Marine Pollution Bulletin 110 (2016) 261–273

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

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

Annett Finger [a,*], Jennifer L. Lavers [b], John D. Orbell [a], Peter Dann [c], Dayanthi Nugegoda [d], Carol Scarpaci [a]

[a] Institute for Sustainability & Innovation, College of Engineering & Science, Victoria University, Hoppers Crossing, Werribee, Victoria 3030, Australia
[b] Institute for Marine and Antarctic Studies, 20 Castray Esplanade, Battery Point, Tasmania 7004, Australia
[c] Research Department, Phillip Island Nature Parks, PO Box 97, Cowes, Victoria 3922, Australia
[d] RMIT University, School of Applied Science, GPO Box 2476, Melbourne, Victoria, Australia

ARTICLE INFO

Article history:
Received 15 April 2016
Received in revised form 9 June 2016
Accepted 13 June 2016
Available online 18 June 2016

Keywords:
Seabird
Bioindicator
Mercury
Port Phillip Bay
Bass Strait

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.

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 and 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

* Corresponding author.
E-mail address: Annett.Finger@live.vu.edu.au (A. Finger).

http://dx.doi.org/10.1016/j.marpolbul.2016.06.055
0025-326X/

(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 (Fig. 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 (Fig. 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.

2. Materials and methods

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 (Fig. 1, Table 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 and 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).

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 and 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 and 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

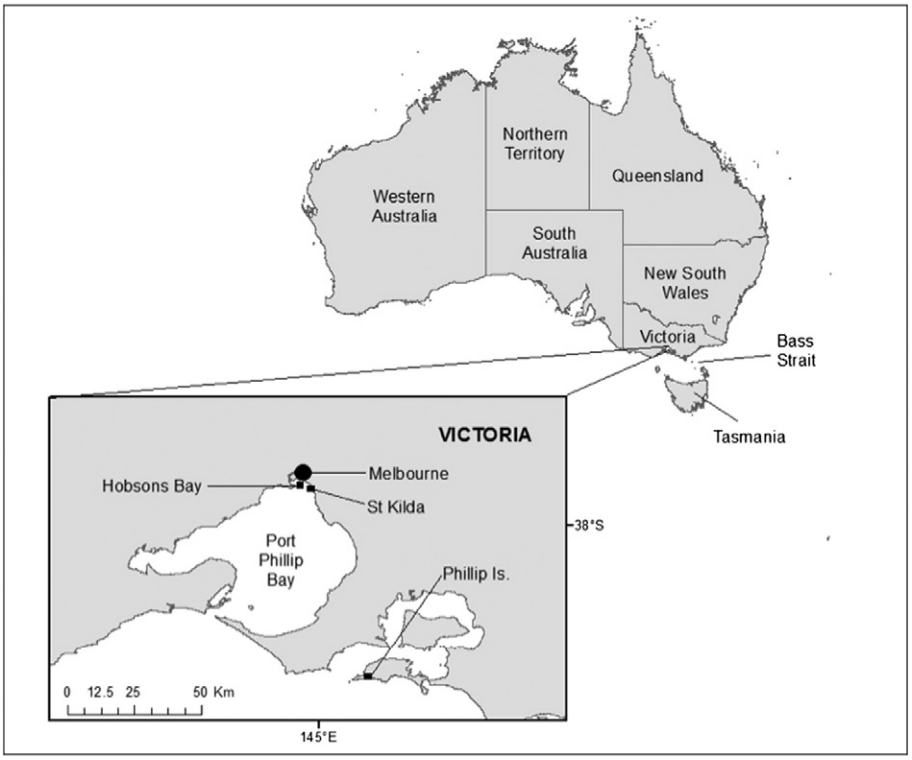

Fig. 1. Little Penguins were sampled at St Kilda and Phillip Island from 2011 to 2013.

Table 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		Aluminium	Arsenic	Boron	Calcium	Copper	Iron	Mercury	Lead	Selenium	Zinc
	Season	Location										
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]
	Breeding	St Kilda	2.37 ± 1.82 (12) [0.80 − 6.20]	2.89 ± 1.35 (13) [0.48 − 5.10]	0.81 ± 0.57 (11) [0.14 − 2.10]	296 ± 35.5 (11) [240 − 340]	2.29 ± 0.42 (13) [1.77 − 2.90]	2309 ± 145.4 (13) [1933 − 2600]	3.77 ± 0.94 (13) [2.65 − 5.55]	0.05 ± 0.01 (13) [0.04 − 0.08]	27.64 ± 12.03 (13) [13.67 − 59.33]	33.81 ± 3.14 (13) [28.00 − 40.67]
		Phillip Island	–	–	–	–	–	–	–	–	–	–

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 and 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).

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 60 °C 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 95 °C. 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 7700× 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.

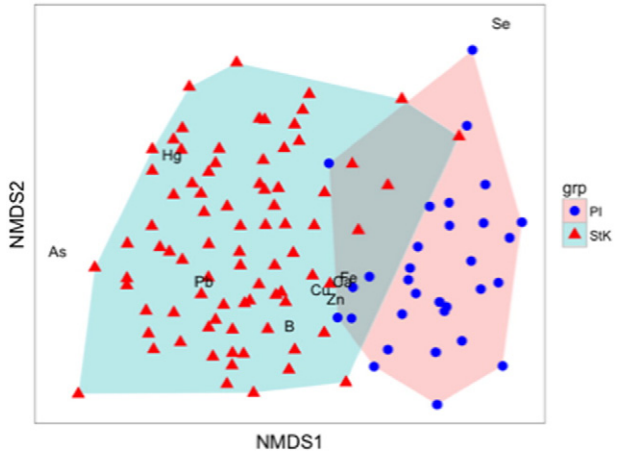

Fig. 2. Two-dimensional NMDS plot with Bray–Curtis distance for Little Penguin blood samples by colony location (StK = St Kilda, PI = Phillip Island, stress = 0.20). Polygon ellipse lines are drawn for each sampling location. Metals and metalloids are displayed by their periodic symbols.

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.

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 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 and 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 and 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 and 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).

