6 mm at event 4, Table 5.2). These represent juvenile and young adult age classes, respectively (Neira *et al.*, 1999). While anchovy and sandy sprat were also caught on two separate occasions and differed in standard lengths between events (Table 5.2), these were pooled in the nMDS plots (Figure 5.2), as their metal(loid) concentrations within the same species between events were not distinctly different (Figure S1, Supplementary Material). Mean As concentrations were highest in young adult pilchards (19.8 mg/kg dw) and lowest in sandy sprat caught at event 4 (8.68 mg/kg dw). Mean Cd

concentrations were uniformly low (< 0.1 mg/kg dw) except for juvenile pilchards at event 2 (0.41 mg/kg dw). Mean Hg and Pb concentrations were highest in anchovy, followed by sandy sprat and young adult pilchard, and were lowest in juvenile pilchard (Table 5.2). Average Se concentrations in whole fish were two to three-fold higher in sandy sprat and pilchard than in anchovy (Table 5.2). These differences are reflected in the NMDS analysis (Figure 5.2), with the strongest dissimilarity evident between juvenile pilchards (highest in Cd) and anchovies (highest in Hg and Pb). Mean Hg concentrations were positively correlated with fish standard length in all three species, suggesting bioaccumulation as a primary driver of this trend (Table 5.3). Mean As and Se concentrations were also positively correlated with fish standard length in sandy sprat, while all metal(loid)s measured in pilchards showed positive correlations (Table 5.3).

5.3.2. Metal(loid)s in blood and faeces of Little Penguins at Port Phillip Bay

Table 5.4 shows metal(loid) concentrations in blood and faeces of Little Penguins at St Kilda during the four events. Cd concentrations were below the limit of reporting for all but one blood sample (0.015 mg/kg dw) and ranged from 0.21 mg/kg dw to 1.35 mg/kg dw in faeces. Concentrations of As and Pb were also higher in faeces than blood samples. For As, the ratio of faeces to blood was on average four to ten, and for Pb it was five to 26. Hg and Se concentrations were higher in blood than faeces, ranging in blood to faeces ratio from 7.6 to 20.6 for Hg, and from 1.7 to 5.2 for Se. This difference in metal(loid) distribution among the three matrices is highlighted by Figure 5.3,

which shows an overlap of fish and faecal samples near As and Pb 'peaks' on one side, while the blood samples are concentrated around the Hg 'peak' on the other, and Se is situated between these two groups. Correlations between paired data of blood and faeces metal(loid) concentrations of St Kilda penguins were significant only for Se (r_t = 0.43, t_{39} = 3.84, p < 0.001, Figure S2, Supplementary Material). We also found that heavier penguins had higher faecal Se concentrations (r_t = 0.25, t_{39} = 2.19, p < 0.05). No other faecal metal(loid) showed any significant correlation with penguin body mass. For correlations between blood metal(loid) concentrations and penguin body mass, see the analysis of a larger data set presented in Finger *et al.* (2016).

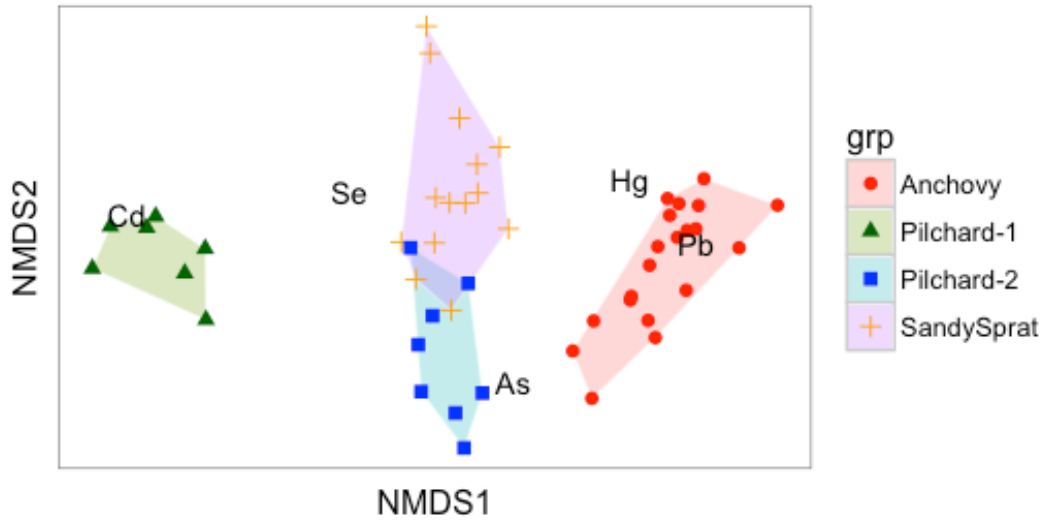

Figure 5.2: Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin fish prey items (stress = 0.09). Polygon ellipse lines are drawn for each fish prey species: Anchovy (red full circle), Pilchard-1 (juvenile, green full triangle), Pilchard-2 (young adult, blue full square), Sandy Sprat (orange cross). Trace elements are displayed by their periodic symbols: As, Cd, Hg, Pb and Se.

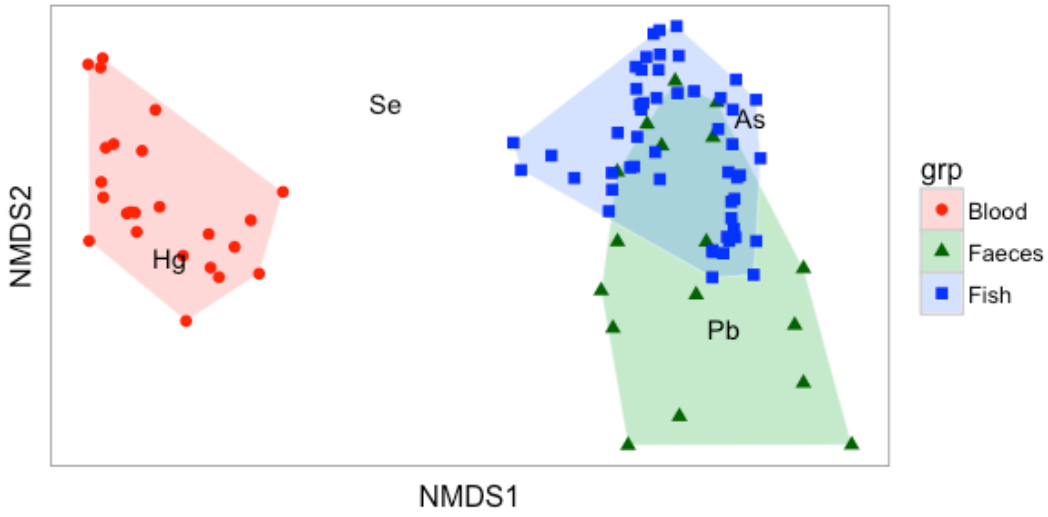

Figure 5.3: Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin blood, faeces and fish prey items (all fish species pooled) for the four events (stress = 0.04). Polygon ellipse lines are drawn for each matrix: Blood (red full circle), Faeces (green full triangle), Fish (blue full square). Trace elements are displayed by their periodic symbols: As, Hg, Pb and Se. Cadmium was excluded since it was under the limit of reporting for all but one blood sample.

5.3.3. Diet-Adjusted Metal(loid) Blood Concentrations

To account for dietary shifts on Little Penguin blood Hg concentrations, the relationships between Hg concentrations and blood nitrogen isotope ratios ($\partial^{15}N$) values for 16 individual penguins, jointly sampled in 2012 (Table S1, Supplementary Material) were assessed. Within this limited dataset, Hg concentrations positively correlated with blood $\partial^{15}N$ values (Figure 5.4, R^2 = 0.25, r = 0.50, $p < 0.05$). Using equation (1), diet-adjusted Hg concentrations were calculated and are presented with their unadjusted values in Figure 5.5. After adjusting for trophic shifts, median Hg concentrations were lower in January 2012 and unchanged in September and November 2012, however, variation in November 2012 increased (Figure 5.5 right panel).

Table 5.2: Morphometric data and metal(loid) concentrations in whole fish [mg/kg dry weight] collected from Port Philip Bay during 2011 to 2013, given as mean ± SD with ranges in square brackets. Sample numbers are 10 for each species / event, unless where indicated differently in round brackets (due to extreme outlier removal, see materials and methods).

Event	Fish species	Mean Standard Length [mm]	Mean Drying Factor	Arsenic	Cadmium	Mercury	Lead	Selenium
#1	Sandy sprat *Hyperlophus vittatus*	62.5	4.05	11.88 ± 2.67 [8 - 16]	0.09 ± 0.02 [0.05 - 0.12]	0.13 ± 0.01 (7) [0.13 - 0.14]	0.09 ± 0.03 (7) [0.06 - 0.16]	4.97 ± 1.24 [3.0 - 6.9]
#2	Pilchard-1 (juvenile) *Sardinox sagax*	57.4	3.99	11.31 ± 3.03 [6.4 - 15.0]	0.37 ± 0.09 (9) [0.26 - 0.56]	0.04 ± 0.01 [0.03 - 0.05]	0.05 ± 0.01 (8) [0.04 - 0.06]	4.94 ± 0.78 [3.5 - 5.9]
#3	Anchovy *Engraulis australis*	69.2	3.47	13.5 ± 2.07 [11 - 17]	0.07 ± 0.01 [0.06 - 0.09]	0.22 ± 0.04 [0.17 - 0.30]	0.26 ± 0.04 [0.19 - 0.34]	2.37 ± 0.14 [2.1 - 2.5]
#4.1	Anchovy *Engraulis australis*	71.7	3.99	16.4 ± 2.95 [13 - 21]	0.05 ± 0.02 (9) [0.04 - 0.09]	0.22 ± 0.06 [0.16 - 0.38]	0.21 ± 0.11 [0.08 - 0.43]	2.24 ± 0.24 [1.9 - 2.7]
#4.2	Sandy sprat Hyperlophus vittatus	75.9	4.34	8.68 ± 1.84 [6.2 - 12]	0.09 ± 0.01 (8) [0.07 - 0.10]	0.21 ± 0.04 [0.15 - 0.25]	0.11 ± 0.03 (9) [0.07 - 0.14]	6.64 - 1.89 [4.9 - 11]
#4.3	Pilchard-2 (young adult) Sardinox sagax	108.6	3.18	19.8 ± 2.82 [17 - 25]	0.07 ± 0.02 [0.05 - 0.13]	0.13 ± 0.06 [0.06 - 0.22]	0.13 ± 0.05 (8) [0.07 - 0.22]	6.29 ± 1.16 [4.5 - 8]

Table 5.3: Relationships between As, Cd, Hg, Pb and Se concentrations in three Little Penguin fish prey species and their standard lengths (n = 20 for each species).