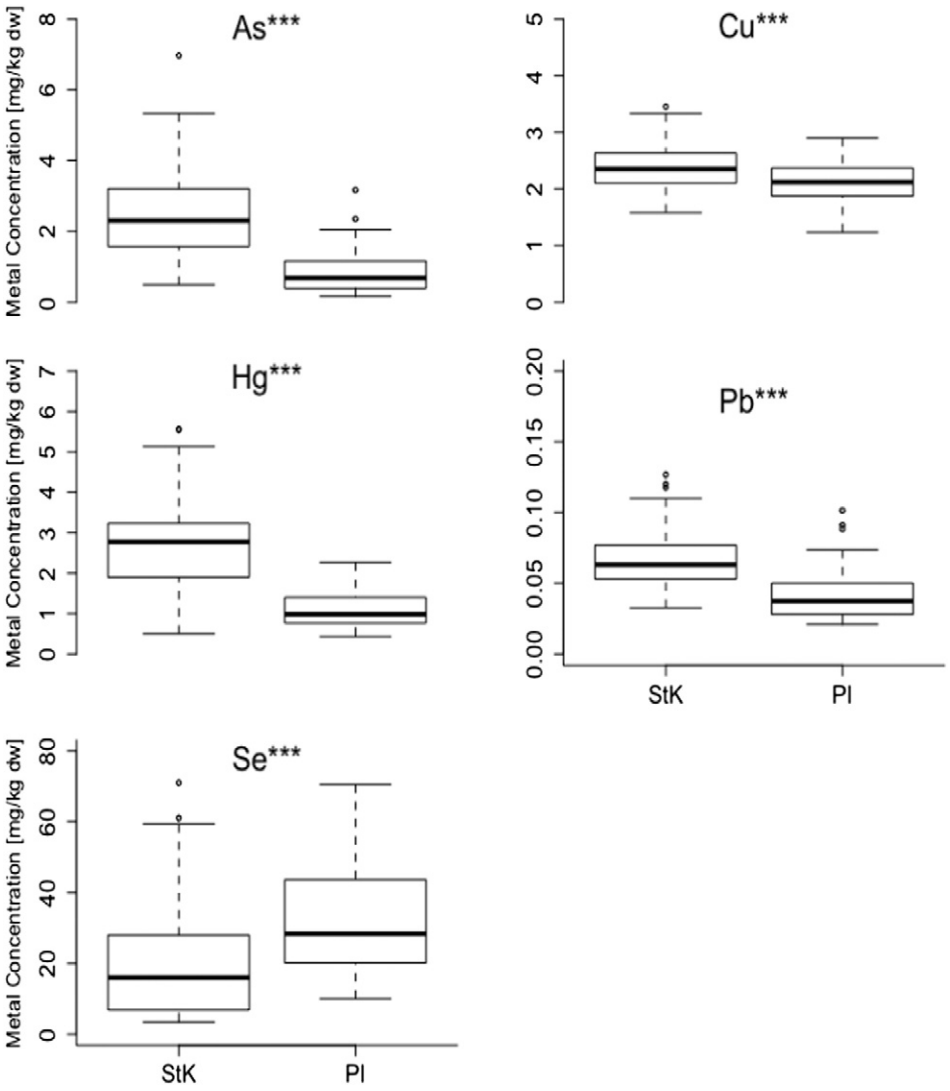

Fig. 3. Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins that were significantly different (pairwise *t*-tests with "Holm" correction) by colony location (StK = St Kilda, PI = Phillip Island). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 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 (°) are defined as between 1.5 and 3 ∗ IQR.

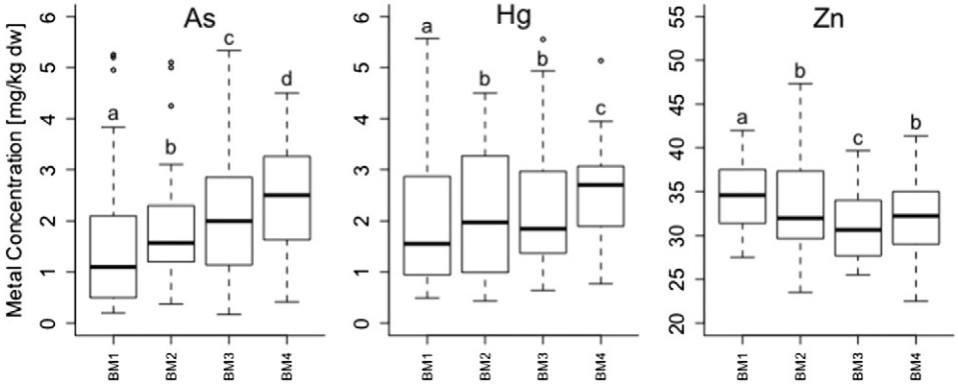

Fig. 4. Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins that were significantly different (lower case letters, Tukey HSD, $p < 0.05$) by body mass category. BM1: 900–1080 g (n = 44), BM2: 1090–1170 g (n = 35), BM3: 1180–1309 g (n = 44), BM4: 1320–1870 g (n = 34). Data shown 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 (°) are defined as between 1.5 and 3 ∗ IQR.

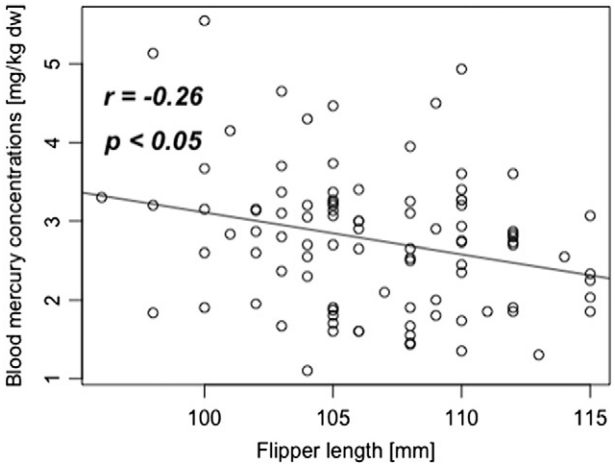

Fig. 5. Pearson correlation between Little Penguin blood mercury concentrations and flipper length at St Kilda (n = 96).

3. Results

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 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 (Fig. 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 (Fig. 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, Fig. 3). Mean blood As concentrations were almost 3× 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× 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 $\lambda = 0.55$; partial $\eta^2 = 0.18$. 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 (Fig. 4).

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 $d = -0.47$). 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

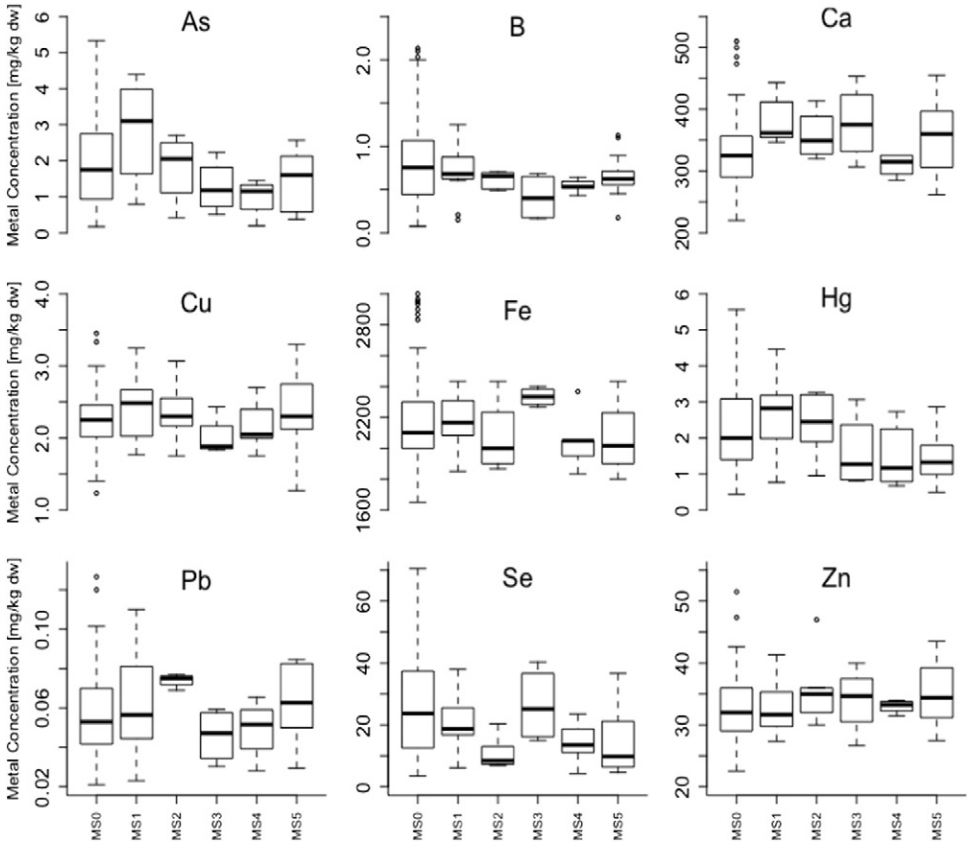

Fig. 6. Metal and metalloid concentrations (mg/kg dry weight) in whole blood of Little Penguins by moult stage. MS0 means sample was taken outside of moult. M1 to MS5 are progressive moult stages, see methods for details. Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined 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 (°) are defined as between 1.5 and 3 * IQR.