Species	Arsenic		Cadmium		Mercury		Lead		Selenium	
	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*
Anchovy	0.08	0.22	0.04	0.39	**0.27**	**0.02**	0.02	0.58	0.00	0.91
Sandy Sprat	**0.23**	**0.03**	0.02	0.59	**0.60**	**<0.001**	0.12	0.20	**0.30**	**0.01**
Pilchard	**0.76**	**<0.001**	**0.79**	**<0.001**	**0.49**	**<0.001**	**0.53**	**<0.01**	**0.36**	**<0.01**

Table 5.4: Mean metal(loid) concentrations in blood and faeces of Little Penguins at St Kilda at times coinciding with fish sample collections (events), given as mean ± SD [mg/kg dry weight]. Sample numbers are given in round brackets and ranges in square brackets. "<LR" represents a value that was determined to be under the limit of reporting.

Event	Penguin life stage	Matrix	Arsenic	Cadmium	Mercury	Lead	Selenium
#1	Moult 2011	Blood	2.02 ± 0.52 (10) [1.2 - 2.7]	0.015 (1)	1.75 ± 0.44 (9) [1.10 - 2.45]	0.07 ± 0.01 (8) [0.06 - 0.08]	6.21 ± 1.24 (10) [4.20 - 7.79]
		Faeces	21.20 ± 7.76 (5) [10.35 - 31.00]	0.39 ± 0.08 (4) [0.29 - 0.45]	0.23 ± 0.15 (5) [0.11 - 0.48]	1.85 ± 1.46 (5) [0.50 - 4.20]	3.55 ± 1.91 (5) [2.03 - 6.45]
#2	Breed 2012	Blood	2.92 ± 1.73 (6) [1.40 - 5.20]	< LR	3.34 ± 0.42 (6) [2.93 - 4.15]	0.17 ± 0.06 (6) [0.05 - 0.20]	26.25 ± 9.65 (6) [13.50 - 41.50]
		Faeces	16.39 ± 8.96 (6) [8.96 - 33.04]	0.73 ± 0.44 (6) [0.24 - 1.35]	0.30 ± 0.13 (6) [0.18 - 0.53]	0.87 ± 0.31 (5) [0.46 - 1.12]	5.10 ± 0.84 (6) [4.00 - 6.10]
#3	Breed 2012	Blood	3.02 ± 0.84 (6) [2.20 - 4.50]	< LR	2.88 ± 0.22 (6) [2.65 - 3.20]	0.05 ± 0.01 (5) [0.03 - 0.06]	44.97 ± 11.57 (6) [28 - 61]
		Faeces	27.00 ± 4.18 (5) [22 - 33]	0.26 ± 0.05 (5) [0.21 - 0.33]	0.14 ± 0.02 (5) [0.11 - 0.16]	0.27 ± 0.05 (5) [0.22 - 0.34]	10.96 ± 3.10 (5) [6.67 - 14.00]
#4	Breed 2013	Blood	3.34 ± 0.73 (6) [2.50 - 4.23]	< LR	4.20 ± 1.14 (6) [2.90 - 5.55]	0.05 ± 0.01 (6) [0.04 - 0.07]	21.39 ± 4.41 (6) [16.33 - 28.00]
		Faeces	14.43 ± 1.91 (3) [12.30 - 16.00]	0.41 ± 0.17 (4) [0.25 - 0.60]	0.31 ± 0.14 (3) [0.22 - 0.46]	0.68 ± 0.58 (3) [0.19 - 1.50]	11.80 ± 3.20 (3) [9.90 - 15.50]

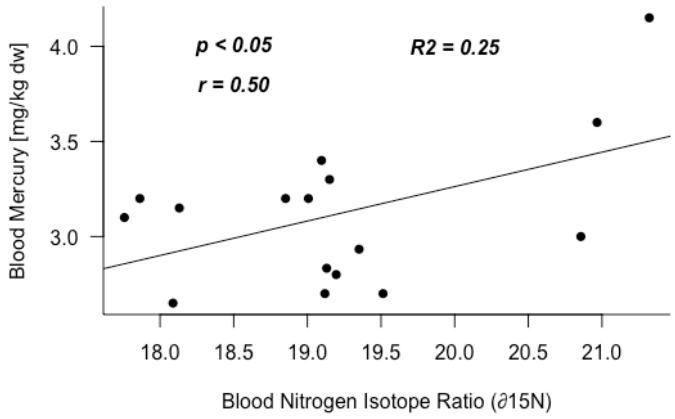

Figure 5.4: Relationship between Little Penguin blood mercury concentrations and blood $\partial^{15}N$ values (paired samples, n = 16), collected in January, September and November of 2012 at St Kilda.

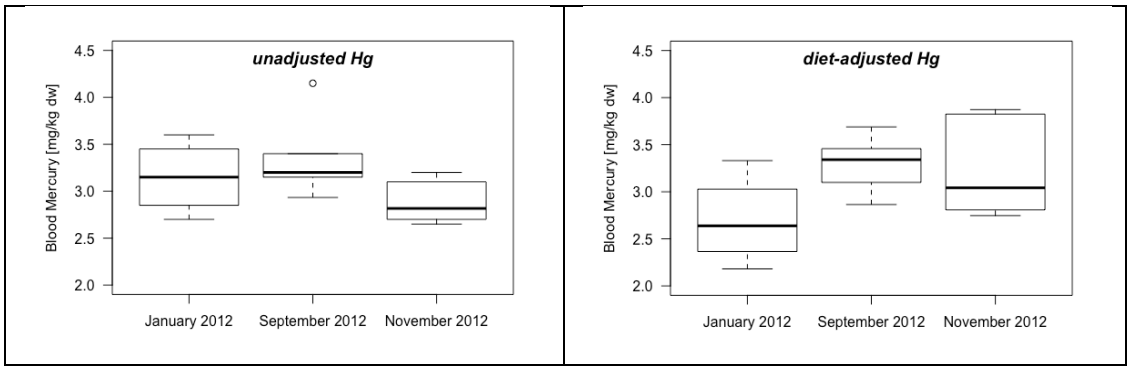

Figure 5.5: Little Penguin blood mercury concentrations; unadjusted (left) and adjusted (right) for diet. Samples were collected at St Kilda in January 2012 (n = 4), September 2012 (n = 6) and November 2012 (n = 6).

5.4. Discussion

High levels of heavy metals have been reported in the sediment, water and biota of Port Phillip Bay since the early 1980's (Harris *et al.*, 1996), but this is the first report of metal(loid) concentrations in anchovy, pilchard and sandy sprat within Port Phillip Bay. Walker (1988) measured Hg in muscle tissue of anchovy and pilchards in Victorian waters < 75 m outside Port Phillip Bay. Mean Hg concentrations in whole anchovy reported here were

approximately double the mean Hg concentrations reported in muscle tissue outside the bay, while whole pilchard Hg concentrations mirrored those reported in pilchard muscle tissue by Walker (1988). Dunlop *et al.* (2013) measured metals in whole fish fed to a captive penguin group and then collected and analysed metals in moulted feathers of both captive and free-ranging Little Penguins in Western Australia. Metal concentrations for whole fish were not differentiated by species in the report, but N. Dunlop kindly provided us with these data (unpublished data, Table S3, Supplementary Material). Cadmium concentrations were relatively high in pilchard from King George Sound, Western Australia (0.60 mg/kg dw), but were otherwise comparable with our results. Mean Se concentrations were generally lower there than those reported in this study, while Pb was in the same order of magnitude in both studies. Hg concentrations in fish from Tweed Head, New South Wales and Oyster Harbour, Western Australia, Australia, were uniformly low (0.04 - 0.09 mg/kg dw). In contrast, anchovy Hg concentrations in our study were up to five times higher (0.22 mg/kg dw), confirming the presence of high concentrations of bioavailable Hg in the Bay.

The only other published fish metal data available for comparison are those measured in other, related anchovy and pilchard species, favoured for human consumption in the Mediterranean and neighbouring seas. Cd, Hg and Pb presented for whole fish in this study were at the same or lower levels as those reported elsewhere (Keskin *et al.*, 2007; Alkan *et al.*, 2016; Bosch *et al.*, 2016), while As concentrations were higher than those reported by Alkan *et al.* (2016) for the Black Sea. The relatively high concentrations of As measured in the three clupeoid species presented in this study are likely due

to As being naturally abundant in Port Phillip Bay (Harris *et al.*, 1996). Seafood often contains high concentrations of As, but primarily in the non-toxic organic form (Fabris *et al.*, 2006; Borak and Hosgood, 2007). Gagnon *et al.* (2016) measured total and inorganic As in white muscle of sand flathead (*Platycephalus bassensis*) from Port Phillip Bay in 2015, and inorganic As was below detection limit for all samples. Like the Little Penguin, sand flathead also prey on anchovy, pilchard and sandy sprat (Officer and Parry, 1996). It is therefore important to look at how their metal(loid) concentrations compare. Hg concentrations were lower in whole fish samples reported in the three species analysed this study (mean 0.04 - 0.22 mg/kg dw), compared to those reported for white muscle of sand flathead (0.41 - 1.19 mg/kg dw, Gagnon *et al.*, 2016), which is in concurrence with Hg's ability to biomagnify with increasing trophic level (Eisler, 2006). Mean As concentrations in whole fish sampled during this study were similar to those in the white muscle of Port Phillip Bay sand flathead (Gagnon *et al.*, 2016), while both Cd and Pb mean concentrations were higher in the whole fish samples reported here. This may be explained by the fact that we report on metal(loid) concentrations in whole fish. In contrast, Gagnon *et al.* (2016) investigated metal(loid) concentrations in muscle tissue, which usually contains lower concentrations of these elements than liver and kidney (Neff, 2002).