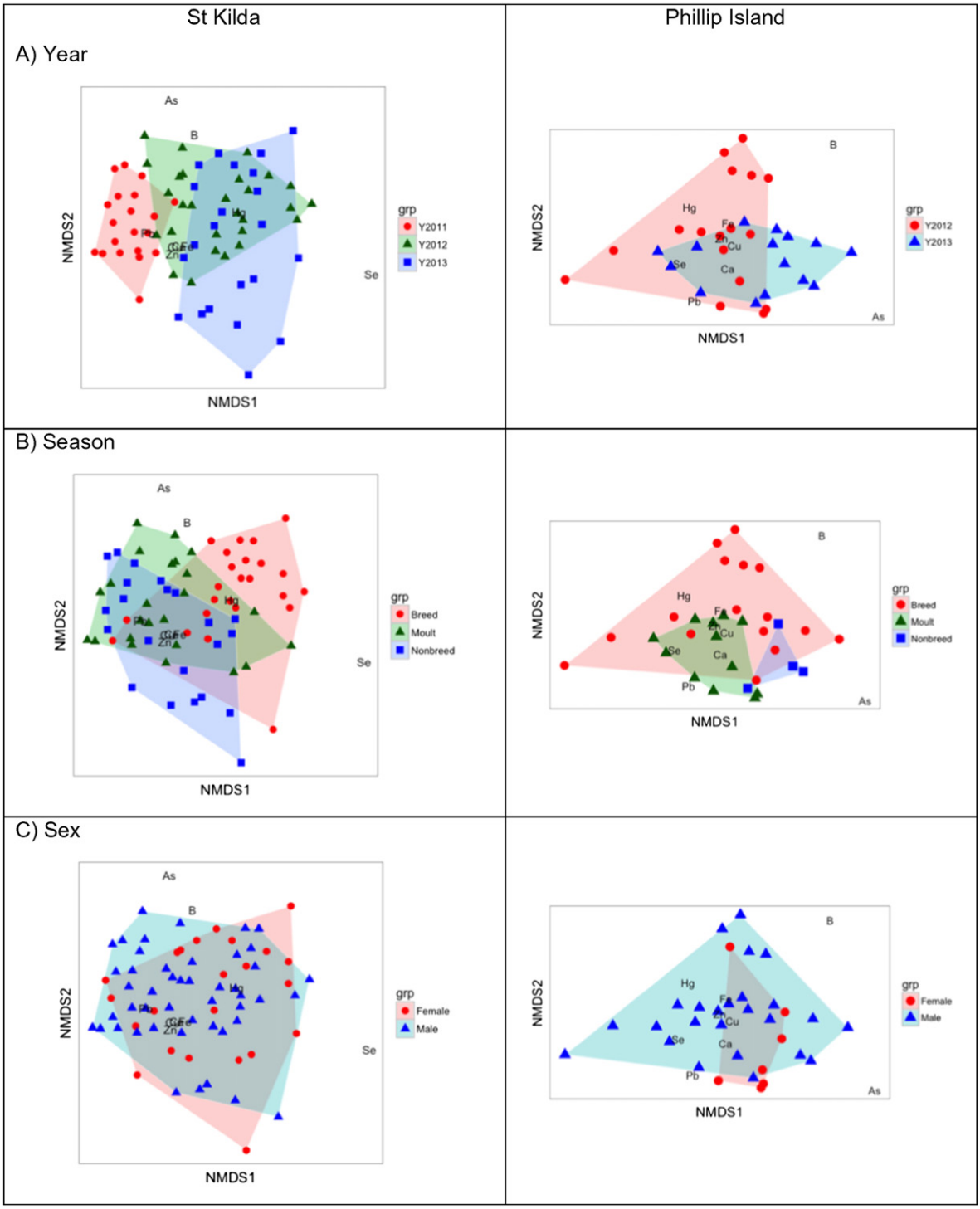

Fig. 7. Two-dimensional NMDS plots with Bray–Curtis distance for Little Penguin blood samples from St Kilda (stress = 0.18, left panels) and Phillip Island samples (stress = 0.23, right panels) by factors A) year, B) season and C) sex. Polygon ellipse lines are drawn for each grouping; see legends for details for each. Metals and metalloids are displayed by their periodic symbols.

length at St Kilda (Pearson correlation, r = -0.26, $t_{94} = -2.59$, $p < 0.05$, r [95%CI] = 0.44, Fig. 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 (Fig. 6).

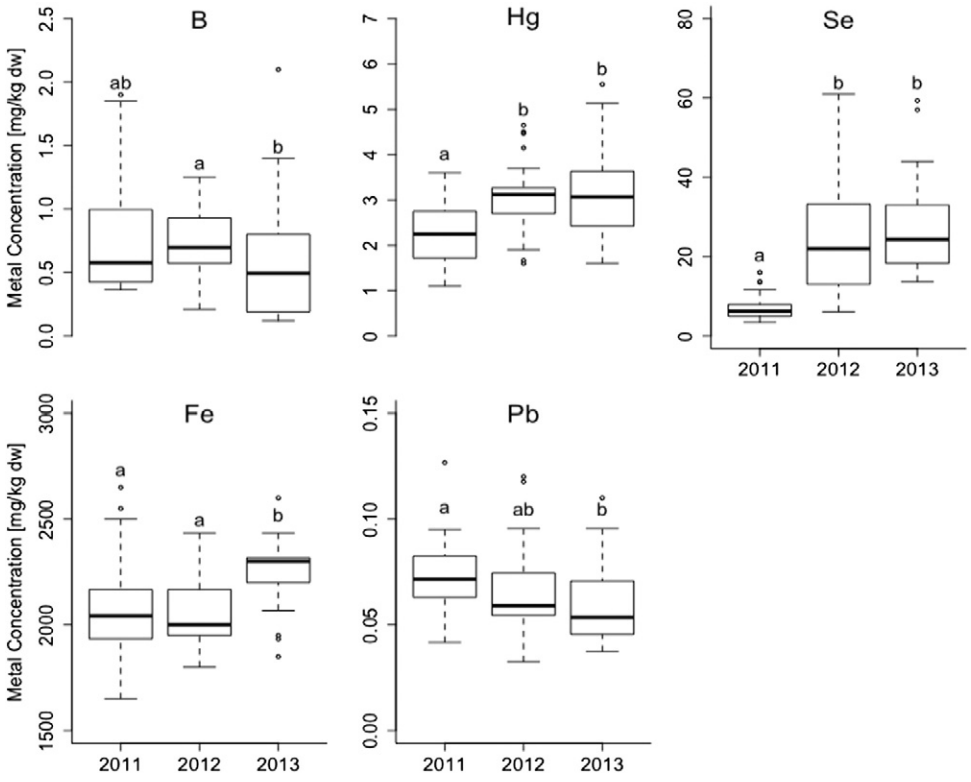

Fig. 8. Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at St Kilda that were significantly different between years (lower case letters, Tukey HSD, $p < 0.05$). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as the 25th and 75th percentiles. 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 (°) are defined as between 1.5 and 3 ∗ IQR.

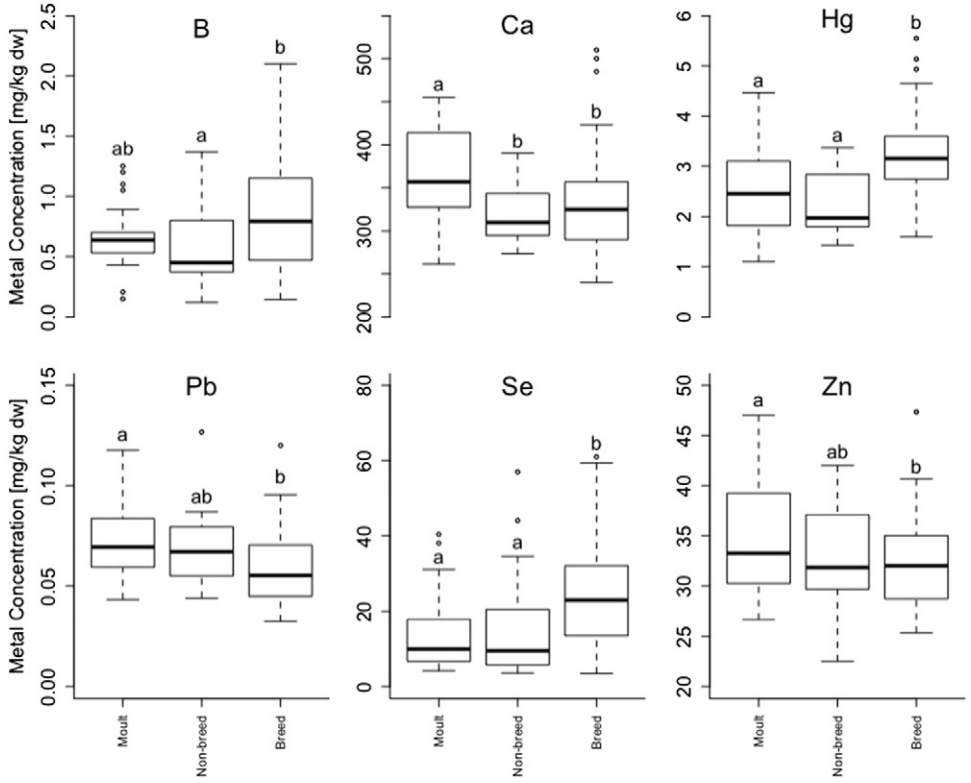

Fig. 9. Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at St Kilda that were significantly different (lower case letters, Tukey HSD, $p < 0.05$) in three distinct seasons (moult, non-breeding and breeding). Data shown are median values with lower (Q1) and upper (Q3) 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 (°) are defined as between 1.5 and 3 ∗ IQR.

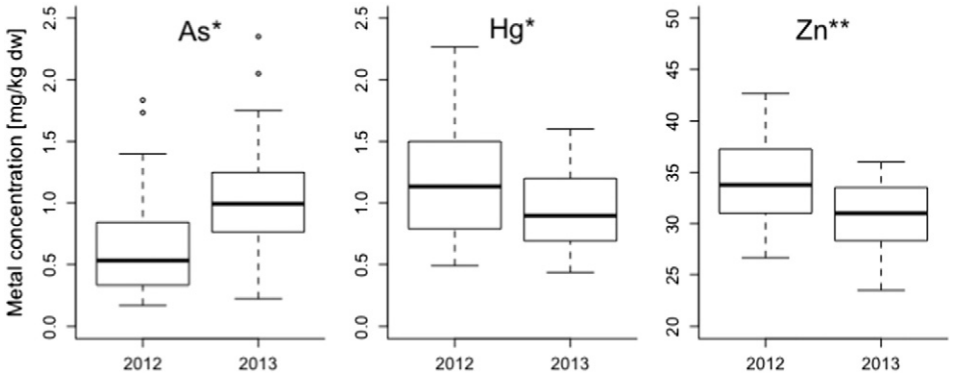

Fig. 10. Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at Phillip Island that were significantly different (univariate ANOVA, $p < 0.025$) between the two years sampled (2012 to 2013). Data shown are median values with lower (Q1) and upper (Q3) quartiles, defined as 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 (°) are defined as between 1.5 and 3*IQR.

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 $\lambda = 0.15$; partial $\eta^2 = 0.61$) and season ($F_{2,67} = 3.92$, $p < 0.001$; Wilk's $\lambda = 0.39$; partial $\eta^2 = 0.37$), 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 (Fig. 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 (Fig. 8 and 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 $d = 0.24$, Fig. 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 $\eta^2 = 0.19$) and season ($F_{2,47} = 22.59$, $p < 0.001$, partial $\eta^2 = 0.31$), but no combined effect year:season ($p > 0.05$, partial $\eta^2 = 0.09$) at St Kilda.

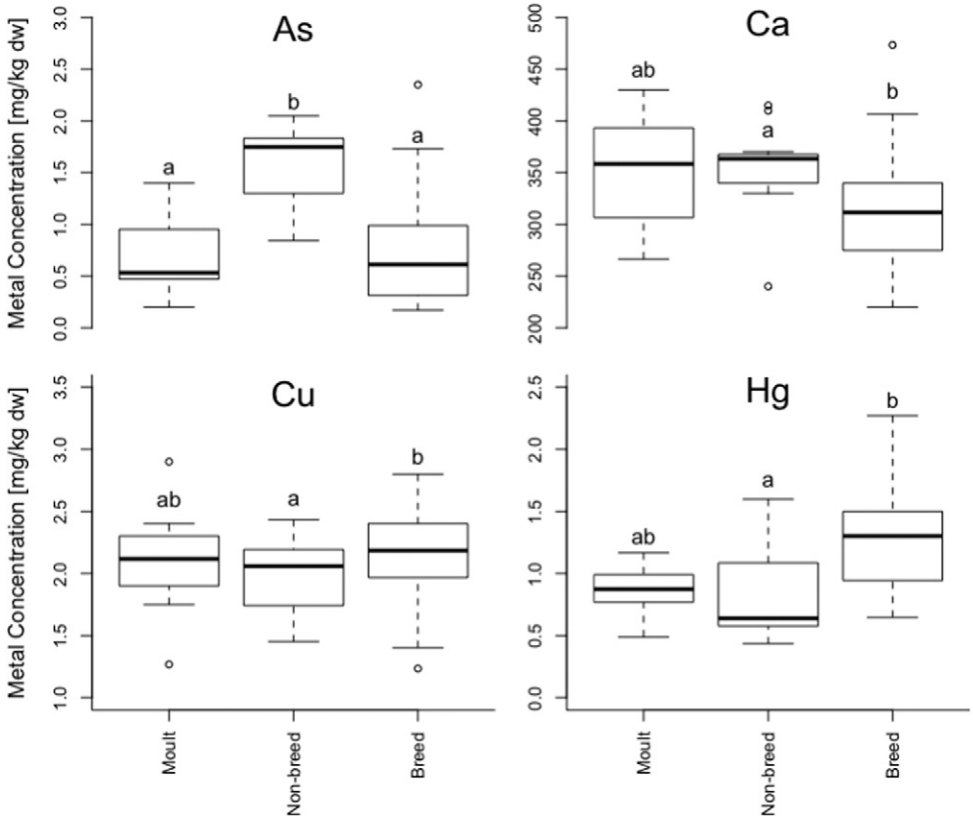

Fig. 11. Metal concentrations (mg/kg dry weight) in whole blood of Little Penguins sampled at Phillip Island that were significantly different (lower case letters, Tukey HSD, $p < 0.05$) in three distinct seasons (moult, non-breeding and breeding). Data shown are median values with lower (Q1) and upper (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 (°) are defined as between 1.5 and 3 ∗ IQR.