The trophic transfer of heavy metals in polluted ecosystems can result in harmful concentrations in tissues of fish (Dallinger *et al.*, 1987). The fish species investigated here are commercially caught for bait and also human consumption. It is therefore important to note that Hg concentrations were below the reference health standard in all samples (0.5 mg/kg fresh weight,

FSANZ, 2004). There are currently no reference health standards defined for As or Pb (FSANZ, 2011), however, as stated earlier, As in fish is primarily in the non-toxic organic form (Borak and Hosgood, 2007), and all samples were below the Pb safe limit set by the European Commission (0.3 mg/kg wet weight, EU, 2006). Food Standards Australia New Zealand (FSANZ) have set a provisional tolerable monthly intake standard of 25 µg / kg body weight for Cd and an upper level daily intake of 60 - 400 µg for Se (FSANZ, 2011). Assuming 150 g of fish to be consumed by a 70 kg person in one meal per day, all fish samples analysed were below food safety standards (maximum) for Cd. The young adult pilchard cohort had the highest Se wet weight concentration (1.98 mg/kg), which if consumed (as a 150 g meal) would make up 74% (297 µg) of the upper level daily intake specified for Se.

Hg was the only element that showed significant positive correlations with fish standard length in all three fish species (Table 5.3). This confirms that Hg concentrations in fish are highly dependent on growth rate variations (Jones *et al.*, 2013). The two cohorts of pilchards caught in this study might have been the same 'generation'; some caught as juveniles and some caught over a year later, as young adults (Neira *et al.*, 1999). Thus, the data presented here can give information on metal(loid) bioaccumulation in this species. The juvenile pilchards, which likely had recently entered Port Phillip Bay (Neira *et al.*, 1999), had a distinctly different combination of metal(loid)s from the young adults (Figure 5.2), potentially reflecting differences in bioavailable metal(loid)s between Bass Strait (higher Cd) and Port Phillip Bay (higher As, Hg and Pb). Anchovy had the highest Hg concentrations, owing to

the fact that they are feeding at a higher trophic level than pilchards and sandy sprat (Kowalczyk *et al.*, 2013).

The three fish species investigated here have been confirmed as Little Penguin prey at St Kilda in previous stomach-flush and stable isotope studies (Preston, 2010; Kowalczyk *et al.*, 2013; Kowalczyk *et al.*, 2015b). Despite not being able to establish exactly what prey the sampled penguins had consumed, valuable information can be drawn from the multidimensional metal(loid) analysis of potential fish prey, blood and faeces (Figure 5.3). This plot presents how fish prey items relate to the penguin blood and faeces in terms of their contaminant concentrations. This is especially valuable as field studies directly linking seabird contaminant loads with prey items are rare (Monteiro *et al.*, 1998; Arcos *et al.*, 2002; Carvalho *et al.*, 2013), and, to our knowledge, no reports exist of paired blood and faecal metal data in seabirds. Metal(loid) concentrations in faeces were closer to those measured in fish prey items than in the blood of Little Penguins (Figure 5.3), indicating a well-functioning detoxification mechanism through faeces for all measured metal(loid)s, except for Hg. Total Hg in blood far exceeded that in faeces by an average factor of 13.2 : 1 (Table 5.2). Selenium has not been shown to biomagnify in most food webs (Outridge *et al.*, 1999).

Both blood and faecal collections are non-destructive, but passive faecal sampling requires considerably less handling and skill, has less impact on the animal and is thus less invasive. Depuration of non-essential elements through faeces is a main way of detoxification. For instance, Lewis and Furness (1991) administered Hg to laboratory-reared Black-headed Gull (*Larus ridibundus*) chicks and reported that between 11% and 38% of it was

excreted through faeces. The authors found no relationship of excretion rates with the administered dose, which was within the range of potential environmental exposure. Faecal Cd concentrations reported here were well below those reported for penguins in Antarctic regions (Szefer *et al.*, 1993; Ancora *et al.*, 2002), the latter likely due to elevated Cd concentrations in squid, an important food component of Antarctic penguins (Honda and Tatsukawa, 1983). Faecal Hg concentrations in Little Penguins were comparable to those reported in South Polar Skua (*Catharacta maccormicki*), Adélie Penguin (*Pygoscelis adeliae*) and Emperor Penguin (*Aptenodytes forsteri*) (Bargagli *et al.*, 1998), while Pb concentrations compared with those found in Gentoo Penguins (*Pygoscelis Papua*) from several Antarctic locations (Celis *et al.*, 2015).

Seabird faeces has been proposed as a suitable metal bioindicator matrix (Yin *et al.*, 2008), but collection methods range from being "taken off the top of a mass of faeces deposited on the ground" (Celis *et al.*, 2015), "taken from unattended nests" (Bargagli *et al.*, 1998), to being collected freshly, meaning "that it's produced by seabirds or animals in the past couple of days" and pooled by species (Yin *et al.*, 2008), or not specified (Ancora *et al.*, 2002). It is questionable how the age, or in fact source, of a faecal deposition can be determined with any certainty, using such methods. The collection method reported herein, albeit time-consuming, ensured the establishment of paired samples and the investigation of matrix correlations. Unfortunately, only Se showed a correlation between these two matrices, and it is thus not recommended to use faecal concentrations for approximations of Little Penguin body burden for As, Cd, Hg or Pb. Perhaps, penguin and other

seabird faeces, if deposited at large quantities, are better utilised as long-term pollution indicators (Sun and Xie, 2001; Blais *et al.*, 2005; Xie and Sun, 2008)?

Previously, concern has been raised over an increasing trend in blood Hg concentrations over three years (2011 to 2013) in St Kilda penguins (Finger *et al.*, 2016). It is important to investigate whether this trend was due to an increase of bioavailable Hg within Port Phillip Bay or due to penguins changing their diet from lower to higher trophic prey. This can be done by incorporating ∂^{15}N values (Braune, 2007; Burgess *et al.*, 2013) Unfortunately, due to the scarcity of paired samples of Hg and stable isotope data, we were able to calculate diet-adjusted Hg concentrations for only 16 penguins, sampled in January, September and November 2012. However, this limited investigation suggests that some of the variation in blood Hg concentrations in Little Penguins is unrelated to changes in trophic position (∂^{15}N) of their prey items, indicating other sources of bioavailable Hg within this semi-enclosed bay. Future studies should include simultaneous stable isotope ratios and metal determinations in species selected as bioindicators for conclusive evidence on trophic transfer of metal(loid)s, especially Hg.

5.5. Conclusions

The three fish prey species investigated in this study contained moderate and comparable concentrations of metal(loid)s, with two exceptions: (1) As, which is naturally present at high concentrations within Port Phillip Bay, and predominantly accumulates in the non-toxic organic form in fish; and (2) Hg, which was highest in anchovy (0.22 mg/kg dw). While this

concentration is still below safe limits set for human consumption (FSANZ, 2004), it may be of long-term concern to St Kilda's Little Penguins, which predominantly feed on that species (Preston, 2010). Despite assurances that input of Hg into Port Phillip Bay has decreased or remained constant over the recent past (Harris *et al.*, 1996) and that large-scale dredging has not caused an increase of metals through re-suspension of contaminated sediments (PoMC, 2010), this and previous studies (Finger *et al.*, 2015; Finger *et al.*, 2016) demonstrate that Hg is bioavailable in Port Phillip Bay, and accumulates in fish and the Little Penguin. These recent studies highlight a critical need for surveillance of the bioavailability of this toxic metal within the Bay. A long-term program, measuring Hg concentrations and $\partial^{15}N$ values in blood and / or feathers of Little Penguins at this urban colony of St Kilda, Melbourne, Australia, would assist in the conservation of this iconic species, as well as help inform future environmental assessments.

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Chapter 6

General discussion

Little Penguin at St Kilda (Photo source: @DELWPPortPhillip)

6. General discussion

6.1. **Summary of findings**

This study investigated the suitability of the Little Penguin as a sentinel of coastal pollution. I was able to show that sufficient quantities of sampling materials can be collected using moderately invasive and non-destructive methods. The data presented in this thesis advance previously scarce ecotoxicological data on this species, and confirm its utility as a bioindicator of metal and metalloid pollution within its foraging zone. With an exponentially growing human population and advancing climate change, there is an urgent global need to monitor changes in the condition of coastal habitats. The information contained in this thesis provides an empirical base for our understanding of this resident, urban, high trophic feeder and can be used to inform future monitoring programs. Below is a summary of this information, presented as answers to the research questions posed in Chapter 1.

QUESTION: Can the Little Penguin nesting at St Kilda be utilised as an effective bioindicator for metal pollution in Port Phillip Bay?

ANSWER: Yes, the Little Penguin is a suitable model species to be utilised as an effective bioindicator of metal and metalloid pollution in Port Phillip Bay. It is possible to non-destructively collect sufficient quantities of materials at biologically relevant intervals from St Kilda, as well as from suitable reference colonies, with relatively low impact on the animal and at comparatively low cost. Other seabird species that may be considered as bioindicators, such as the Short-tailed Shearwater (*Puffinus tenuirostris*) and the Crested Tern (*Thalasseus bergii*), are migratory, feed within and outside the bay, and few

data exist on seasonal variations in distribution or detailed movement patterns (Norman, 1992; Dann *et al.*, 2003). The Australasian Gannet (*Morus serrator*) nest within Port Phillip Bay and has been extensively researched (Norman *&* Menkhorst, 1988; Bunce *&* Norman, 2000; Bunce, 2001), but with an average foraging range of approximately 94 km during the breeding period (Bunce, 2001), is feeding at least partially outside of the bay. Furthermore, the level of data on Little Penguin toxicology presented in this thesis does not exist for any other coastal high trophic feeder in Port Phillip Bay and perhaps even worldwide - I would therefore encourage this work to be continued with the Little Penguin rather than a new model species being selected for the same purpose.

QUESTION: Do Little Penguins at St Kilda carry significantly higher concentrations of metals than Little Penguins feeding at more pristine feeding grounds?