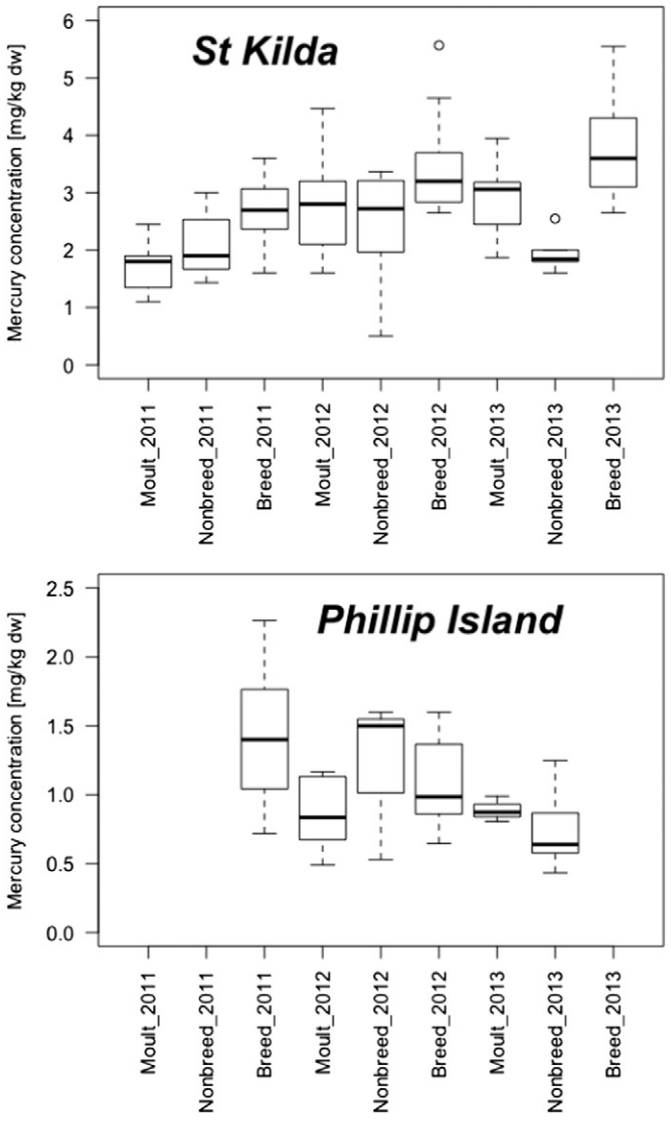

Fig. 12. Mercury concentrations (mg/kg dw) in blood of Little Penguins, sampled from March 2011 to December 2013 at St Kilda and from November 2011 to May 2013 at Phillip Island.

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 $\lambda = 0.39$; partial $\eta^2 = 0.61$) and season ($F_{2,29} = 4.36$, $p < 0.001$; Wilk's $\lambda = 0.12$; partial $\eta^2 = 0.65$). One-way MANOVA found an overall significant effect for sex at Phillip Island ($F_{1,30} = 2.59$, $p < 0.05$; Wilk's $\lambda = 0.49$; partial $\eta^2 = 0.52$), however, pairwise *t*-tests with "Holm" correction found no metals or metalloids were significantly different by sex. NMDS analyses plots are presented in Fig. 7 (right panels). 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 (Fig. 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 (Fig. 11). Over the two years of sampling at Phillip Island, penguins carried decreasing concentrations of Hg ($t = -3.44$, $p < 0.01$, Cohen's $d = -0.60$) (Fig. 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 $\eta^2 = 0.26$), but not for year or year:season interaction (both $p > 0.05$, partial $\eta^2 = 0.12$ and 0.05, respectively).

4. Discussion

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 (Fig. 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 and 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 (Fig. 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 and 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 $\eta^2 = 0.03$). 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.

4.2. Seasonal variation

Overall, the seasonal variation of blood metal concentrations was different at St Kilda and Phillip Island (Fig. 7, B panels). Blood Hg concentrations, however, followed a distinct seasonal pattern at both locations: Non-breeding < Moulting < Breeding (Figs. 9 and 11). This pattern is also visible during each individual sampling year at both locations (Fig. 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 (Fig. 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 Fig. 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 and Gaskin, 1987; Monteiro and Furness, 2001a and 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 (Fig. 11). Of those elements, As displayed the most pronounced variation between seasons. The data are influenced by low sample numbers (Table 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 and Perrins, 2010). While inter-seasonal variation of Ca at St Kilda (Moult > Non-breeding ~ Breeding, Fig. 9) was different to Phillip Island (Moult ~ Non-breeding > Breeding, Fig. 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 and 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 and 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 (Fig. 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.

4.3. Inter-annual variation

We found a trend of increasing blood mercury concentrations in Little Penguins nesting at St Kilda from 2011 to 2013 (Fig. 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 and Lannig, 2008; Millero et al., 2009), but it is unlikely that this alone could have a major effect over such a short timeframe. 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 (Fig. 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.

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.

Acknowledgements

Victoria University for a Return-To-Study-Scholarship to AF, Holsworth Wildlife Research Endowment, Port Phillip EcoCentre, Phillip Island Nature Parks and Birds Australia generously provided funding. Thanks are due to the National Measurement Institute (NMI) for assisting with the metal analyses and to S. Tzardis (NMI) for his expert advice. Research was conducted under scientific permits issued by the Victorian Department of Environment and Primary Industries (10005200) and Victoria University's Animal Experimentation Ethics Committee (permit 05/09). Parks Victoria kindly granted permission to work along the St Kilda breakwater. We gratefully received in-kind support from N. Blake, M. Booth, the late S. Caarels, M. Cowling, A. Fabijanska, N. Filby, P. Guay, T. Haase, Z. Hogg, N. Kowalczyk, T. Preston, L. Renwick, B. Robertson, C. Wallis, P. Wasiak and various volunteers. We thank the anonymous reviewer who improved a previous draft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.06.055.

References

ABS, 2011. Census QuickStats Greater Melbourne. Australian Bureau of Statistics retrieved from http://www.censusdata.abs.gov.au.
Aly, W., Williams, I.D., Hudson, M.D., 2013. Metal contamination in water, sediment and biota from a semi-enclosed coastal area. Environ. Monit. Assess. 185, 3879–3895. http://dx.doi.org/10.1007/s10661-012-2837-0.
Arnould, J.P.Y., Dann, P., Cullen, J.M., 2004. Determining the sex of Little Penguins (*Eudyptula minor*) in northern Bass Strait using morphometric measurements. Emu 104, 261–265.
Bearhop, S., Ruxton, G.D., Furness, R.W., 2000. Dynamics of mercury in blood and feathers of great skuas. Environ. Toxicol. Chem. 19 (6), 1638–1643. http://dx.doi.org/10.1002/etc.5620190622.
Becker, P.H., 2003. Biomonitoring with Birds. In: Markert, B.A., Breure, A.M., Zechmeister, H.G. (Eds.), Bioindicators and biomonitors. Elsevier, San Diego.
Bond, A.L., Hobson, K.A., Branfireun, B.A., 2015. Rapidly increasing methyl mercury in endangered ivory gull (*Pagophila eburnea*) feathers over a 130 year record. Proc. R. Soc. Lond. B 282, 20150032.
Braune, B.M., Gaskin, D.E., 1987. A mercury budget for the Bonaparte's Gull during autumn moult. Ornis Scand. 18 (4), 245.
Burger, J., 1993. Metals in avian feathers: bioindicators of environmental pollution. Rev. Environ. Toxicol. 5, 203–311.
Carravieri, A., Cherel, Y., Blévin, P., Brault-Favrou, M., Chastel, O., Bustamante, P., 2014. Mercury exposure in a large subantarctic avian community. Environ. Pollut. 190, 51–57.
Carravieri, A., Cherel, Y., Jaeger, A., Churlaud, C., Bustamante, P., 2016. Penguins as bioindicators of mercury contamination in the southern Indian Ocean: geographical and temporal trends. Environ. Pollut. 213, 195–205.
Carvalho, P.C., Bugoni, L., McGill, R.A., Bianchini, A., 2013. Metal and selenium concentrations in blood and feathers of petrels of the genus *Procellaria*. Environ. Toxicol. Chem. 32, 1641–1648.
Chiaradia, A., Forero, M.G., Hobson, K.A., Cullen, J.M., 2010. Changes in diet and trophic position of a top predator 10 years after a mass mortality of a key prey. ICES J. Mar. Sci. 67 (8), 1710–1720.
Chiaradia, A., Forero, M.G., Hobson, K.A., Swearer, S.E., Hume, F., Renwick, L., Dann, P., 2012. Diet segregation between two colonies of little penguins *Eudyptula minor* in southeast Australia. Austral Ecol. 37, 610–619.
Cohen, J., 1988. Statistical Power Analysis for the Behavioral Sciences. second ed. Erlbaum, Hillsdale, NJ.
Collins, M., Cullen, J.M., Dann, P., 1999. Seasonal and annual foraging movements of little penguins from Phillip Island, Victoria. Wildl. Res. 26, 705–721.
R Core Team, 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (URL https://www.R-project.org/).
Del Re, A.C., 2013. compute.es: Compute Effect Sizes. R package version 0.2-2. (URL http://cran.r-project.org/web/packages/compute.es).
Dunlop, J.N., McNeill, S., Cannell, B., 2013. Seabird Feathers as Indicators of Mercury & Selenium Contamination in the Coastal Waters of South Western Australia. Conservation Council of Western Australia.
Eagles-Smith, C.A., Ackerman, J.T., Adelsbach, T.L., Takekawa, J.Y., Miles, A.K., Keister, R.A., 2008. Mercury correlations among six tissues for four waterbird species breeding in San Francisco Bay, California, USA. Environ. Toxicol. Chem. 27 (10), 2136–2153.
Edge, K., Dafforn, K., Simpson, S., Ringwood, A., Johnston, E., 2015. Resuspended contaminated sediments cause sublethal stress to oysters: A biomarker differentiates total suspended solids and contaminant effects. Environ. Toxicol. Chem. 34, 1345.
EPA, 2007. Yarra and Maribyrnong Estuaries: Investigation of Contamination in Fish. Publication 1094EPA Victoria, Melbourne, Australia.
EPA, 2009. Lower Yarra Fish Study: Investigation of Contaminants in Fish. Publication 1283EPA Victoria, Melbourne, Australia.
EPA, 2013. The Origin, Fate and Dispersion of Toxicants in the Lower Sections of the Yarra River. EPA Victoria, Carlton, Victoria, Australia.
EPA, 2015. Marine data for Hobsons Bay and Newport. Applied Science Front Desk. EPA Victoria.
Evers, D.C., Kaplan, J.D., Meyer, M.W., Reaman, P.S., Braselton, W.E., Major, A., Burgess, N., Scheuhammer, A.M., 1998. Geographic trend in mercury measured in common loon feathers and blood. Environ. Toxicol. Chem. 17, 173–183.
Evers, D.C., Savoy, L.J., DeSorbo, C.R., Yates, D.E., Hanson, W., Taylor, K.M., Siegel, L.S., Cooley Jr., J.H., Bank, M.S., Major, A., 2008. Adverse effects from environmental mercury loads on breeding common loons. Ecotoxicology 17, 69–81.
Fabris, G.J., Monahan, C.A., Batley, G.E., 1999. Heavy metals in waters and sediments of Port Phillip Bay, Australia. Mar. Freshw. Res. 50 (6), 503–513.
Fetters, K.J., Costello, D.M., Hammerschmidt, C.R., Burton, G.A., 2015. Toxicological effects of short-term resuspension of metal-contaminated freshwater and marine sediments. Environ. Toxicol. Chem. 35, 676–686.
Finger, A., Lavers, J.L., Dann, P., Nugegoda, D., Orbell, J.D., Robertson, B., Scarpaci, C., 2015. The Little Penguin (*Eudyptula minor*) as an indicator of coastal trace metal pollution. Environ. Pollut. 205, 365–377.
Fukushima, K., Saino, T., Kodama, Y., 1992. Trace metal contamination in Tokyo bay, Japan. Sci. Total Environ. 125, 373-–389.
Harada, M., 1995. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. Crit. Rev. Toxicol. 25, 1–24.
Harrell Jr., F.E., Dupont, M., 2013. Hmisc: Harrell Miscellaneous R package version 3 pp. 10–11.
Harris, G., Batlety, G., Fox, D., Hall, D., Jernakoff, P., Molloy, R., Murray, A., Newell, B., Parslow, J., Skyring, G., Walker, S., 1996. Port Phillip Bay Environmental Study: Final Report. CSIRO, Canberra, Australia.
Hedge, L., Knott, N., Johnston, E., 2009. Dredging related metal bioaccumulation in oysters. Mar. Pollut. Bull. 58, 832–840.