ANSWER: The results of this study provide strong evidence of a clear link between non-essential trace element concentrations in Little Penguins and the level of industrialisation of their foraging zones. In particular, Little Penguin blood concentrations of arsenic, mercury and lead were significantly higher in the metropolitan St Kilda population compared to more remote Phillip Island and Notch Island.

QUESTION: Which sampling matrices (blood, feathers or faeces) are preferential for which metals?

ANSWER: Considering the impact on the study animal, as well as the effort and success rate of collecting the sample and the associated bias due to sample contamination, I recommend the preferential use of blood for all metals and metalloids. Little Penguin feathers can be used as a suitable matrix for determining mercury, lead and iron; however, standard deviations were larger than in blood, most likely due to external contamination (discussed further below). Little Penguin faecal samples can be used as an approximation only for selenium.

QUESTION: What are the inter-annual and inter-seasonal variations in metal concentrations in Little Penguins at St Kilda and Phillip Island?

ANSWER: Overall, inter-annual and inter-seasonal variation in metal and metalloid concentrations in Little Penguins differed between St Kilda and Phillip Island. However, blood mercury concentrations followed a distinct seasonal pattern of non-breeding < moult < breeding at both locations, suggesting some physiological mechanism, possibly linked to the increased accumulation during intense periods of feeding (while providing for offspring during the breeding season), as well as the mobilisation of mercury from internal organs during fasting and the depuration of mercury into growing feathers during moult. Notably, I found an increasing trend of blood mercury concentrations in Little Penguins nesting at St Kilda, contrary to a decreasing trend at Phillip Island during part of the same period.

QUESTION: Which of the metals measured in this study are of most concern to the Little Penguin within Port Phillip Bay?

ANSWER: Of the metals and metalloids measured in this study, mercury is of most concern to the welfare of the Little Penguin at St Kilda, as it came closest to levels of concern established in previous studies. Mercury contamination thresholds that lead to adverse effects are poorly understood in seabirds, especially for feather and blood matrices, and comparisons between studies are often hampered by differences in methodology. The maximum whole blood mercury concentration measured in this study (5.57 mg/kg dry weight, equals 0.88 mg/kg wet weight) is below the minimum concentration found to cause detrimental impacts on reproduction in the Common Loon (*Gavia immer*) (3 mg/kg wet weight, Evers *et al.*, 2008). However, it is higher than mean concentrations found in red blood cells of Black-legged Kittiwakes (*Rissa tridactyla*) that skipped breeding (2.07 mg/kg, Tartu *et al.*, 2013). Unfortunately, no conversion factors exist between whole blood and red blood cell mercury concentrations, impeding a direct comparison.

QUESTION: How do metal concentrations in the Little Penguin's main fish prey species relate to Little Penguin blood and faecal concentrations?

ANSWER: Fish prey species and penguin faecal samples had quantitively similar concentrations of arsenic and lead, suggesting faeces as a primary route of detoxification for these elements. Mercury was highest in the blood of Little Penguins, confirming its propensity to biomagnify in trophic chains. Mercury concentrations in Australian anchovy (*Engraulis australis*) were up to five times higher than measured elsewhere in Australia. While those concentrations were still below safe limits set for human consumption, they

may be of concern for St Kilda Little Penguins, which predominantly feed on this species.

QUESTION: What sampling protocol would be suitable to survey long-term metal pollution within Port Phillip Bay?

ANSWER: The sampling protocol introduced in this study is recommended for long-term monitoring of contamination within Port Phillip Bay. In short, between ten and fifteen blood samples should be collected at St Kilda and Phillip Island at three distinct sampling seasons (breeding, moult and non-breeding). Additionally, feather samples from ten individuals should be collected at moult from both locations. The sampling effort should include the taking of morphological measurements, in particular body weight and flipper length. Each sample should be analysed for (inorganic) arsenic, mercury, lead and selenium. Stable isotope ratios should be determined in all samples to investigate and adjust for diet-related mercury variations.

6.2. **Conservation and management**

Little Penguins have a large amount of public appeal, and receive strong support for their health and conservation. This study contributes to scientific knowledge by filling gaps that exist due to the irregularity of surveys. The Little Penguin has the potential to be used as an early warning indicators of environmental change within Port Phillip Bay. The data and analyses presented in this study can inform the management of not only the Little Penguin, but other piscivores that forage in the bay at various times of the year (e.g. Australasian Gannets and Burrunan dolphins *Tursiops autralis*)

(Bunce & Norman, 2000; Charlton-Robb *et al.*, 2011). For the sake of the penguins and the bay, it appears essential to develop a long-term program, aiming at monitoring changes of contamination within Port Phillip Bay. As a priority, mercury concentrations should be measured annually in moulted Little Penguin feathers at both St Kilda and Phillip Island. While this will not yield any information on inter-seasonal changes, it will provide a cost-effective and minimally invasive, non-destructive method to monitor inter-annual variability of this pervasive toxic pollutant. Last moult, I collaborated with an honour's student (F. Sperring, Monash University) to collect 10 samples of moulted feathers from St Kilda. I propose to St Kilda Earthcare and Phillip Islands Nature Parks to add moulted feather collection to their animal ethics permit, so that a time-line of penguin feather samples can be established.

6.3. Future research

Several questions have emerged during this work, which need to be addressed in the future. Firstly, what is the long-term trend for mercury in Port Phillip Bay? A longer time series is needed to confirm or contradict the inter-annual mercury pattern found in this study. This is a particularly important consideration for potential future dredge works. Secondly, what if any effects does mercury have on the Little Penguin at a population level? Chronic mercury toxicity in birds has been associated with reproductive effects, such as lower egg weight, decreased hatchability, impaired egg laying and territorial fidelity (see review in Scheuhammer, 1987; Monteiro & Furness, 1995). More recently, the research presented by Tartu *et al.* (2013; 2015) might inspire studies looking into differences in penguin nesting behaviour

and parental investment linked to blood mercury concentrations. Thirdly, what population markers can be used to compare penguins nesting at St Kilda and at Philip Island? As discussed in Chapter 4, due to the different burrow types and the resulting differences in penguin accessibility at St Kilda and Phillip Island, we currently cannot compare common population markers, such as fledging success between these two colonies. I suggest the monitoring of other markers, which can be assessed at both colonies equally, for instance morphological measurements or recapture rates. Fourthly, are there any other markers, on an individual level, that can be used to measure the effect of high mercury concentrations in the Little Penguin? Future studies should give strong consideration to the role of mercury as an endocrine disruptor (Perez-Cadahia *et al.*, 2008; Tan *et al.*, 2009; Tartu *et al.*, 2014). And finally, can we improve the cleaning process of penguin feathers to decrease external contamination, and thus establish a more robust correlation with blood metal and metalloid concentrations? One of the most common methods to clean feathers prior to acid digestion is to wash them repeatedly in deionised water and acetone (Dauwe *et al.*, 2003; Jaspers *et al.*, 2004). I did not use acetone in my feather cleaning method, to be consistent with prior methods applied in Little Penguin feather metal analysis. Mercury concentrations in feathers, which are a main focus of this thesis, are not or only slightly affected by exogenous contamination (Dauwe *et al.*, 2003), but it would be worthwhile to investigate the effectiveness of a range of cleaning methods to highlight the potentially significant issue of external contamination.

The lack of uniform procedures in ecotoxicology impedes the comparison of results, which is paramount for widely drawn conclusions.

Throughout this work, I have strived to implement the most acceptable, most widely used methods for sample collection, preparation and analysis. This was done, both to be able to compare my results to previous studies, but also with a look ahead – to provide a relatively simple, yet valid and effective framework for future monitoring and surveillance works. Such studies, if undertaken consistently over several years, have the potential to fill further knowledge gaps in high-trophic feeder toxicology, such as pollutant bioaccumulation, biomagnification, detoxification and potential impacts of major dredge works in contamination hot spots. These are very important aspects of ecotoxicology because they have the potential to answer direct questions as to the impact of anthropogenic activities and the effect these have on an urban dweller such as the Little Penguin. Findings from such studies should then be used to inform environmental impact studies, as well as conservation management initiatives. Furthermore, this study could easily be adapted to be used to monitor organic pollution within Port Phillip Bay. With growing concerns about the impacts of plastics in our oceans (Jambeck *et al.*, 2015), only available funds limit this extension.

6.4. Concluding remarks

The most notable results of this study include Little Penguins carrying significantly higher concentrations of arsenic, mercury and lead at St Kilda than penguins from more remote and less urban colonies, with mercury exhibiting an increasing trend over the three years measured. Phillip Island is a suitable reference study site and showed a declining trend of mercury during part of this time, despite a foraging area overlap with St Kilda

penguins. Mercury was highest in the penguins' favourite fish prey item within Port Phillip Bay, the Australian anchovy, and was found to accumulate in the blood of the Little Penguin, while arsenic and lead were readily depurated through faeces.

The findings presented in this thesis significantly advance the knowledge base of seabird ecotoxicology. To my knowledge, this study provides the first data on metal and metalloid concentrations in the blood of any penguin species, as well as the first investigation of inter-seasonal metal variation. More novel reports include metals and metalloid concentrations in the Little Penguin, first blood / feather and blood / faeces metal concentration correlations in penguins, as well as the first presentation of within-moult metal and metalloid concentrations of any seabird. Due to the size and comprehensive nature of the data set presented in this dissertation, I believe the Little Penguin to now be the most ecotoxicologically researched resident coastal high trophic feeder in the southern hemisphere, or perhaps in the world. This is important information for the management of Port Phillip Bay, a semi-enclosed bay facing considerable pressures, such as dredging activities.

The environmental impact assessments accompanying the last major dredging activity executed within Port Phillip Bay in my opinion did not appropriately ascertain whether highly contaminated particles in the dredged soils had in fact entered the marine food web and affected an increase of toxicants in high-trophic feeders. Metal concentrations determined in resident local fish (Black bream *Acanthopagrus butcheri*) before dredge activities commenced were compared with data collected from the same species midway through the dredge activities. To date, it is unknown how long it would

take for toxicants, introduced at the bottom of the marine food web as sediment particles, to reach the trophic level of fish the size of Black bream (>26 cm), but it is safe to assume it would be in the order of years rather than months. While I have not been able to conclusively connect the increasing trend in Hg with the timing of the dredging, due to the limited timeline and the lack of pre-dredge penguin blood metal concentration data, I raise concern and provide a solid database for future assessments. The data contained in this thesis present an ideal platform from which to gauge future impacts of industrial developments on, and perhaps even elucidate complex issues such as contaminant mobilisation and trophic uptake. I hope this will also ensure the adaptive management and long-term conservation of this feisty urban dweller.

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Appendix 1

Standard Operating Protocol - sample digestion

PRE-OPERATIONAL SAFETY CHECKS
Equipment:

1. Ensure that the Dry Block Heater, ‘Memmert Oven – Selby’, 'Vortex Mixer' and Analytical Balance all have been electrically tested and tags attached.
2. Read and understand the ‘Dry Block Heater’, ‘Memmert Oven – Selby’, Vortex Mixer’ and ‘Biohazardous Waste’ SOPs.

OPERATIONAL SAFETY CHECKS
Dry Block Heater:

1. Ensure that the power switch (situation on rear panel) is on ‘off’ position before plugging in the cord into the power point.

PRINCIPLE
Sample is oven-dried at 60ºC, then digested with 65% Nitric Acid (HNO_3) and 37% Hydrochloric Acid (HCl) in a heating block at 95ºC.

REAGENTS AND STANDARDS

1. SUPRAPUR Nitric acid HNO_3, 65%, trace metal grade, by Merck
2. EMSURE Hydrochloric acid HCl, 37%, trace metal grade, by Merck
3. Deionised water from Milli-RO / Milli-Q system (Millipore)
4. NMI In-house reference material AGAL3 (prawn tissue)
5. NMI In-house reference material AGAL4 (Bovine liver tissue)

APPARATUS

1. Memmert Oven - Selby
2. Vortex Mixer "Vormix"
3. Analytical Balance
4. Dry block heater Model DBH30D by Ratec

SAMPLES
Biological samples of little penguin blood are stored frozen at -20ºC and are thawed at room temperature under the fume hood prior to executing this SOP.

GLASSWARE AND LABWARE

1. BD 6 mL trace element vacutainers (Royal blue stoppers)
2. Disposable transfer pipettes
3. Eppendorf Pipettes – 1000 and 5000 µL
4. DigiTubes 50 mL (PP) made by SCP Science plus lids
5. Dispensing bottle (for deionised water)
6. Stainless steel spatulas
7. 10 mL Terumo syringes with luer lock
8. 14 mL Falcon tubes with screw tops
9. Syringe filter (28 mm diam., 0.45 µm pore size, non-sterile)
10. Neoprene gloves
11. Heat-proof gloves
12. Tightly fitting safety goggles
13. Face shield

STANDARD OPERATING PROTOCOL

1. Allow frozen samples to thaw at room temperature in vacutainers on tray under fume hood.
2. Label DigiTubes "AF#-1" to “AF#-3” where "#" is the unique sample number based on data sheet and label on vacutainer. These are for triplicate samples. Sometimes, only duplicates will be processed, depending on the amount of blood contained in the individual vacutainer.
3. Place each DigiTube with lid into an analytical balance and record the exact weight of the empty container.
4. Remove the lid and tare analytical balance to zero.
5. Mix blood well with the help of a disposable pipette and / or place on vortex mixer for a few seconds.
6. Transfer ~0.5 g of blood into the DigiTube using the same disposable pipette and record exact wet weight of sample.
7. ***Repeat steps 2 to 6 for another 1 or 2 replicates, depending on available sample volume.***
8. Discard disposable pipette and if all sample material used, empty vacutainer into a biohazardous waste container after dispensing. Recap vacutainer and put back into freezer if any sample remains.

9. Loosely cap (thread engaged but not fully closed) the DigiTubes so that moisture can escape during the drying process.

10. ***Repeat steps 2 to 9 for another 2 - 3 samples until there are 9 DigiTubes with sample replicates of ~ 0.5 mg.***

11. Dispense ~0.5 g of deionised water into a DigiTube labelled "Blank".

12. Place a DigiTube labelled "AGAL3" into an analytical balance.

13. Tare analytical balance to zero.

14. Dispense ~0.2 g of the reference material AGAL3 (certified reference material, CRM) into the DigiTube using a clean stainless steel spatula.

15. Record the exact dispensed weight of AGAL3.

16. Place a DigiTube labelled "AGAL4" into an analytical balance.

17. Tare analytical balance to zero.

18. Dispense ~0.2 g of the reference material AGAL4 into the DigiTube using a clean stainless steel spatula.

19. Record the exact dispensed weight of AGAL4.

20. Add ~0.5 mL of deionised water to the "Blank" and both the AGAL3 and AGAL4 DigiTubes.

21. Place AGAL3 DigiTubes on vortex mixer for a few seconds to mix with dry reference material.

22. ***Repeat step 21 for AGAL4 DigiTube.***

23. There should now we 12 DigiTubes in one rack = one batch. Each batch shall include one blank and two reference materials.

Using heatproof gloves:

24. Place rack with the 12 DigiTubes into oven at 60˚C for at least 48 hrs.

25. ***Repeat for each DigiTube****:* remove a DigiTube from oven, tighten cap, place it into analytical balance and record exact weight of sample. Loosen cap and place DigiTube back into oven to continue drying process.

26. ***Repeat step 11*** after another 2 hrs in the oven for all DigiTubes.

27. ***Repeat step 12*** until 2 consecutive weight measurements are identical to within +/- 0.5 mg.

28. Place rack with DigiTubes under the fume hood and allow samples to cool to room temperature.

29. Add ~0.5 mL of deionised water to all DigiTubes. Place each DigiTube on vortex mixer for a few seconds to mix with dried sample materials.

Using the fume hood, wearing tightly fitting goggles:

30. Using an Eppendorf Automatic Pipette, measure out 3 mL of nitric acid and add to each DigiTube.

31. Using another Eppendorf Automatic Pipette, measure out 0.5 mL of hydrochloric acid and add to each DigiTube.

32. Place lids tightly on all DigiTubes and mix each sample thoroughly using the Vortex Mixer.

33. **Optional**: Keep loosely capped DigiTubes (thread engaged but not fully closed so built-up pressure during digestion can escape) overnight under the fume hood at room temperature. This will start the digestions and decrease the time needed in the dry block heater. Repeat step 32 before continuing with the next step.

34. Loosely cap all DigiTubes (thread engaged but not fully closed so built-up pressure during digestion can escape) and place into the dry block heater, set at 95˚C.

Using the fume hood, wearing tightly fitting goggles and heatproof gloves:

35. After 60 minutes, carefully take out one DigiTube, tightly close the cap and place on the Vortex Mixer for a few seconds. Loosen cap and place DigiTube back into heating block.

36. ***Repeat step 34 for each DigiTube.***

Using the fume hood, wearing tightly fitting goggles and heatproof gloves:

37. ***Repeat for steps 34 and 35 after 120 minutes of 95ºC heating process***.

38. Continue heating process until the analyte has turned clear.

39. Place DigiTubes in a rack in an ice bath, under fume hood to allow samples to cool for >15 minutes. **DO NOT** open the DigiTubes until they have cooled to room temperature! This is done to prevent loss of more volatile compounds, e.g. mercury.

40. Label one falcon tube for each DigiTube, copying the labels appropriately.

41. **Using the fume hood:**

42. Add ~1 mL of deionised water to the contents of the DigiTube, recap tightly and place on the Vortex Mixer for a few seconds. **Note:** This is done to prevent the filter from 'locking up', which can happen if the analyte is highly acidic.

43. Attach a filter to the luer lock of a syringe. Remove plunger from syringe.

44. While holding the filter over the falcon tube, transfer the content of the matching DigiTube using a disposable transfer pipette.

45. Carefully rinse the DigiTube by adding ~2 mL of deionised water, placing the lid on tight and gently shaking the DigiTube.

46. Carefully transfer the content of the DigiTube into the syringe using the same disposable transfer pipette.

47. Repeat steps 44 and 45 another 1 - 2 times or until the syringe is almost filled.

48. Insert plunger into syringe and carefully push solution through the filter into the falcon tube.

49. Top up with deionised water to precisely 14 mL (labelled on falcon tube) using a transfer pipette.

50. Place the screw top tightly on the falcon tube; seal the lid using Parafilm^{R} and store at room temperature until analysis.

51. ***Repeat steps 41 to 49 for each DigiTube.***

HOUSEKEEPING

Safely discard DigiTubes, disposable pipettes, pipette tips, syringes and filters as per *'Biohazardous Waste SOP'*.

HAZARDS

- Heat burns
- Zoonosis
- Hazardous Substances
- Fume hood failure.

Appendix 2

Supplementary material to Chapter 3

SUPPLEMENTARY MATERIAL

The Little Penguin (*Eudyptula minor*) as an indicator of coastal trace metal pollution

To test for loss due to sublimation in the oven-drying process, we acid-digested a test batch of 12 aliquots of Standard Reference Materials (SRM) provided by the National Measurement Institute (NMI), Melbourne, Australia: AGAL3 (prawn tissue) and AGAL4 (bovine liver tissue).

We mixed five aliquots of Agal3 and five aliquots of Agal4 (~0.2 mg each) with ~0.5 mL milliQ water, then dried them at 60ºC for 48 hrs in the exact same oven all the blood and feather samples were dried in. After that, we added one aliquot of Agal3 and one aliquot of Agal4 (~0.2 mg each), in exactly the same way as was done during blood and feather sample preparation. All samples were then digested, filtered and delivered to NMI, where they were analysed in the exact same manner as during the blood and feather analysis. The results, as percentage recoveries, are given in Table S1.

Kruskal Wallis rank sum tests found no significant difference in recovery for any element between the two groups; SRMs oven-dried and SRMs not oven-dried (Table S2).

Table S1: Percentage recoveries of a sublimation test batch of standard reference materials (SRM); ten of the SRM were oven-dried at 60ºC for 48 hrs and two SRM were not oven-dried.

% Recovery	Al	As	B	Ca	Cd	Cu	Fe	Hg	Pb	Se	Zn
Agal3-1	75	82	104	97	89	93	91	89	93	101	89
Agal3-2	67	82	106	95	93	102	79	86	93	102	89
Agal3-3	67	82	103	95	83	100	79	84	87	99	90
Agal3-4	68	80	110	95	90	87	82	85	83	97	90
Agal3-5	70	86	107	90	89	102	88	87	105	102	88
Agal4-1	84	92	109	94	98	104	107	85	113	86	88
Agal4-2	76	89	111	94	100	104	117	84	85	85	87
Agal4-3	91	100	111	94	99	106	103	85	83	90	90
Agal4-4	107	93	110	118	95	101	105	81	91	86	98
Agal4-5	100	98	114	98	103	104	108	84	96	87	89
Agal3 not dried	70	81	111	97	96	93	111	87	130	96	88
Agal4 not dried	79	88	100	90	94	98	97	81	96	83	85

Table S2: Test statistics of the Kruskal Wallis rank sum tests of the sublimation test batch, grouped by whether they were oven-dried or not.

Statistics	**Al**	**As**	**B**	**Ca**	**Cd**	**Cu**	**Fe**	**Hg**	**Pb**	**Se**	**Zn**
Chi-squared	0.01	0.75	0.19	0.19	0.05	1.99	0.74	0.05	2.62	1.16	3.08
p value	0.91	0.39	0.66	0.66	0.83	0.16	0.39	0.83	0.11	0.28	0.08

Appendix 3

Supplementary material to Chapter 4

SUPPLEMENTARY MATERIAL

Seasonal variation and annual trends of trace metals and metalloids in the blood of the Little Penguin (*Eudyptula minor*)

Table S1: Standard reference material (SRM) recovery rates for all elements measured

SRM / Element	Al	As	B	Cd	Cr	Cu	Hg	Pb	Se	Sn	Zn	Ca	Fe
Mean AGAL3 [%]	72	80	97	83	83	111	92	80	83	93	86	96	92
Mean AGAL4 [%]	87	80	100	90	134	94	82	88	85	NA	98	101	96

Table S2: Details of data transformations executed and outliers removed to conform to analysis assumptions - complete data set

Factor / Element	**As**	**B**	**Cu**	**Hg**	**Pb**	**Se**	**Zn**	**Ca**	**Fe**
Location	Sqrt	Sqrt	None	Sqrt	Sqrt	Log	Log	Log	1/Fe
BM_code	Sqrt	Sqrt	None	Sqrt	Sqrt	Sqrt	None	Log	1/Fe
MoultStage	Sqrt	Sqrt	None	Sqrt	Sqrt	Sqrt	Sqrt	Sqrt	1/Fe
Outliers removed	AF571	AF292, AF291, AF879, AF617, AF880	AF349	None	AF389, AF681, AF391, AF683, AF292, AF865	None	None	AF452, AF456, AF339, AF454, AF845, AF445, AF513	AF499
Shapiro ($p > 0.01$)	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Bartlett ($p > 0.01$)	Passed	Passed	Passed	Passed	Passed	Passed	Passed	BM_code^{Ok} ($p < 0.001$)	Passed

Note: Ok - means Manovas were executed with and without that element and there was no real difference, so compliance with assumptions was deemed sufficient.

Table S3: Details of data transformations executed and outliers removed to conform to analysis assumptions - St Kilda

Factor / Element	As	B	Cu	Hg	Pb	Se	Zn	Ca	Fe
Year	Sqrt	Log	None	None	Sqrt	Log	None	Log	None
Season	Sqrt	Log	None	None	Sqrt	Log	None	Log	None
Sex	Sqrt	Log	None	None	Sqrt	Log	None	Log	None
Outliers removed	None	AF292, AF291, AF879, AF880	AF349, AF334	AF792, AF597	AF389, AF681, AF391, AF683, AF292, AF865	AF820	AF494, AF495	AF339, AF513, AF873, AF880, AF684	AF488, AF492, AF493, AF490
Shapiro ($p > 0.01$)	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Bartlett ($p > 0.01$)	Passed	Year[ok] ($p < 0.001$)	Passed	Passed	Passed	Passed	Passed	Passed	Season[ok] ($p < 0.01$)

Note: Ok - means Manovas were executed with and without that element and there was no real difference, so compliance with assumptions was deemed sufficient.

Table S4: Details of data transformations executed and outliers removed to conform to analysis assumptions - Phillip Island

Factor / Element	As	B	Cu	Hg	Pb	Se	Zn	Ca	Fe
Year	Sqrt	Sqrt	None	None	Log	None	None	None	1/Fe
Season	Sqrt	Sqrt	None	None	Log	Sqrt	None	None	1/Fe
Sex	Sqrt	Sqrt	None	None	Log	None	None	None	1/Fe
Outliers removed	AF571, AF617	AF617	None	None	None	None	None	AF452, AF456, AF845, AF454,	None
Shapiro ($p > 0.01$)	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Bartlett ($p > 0.01$)	Passed	Year[ok] ($p < 0.01$)	Passed	Passed	Passed	Passed	Passed	Sex[ok] ($p < 0.01$)	Passed

Note: Ok - means Manovas were executed with and without that element and there was no real difference, so compliance with assumptions was deemed sufficient.

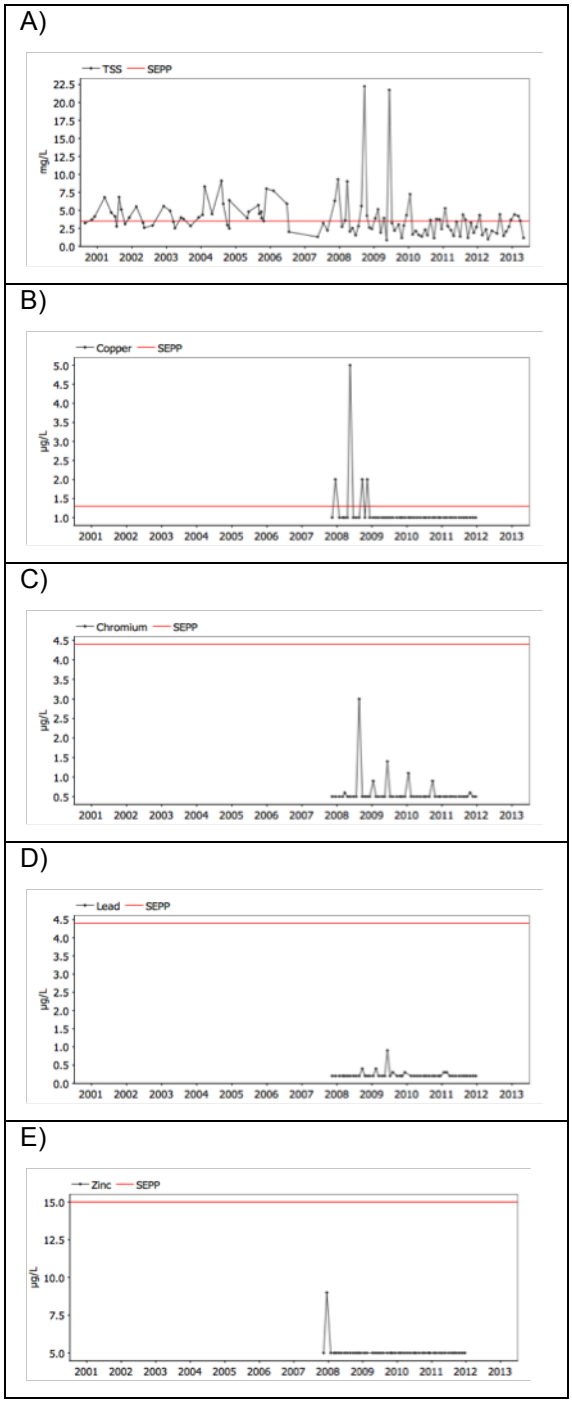

Figure S1: Monthly monitoring data for Hobsons Bay, retrieved from http://www.cleaneryarrabay.vic.gov.au/ "Cleaner Yarra and Bay" report card. A) total suspended solids (TSS), B) copper, C) chromium, D) lead, E) zinc. Data are shown back to 2000 and compared to environmental objectives in the *State environment protection policy (Waters of Victoria; SEPP)* and its *Schedule F6 (Waters of Port Phillip Bay)*. The objectives are shown as red lines and referred to as SEPP.

Appendix 4

Supplementary material to Chapter 5

SUPPLEMENTARY MATERIAL

Metals and metalloids in Little Penguin (*Eudyptula minor*) prey, blood and faeces

Table S1: Subset of Little Penguins sampled collectively for Hg and d15N determination. Hg_adj calculated using correlation coefficient Hg / d15N = 0.4983 and d15N_mean = 19.2125.

Id	Sex	Date	Season	Year	Tissue	d15N	Hg	Hg_adj
AF492	Female	12/01/2012	Breeding	2011	Whole blood	19.5135	2.7000	2.5500
AF493	Male	12/01/2012	Breeding	2011	Whole blood	20.8566	3.0000	2.1807
AF494	Female	12/01/2012	Breeding	2011	Whole blood	19.1506	3.3000	3.3309
AF495	Male	12/01/2012	Breeding	2011	Whole blood	20.9665	3.6000	2.7260
AF678	Male	10/09/2012	Breeding	2012	Whole blood	18.1308	3.1500	3.6890
AF680	Female	10/09/2012	Breeding	2012	Whole blood	19.0070	3.2000	3.3024
AF681	Female	10/09/2012	Breeding	2012	Whole blood	19.0960	3.4000	3.4580
AF682	Male	10/09/2012	Breeding	2012	Whole blood	19.3510	2.9333	2.8643
AF683	Female	10/09/2012	Breeding	2012	Whole blood	21.3210	4.1500	3.0993
AF684	Female	10/09/2012	Breeding	2012	Whole blood	18.8520	3.2000	3.3796
AF702	Male	7/11/2012	Breeding	2012	Whole blood	18.0880	2.6500	3.2103
AF703	Male	7/11/2012	Breeding	2012	Whole blood	19.1959	2.8000	2.8083
AF704	Female	7/11/2012	Breeding	2012	Whole blood	19.1190	2.7000	2.7466
AF705	Female	7/11/2012	Breeding	2012	Whole blood	19.1310	2.8333	2.8739
AF706	Male	7/11/2012	Breeding	2012	Whole blood	17.7580	3.1000	3.8248
AF707	Female	7/11/2012	Breeding	2012	Whole blood	17.8630	3.2000	3.8725

Table S2: Details of outliers removed in Little Penguin blood, faeces and fish prey species

Metal(loid)	**As**	**Cd**	**Hg**	**Pb**	**Se**
Blood data outliers removed	None	NA	None	AF681	None
Faeces data outliers removed	None	AF678, AF684	None	AF295, AF678	None
Fish data outliers removed (for each species separately)	None	AF771, AF1007, AF1003	None	AF985, AF981, AF759, AF768, AF716, AF720, AF721, AF1006	None

Table S3: Details of paired samples for blood-faeces correlations

Season and Year	**# Samples collected**	**# Samples with viable measurements**				
		As	**Cd**	**Hg**	**Pb**	**Se**
Moult 2011	5	5	1	4	4	5
Non-breed 2011	6	6	0	6	6	6
Breed 2011/12	6	4	0	6	6	6
Non-breed 2012	3	3	0	3	3	3
Breed 2012/13	15	14	0	14	14	14
Breed 2013/14	6	5	0	5	5	5
Total	41	37	1	38	38	39

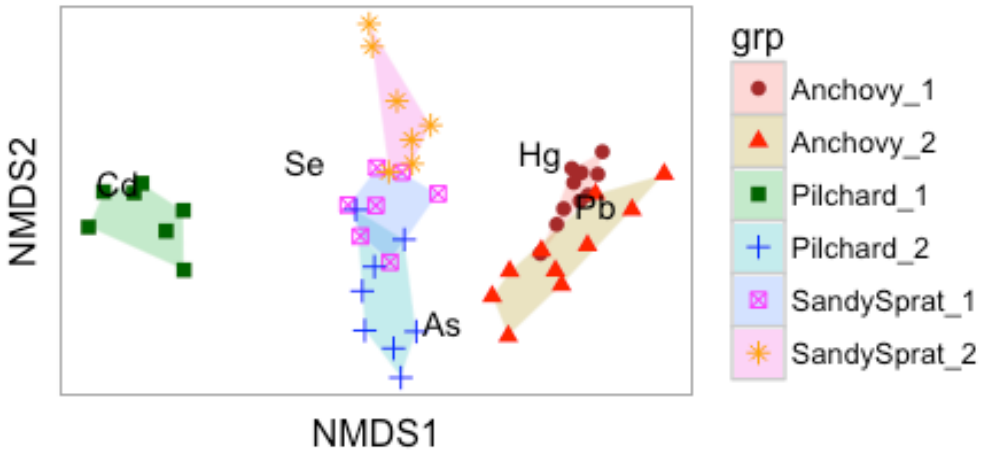

Figure S1: Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin fish prey species, separate for each sampled cohort (stress = 0.09): Anchovy_1 (first cohort, brown full circle), Anchovy_2 (second cohort, red full triangle), Pilchard_1 (first cohort, green full square), Pilchard_2 (second cohort, blue cross), SandySprat_1 (first cohort, magenta cross inside square), SandySprat_2 (second cohort, yellow star). Trace elements are displayed by their periodic symbols: As, Cd, Hg, Pb and Se.

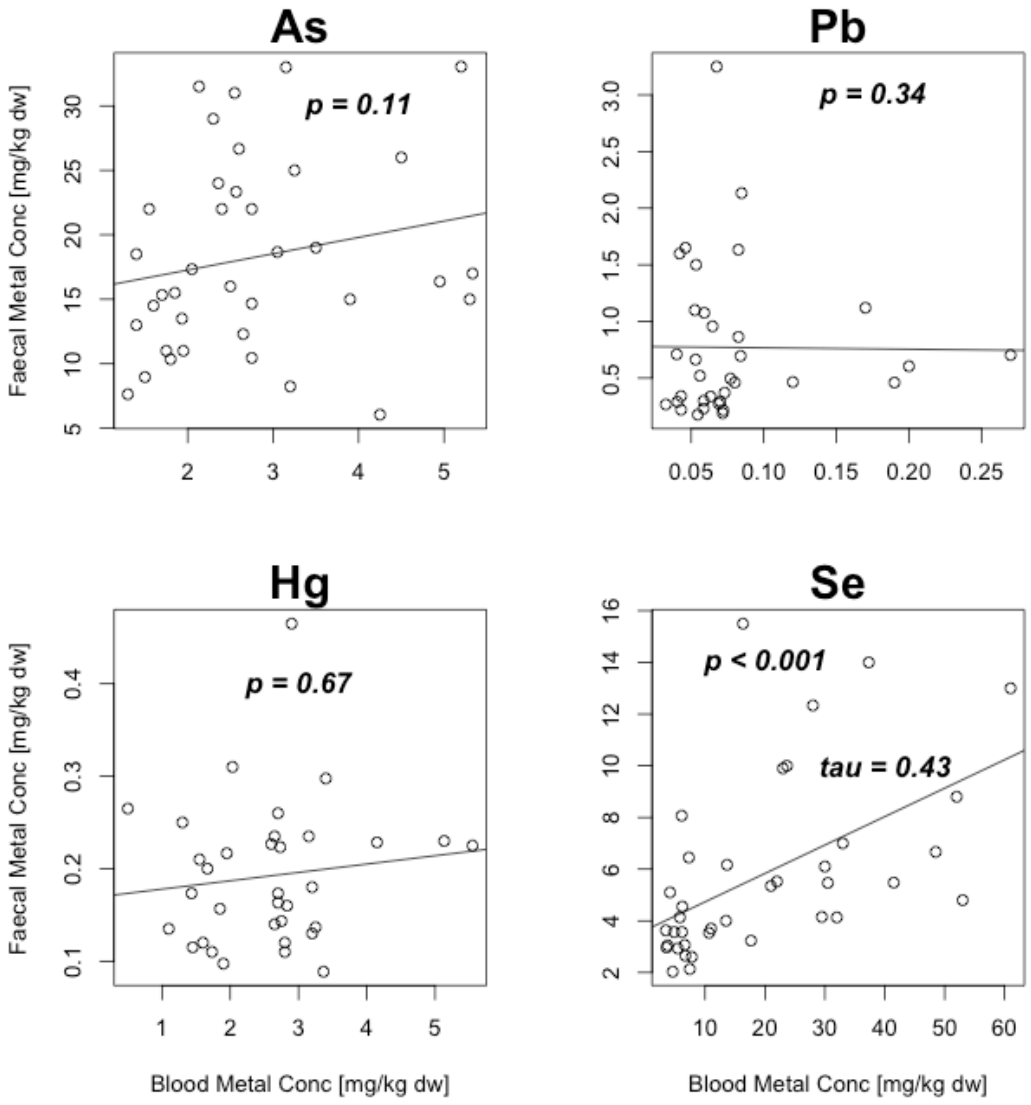

Figure S2: Relationships between metal(loid) concentrations in blood and faeces of Little Penguins from St Kilda, Melbourne, Australia (paired data, blood and faeces collected from the same individual). Arsenic (n = 37), Mercury (n = 38), Lead (n = 38) and Selenium (n = 39).

Table S4: Mean metal and selenium concentrations in whole fish (data provided by N. Dunlop, metal analysis performed at ChemCentre WA), measured in mg/kg dry weight. SD = standard deviation.

			Cd		Hg		Pb		Se	
Species	**Location**	**n**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Sandy sprat *Hyperlophus vittatus*	Tweed Heads, NSW	3	0.25	0.06	0.04	0.01	0.05	0.04	1.52	0.97
Anchovy *Engraulis australis*	Tweed Heads, NSW	3	0.13	0.06	0.04	0.01	0.02	0.01	0.77	0.15
Anchovy *Engraulis australis*	Oyster Harbour, WA	3	0.03	0.005	0.09	0.011	0.37	0.124	1.66	0.2
Pilchard *Sardinox sagax*	King George Sound, WA	10	0.60	0.157	0.03	0.005	0.03	0.175	4.35	0.51

Appendix 5

Adverse incident report and investigation outcomes

VICTORIA UNIVERSITY

ANIMAL EXPERIMENTATION ETHICS COMMITTEE

ADVERSE INCIDENT REPORT

Forwarding Details

All hard copy applications to be delivered to:	Electronic applications are to be forwarded to
Ethics Secretary **The Victoria University** **Animal Experimentation Ethics Committee** Office for Research Victoria University PO Box 14428 Melbourne VIC 8001 **Or** deliver in person to: Ethics & Biosafety Administration Group Office for Research Building C, Room C302 Footscray Park campus.	**The Victoria University** **Animal Experimentation Ethics Committee:** E-mail: aeec@vu.edu.au

As per the Australian Code of Practice for the care and use of animals for scientific purposes 2.2.27 & 2.2.28 it is understood that all adverse incidents are to be dealt with immediately by the responsible researchers, Animal Facility staff and the Animal Welfare Office.

The AEEC should be notified immediately.

This report summarises, for the AEEC the incident and the actions taken.

1. AEEC approval details.

Project Title	Trace metals in little penguin (Eudyptula minor) populations along the Victorian Coastline of Australia
AEETH Number	05-09
Chief Investigator	Dr. Carol Scarpaci
Approval Period	22/01/2010 To 31/12 /2013

2. Number and type of animals affected by the adverse incident

Species	Breed / Strain	Sex (M/F)	Age	Total Number
Little Penguin (*Eudyptula minor*)	N/A (wild)	F	adult	1

3. Description of adverse incident

On Thursday the 21st of February 2013 a fatality of one little penguin (*Eudyptula minor*) occured whilst researchers followed the protocol for sampling for blood and feathers of little penguins at Phillip Island, Victoria, as described in section 3.3.6 of the animal ethics permit AEETH05-09. The fatality occurred post morphometric data collection and pre blood sampling. The details of the event are enclosed below.

Annett Finger (PhD candidate) and 2 volunteers (Mat Booth and Bec Cross, both students at VU) arrived at Phillip Island Nature Park (PINP) at 8:15am. Paula Wasiak and Leanne Renwick, from the Phillip Island Nature Park (hereafter, PINP) advised on the appropriate location to sample and provided directions via the use of a map. An additional volunteer (Mary Cowling, VU Masters candidate) from the PINP volunteer house joined the group.

By 9am the research group reached the suggested location. Prior to selecting a field date to sample, Annett Finger had consulted with regional weather forecast for Phillip Island at www.willyweather.com.au, which predicted a maximum of 23ºC and no rain. The conditions at the onset of sampling were partly overcast with slight wind. As a result Annett and her crew set up their gear under a low tree/bush (for shading) and started checking nesting boxes and bushes for penguins. The area has a mixture of artificial wooden nesting boxes and natural burrow under shrubs of vegetation. They caught and sampled penguins according to the activities detailed in the ethics permit with emissions (no placing in boxes for faecal collection, no insertion of microchips). The first penguin was captured at 9:25 am and the penguin of interest was collected at 11:10 am. Annett was following the guidelines proposed by PINP on bleeding moulting penguins depending on the moult stage they are in: M1= min. 1400g; M2 = min 1300g; M3 = 1100g; M4 = 1000g; M5 = 900g. Only birds exceeding these weight thresholds were selected for bleeding. Birds with lower weights were measured and feathers sampled only.

The exact details of events for the penguin that died are detailed below (please note times are approximate and to our best knowledge). Annett Finger has timed herself previously on several occasions to ensure the procedure of manual handling and bleeding does not exceed a 15 min timeframe.

- 11:00 am Bec and Mary found an artificial nesting box (wood) with a pair of penguins in it. First they took the male, a stage M5 moulter (end of moult, new feathers fully grown, almost all old feathers lost). They brought this bird to Annett Finger at "camp", where it was weighed, scanned, measured and bled. Mat held the bird while Annett bled it and Mary scribed. There were no incidences.
- 11:10 am Annett and Mat returned the male to the location of the nesting box. Bec took out the female. Mat collected loose feathers from the nesting box into a zip-lock bag and Annett returned the male bird into the nesting box. The female (also stage M5) was placed in a cotton bag and was taken

to the "camp" by Annett. Mat and Bec continued scouting for more penguins to sample. Approximate walking distance from nest to camp was 2 min.

- 11:12 am Annett weighed the bird while in the bag using a spring balance (weight of bird = 940 g). Mary scribed. Annett scanned for and found a microchip (6E70145) and Mary scribed. Annett opened the cotton bag slightly to expose the rear of the animal and collected remaining loose moulted feathers and placed them into a zip-lock bag. Mary labeled and scribed.
- 11:13 am Annett took the bird out of the bag, holding the bird by the neck with her left hand. She placed the cotton bag around the bird's body to immobilise its wings, moved her left hand to place index and middle finger above and below the beak with her left thumb at the back of the bird's head. Annett held the bird, similar to a football hold, between her lower left arm and left side of her torso. This is the common grip used by PINP and Earthcare St Kilda to take morphometric measurements. Annett used plastic digital calipers to measure total head length, beak length and beak depth. Placing the bird on her lap, again holding its neck with her left hand, Annett spread out its right wing. Mary assisted with holding out a metal ruler. Annett placed the ruler under the wing, placed the wing along the ruler and read out the length. Mary scribed. This activity took between 3 - 5 min. During the handling, the bird behaved normally for a wild penguin, it was bright, alert and feisty. No gaping or panting was observed that would indicate heat stress. Mary later recalled that she noticed the bird's white feet while Annett took these measurements. She did not voice that observation at the time.
- 11:17 am Annett placed the penguin back into the cotton bag and held it loosely on her lap. Annett asked Mary to find Mat and ask him to come to camp to help bleed the bird. Mat is currently Annett's most skilled and experienced penguin holder while blood collection.
- 11:18 am Mat arrived and Mary departed for the research office to continue her research work. Annett handed the bird in the bag over to Mat (positioned opposite to Annett). Mat positioned the bird (still in bag) upright on his lap while exposing one foot to Annett to check for veins, standard protocol for birds that are to be sampled for blood. Mat remembered that the bird actively shifted its weight while on his lap. Annett noticed the foot was unusually white and asked to see the other foot. The other foot was the same. Annett asked Mat to massage both feet gently to get the blood flowing. Annett prepared her needle and syringe while Mat massaged the bird's feet.
- 11:19 am Annett checked on the feet to see if the colour had improved and she would be able to proceed with the bleeding. The feet still looked white and felt cold. Annett and Mat got alarmed at the same time, they quickly took the bird out of the bag and noticed that it was lifeless. They checked its mouth, which had a couple of feathers in it, but the airways were clear. Mat placed the bird on his lap and did CPR on its chest. The sound of air entry in and out was heard, but the bird remained lifeless. CPR continued for a 2-3 of minutes before calling Paula at PINP to report the incident.
- The deceased bird was placed into a zip-lock plastic bag, placed into the

boot of the car. Annett and her crew drove back to the research office and parked at the volunteer house. Annett removed the deceased bird from the boot and carried it to the research office where she met with Paula and Leanne.

None of the procedures done on this particular bird were unusual in any way or outside the scope of the activities outlined in the animal ethics application. Total handling time was under 10 minutes. The weather was not hot. There was no direct sunlight on the researchers or the bird. The bird did not display any heat stress (panting, gaping, gurgling). Annett had packed extra bottles of water to cool them down should any birds get too hot.

4. Timeline of events

We reported via phone to Paula Wasiak (research assistant at Phillip Island Nature Park - PINP). Paula ordered Annett and her crew to stop sampling, pack up and return to research office with deceased penguin. At the research office, the penguin was initially placed in a freezer for 90 min and then moved to the fridge. Annett informed Dr. Carol Scarpaci, Dr. Patrick Guay and texted Dr. Peter Dann who is Annett's co-supervisor and research manager at PINP. Peter was in meetings. He spoke with Annett upon his return at the research office. He sighted the deceased penguin and got an account of events from Annett. Annett called Alan Hayes to enquire about the possibility of an autopsy. Peter asked Annett to transport the deceased penguin to Melbourne, where it is currently in a fridge, awaiting autopsy.

When was the incident first noticed/reported? Document steps taken from that date to manage the incident by listing dates, times, actions taken and by whom.

5. Cause of the Incident

Cause of death unknown at this early stage. Autopsy planned.

Do you know what caused the incident? If yes, give detail. If no, what is/are the likely cause(s)?

6. Declarations

This report accurately reflects the adverse incident.			
Title	Name	Signature	Date
Chief Investigator	Dr. Carol Scarpaci		_ _ /_ _

			/_ _ _ _

The Victoria University AEEC has reviewed and accepts this adverse incident report.			
Title	Name	Signature	Date
AEEC Chair			_ _ /_ _ /_ _ _ _

Report on the investigation of the adverse incident relating to AEETH 05/09

(Author: Dr. Kerrie Dickson, Chair AEEC, Victoria University)

As part of my review of the adverse incident resulting in the death of one Little Penguin in the field on Philip Island on February 21st 2013, I reviewed the following documents.

- Adverse Incident Report submitted by Dr. Carol Scarpaci on 22nd February 2013
- Diagnostic Testing Report (post-mortem report) prepared by Veterinary Diagnostic Services
- Current application (including all minor amendments) for project AEETH 05/09
- 2011 Annual Report for project AEETH 05/09
- The 2012 Bureau of Animal Welfare Animal Use Return Form for project AEETH 05/09
- Victorian Department of Sustainability and Environment (DSE) Permit # 10005200

The 2012 Annual Report for project AEETH 05/09 was not reviewed as it was not available at the time.

The following are my findings:

- Both the Animal Ethics Approval and DSE permit were current at the time of the adverse incident.
- The DSE permit makes no reference of any requirements of declaring adverse events to DSE
- Condition 13 of the DSE permit clearly states that any dead or dispatched animals must be lodged with Melbourne Victoria within 30 days of death.
- The Adverse Incident Report was very thorough and complete and submitted in a timely manner.
- The post-mortem report indicates that death was the result of hypoxia or suffocation.
- The post-mortem report could not allow determination as to whether the hypoxia was the result of lack of oxygen in the bag in which the birds was carried or the result of accidental strangulation.

- The carrying/keeping of penguins in cotton bags was not approved by the VU AEEC. According to application form reviewed, penguins are to be carried and kept in “plastic animal carry cases” and only transferred into “cotton bags” for weighing.
- Annett Finger has handled (as of the end of 2012) in excess of 250 Little Penguins without any incident.

It is clear that Dr. Carol Scarpaci and Ms Annett Finger have followed the proper protocol in response to the adverse incident and that their detailed and prompt response must be commended. The death was unfortunate, but although the cause of death was established (asphyxia/suffocation), the reason why this occurred remains unclear. Two possibilities could explain this death.

1. Either the Little Penguin could not breathe in the bag
2. Or, it was accidentally strangled during handling.

From the description provided by Ms Annett Finger in the incident report, she was the only person handling the penguin. Therefore, bad handling from an untrained volunteer can be ruled out in this case. Furthermore, it is unlikely that Ms Annett Finger would have accidentally strangled the penguin given that she is experienced and has handled more than 250 Little Penguins without any similar incidents. It is thus possible that the penguin could not breathe in the bag. In ten years of experience working with birds, I have never had any problems with animals asphyxiating in cotton bags. It may be worthwhile investigating what type of cotton was used as thin fabric, although more fragile, may be more permeable to air. Furthermore, when cotton bags become wet, they become somewhat impermeable to air and thus in extreme circumstances anoxic conditions can result. Without any information in the post-mortem report about the state of the windpipe, it will remain impossible to fully explain this death as neither asphyxia nor strangulation can be rule out. Given this information, I would like to make the following recommendations

- The project should be allowed to continue as approved.
- If the carcass of the deceased penguin, or part thereof, is still available, the researcher must contact Wayne Longmore (03 8341 7452) the manager of the Museum Victoria bird collection and enquire as to whether the specimen is needed by the museum.
- Given the lack or requirements by DSE on reporting of adverse events, no action involving DSE needs to be taken, but the permit’s final report should mention the adverse event.
- Ms Annett Finger should use “plastic animal carry cases” to keep and carry Little Penguins and only transfer penguins in bags to weigh; measure and blood sample them as described in AEETH 05/09.

- As I precaution, I should attend her next sampling period to ensure that birds are handled appropriately.
- These recommendations will need to be reviewed if a second incident occurs within the next 12 months.

Signed by Dr. Kerrie Dickson
Chair
AEEC
Victoria University