Hirst, A.J., White, C.A., Green, C., Werner, G.F., Heislers, S., Spooner, D., 2011. Baywide Anchovy Study Sub-program. Milestone Report No. 4. Technical Report No. 150Department of Primary Industries, Queenscliff, Victoria, Australia.
Hirst, A.J., White, C.A., Heislers, S., Werner, G.F., Spooner, D., 2010. Baywide Anchovy Study Sub-program. Milestone Report No. 3. Technical Report No. 114Department of Primary Industries, Queenscliff, Victoria, Australia.
Kowalczyk, N.D., Chiaradia, A., Preston, T.J., Reina, R.D., 2015b. Fine-scale dietary changes between the breeding and non-breeding diet of a resident seabird. 2. Royal Society Open Science, p. 140291.
Kowalczyk, N., Reina, R., Preston, T., Chiaradia, A., 2015a. Selective foraging within estuarine plume fronts by an inshore resident seabird. Front. Mar. Sci. 2, 42.
Kowalczyk, N.D., Reina, R.D., Preston, T.J., Chiaradia, A., 2015b. Environmental variability drives shifts in the foraging behaviour and reproductive success of an inshore seabird. Oecologia 178 (4), 967–979. http://dx.doi.org/10.1007/s00442-015-3294-6.
Kowalczyk, N.D., Chiaradia, A., Preston, T.J., Reina, R.D., 2013. Linking dietary shifts and reproductive failure in seabirds: a stable isotope approach. Funct. Ecol. 28 (3), 755–765.
Lamborg, C.H., Hammerschmidt, C.R., Bowman, K.L., Swarr, G.J., Munson, K.M., Ohnemus, D.C., Lam, P.J., Heimbürger, L.-E., Rijkenberg, M.J., Saito, M.A., 2014. A global ocean inventory of anthropogenic mercury based on water column measurements. Nature 512, 65–68.
Lavers, J.L., Bond, A.L., Hutton, I., 2014. Plastic ingestion by flesh-footed shearwaters (*Puffinus carneipes*): implications for fledgling body condition and the accumulation of plastic-derived chemicals. Environ. Pollut. 187, 124–129.
Lavoie, R.A., Jardine, T.D., Chumchal, M.M., Kidd, K.A., Campbell, L.M., 2013. Biomagnification of mercury in aquatic food webs: a worldwide meta-analysis. Environ. Sci. Technol. 47, 13385–13394.
Logan, M., 2011. Biostatistical Design and Analysis Using R: A Practical Guide. John Wiley & Sons.
McCutcheon, C., Dann, P., Salton, M., Renwick, L., Hoskins, A., Gormley, A., Arnould, J., 2011. The foraging range of Little Penguins (*Eudyptula minor*) during winter. Emu 111, 321–329.
Millero, F.J., Woosley, R., DiTrolio, B., Waters, J., 2009. Effect of ocean acidification on the speciation of metals in seawater. Oceanography 22 (4), 20–33.
Monteiro, L.R., Furness, R.W., 2001a. Kinetics, dose-response, and excretion of methylmercury in free-living adult Cory's shearwaters. Environ. Sci. Technol. 35, 739–746.
Monteiro, L.R., Furness, R.W., 2001b. Kinetics, dose-response, excretion, and toxicity of methylmercury in free-living Cory's shearwater chicks. Environ. Toxicol. Chem. 20, 1816–1823.
Neira, F.J., Sporcic, M.I., Longmore, A.R., 1999. Biology and fishery of pilchard, *Sardinops sagax* (Clupeidae), within a large south-eastern Australian bay. Mar. Freshw. Res. 50, 43–55.
Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ. Int. 35, 971–986.
Oksanen, J., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Simpson, G., Solymos, P., Stevens, M., Wagner, H., 2013. Vegan: Community Ecology Package (R package ver. 2.0–10).
Olmedo, P., Hernández, A., Pla, A., Femia, P., Navas-Acien, A., Gil, F., 2013. Determination of essential elements (copper, manganese, selenium and zinc) in fish and shellfish samples. Risk and nutritional assessment and mercury–selenium balance. Food Chem. Toxicol. 62, 299–307.
Orians, G.H., Pearson, N.E., 1979. On the Theory of Central Place Foraging. Analysis of Ecological SystemsOhio State University Press, Columbus, pp. 155–177.
Overeem, R.L., Peucker, A.J., Austin, C.M., Dann, P., Burridge, C.P., 2008. Contrasting genetic structuring between colonies of the world's smallest penguin, *Eudyptula minor* (Aves: Spheniscidae). Conserv. Genet. 9, 893–905.
Phillips, D., Richardson, B., Murray, A., Fabris, J., 1992. Trace metals, organochlorines and hydrocarbons in Port Phillip Bay, Victoria: a historical review. Mar. Pollut. Bull. 25, 200–217.
PoMC, 2010. EMP Close-out Report — February 2010. Port of Melbourne Corporation, Melbourne.
Preston, T.J., Ropert-Coudert, Y., Kato, A., Chiaradia, A., Kirkwood, R., Dann, P., 2008. Foraging behaviour of little penguins *Eudyptula minor* in an artificially modified environment. Endanger. Species Res. 1 (4), 95–103.
Quinn, G.P., Keough, M.J., 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press.
Reilly, P.N., Cullen, J.M., 1981. The Little Penguin *Eudyptula minor* in Victoria, II: breeding. Emu 81 (1), 1–19. http://dx.doi.org/10.1071/MU9810001.
Reilly, P.N., Cullen, J.M., 1983. The Little Penguin *Eudyptula minor* in Victoria, IV: moult. Emu 83 (2), 94–98. http://dx.doi.org/10.1071/MU9830094.
Reynolds, S.J., Perrins, C.M., 2010. Dietary calcium availability and reproduction in birds. Current Ornithology 17. Springer, New York, pp. 31–74.
Robinson, S.A., Lajeunesse, M.J., Forbes, M.R., 2012. Sex differences in mercury contamination of birds: testing multiple hypotheses with meta-analysis. Environ. Sci. Technol. 46, 7094–7101.
Shaw, T.R., 2008. Sexual Differences in Diet of Little Penguins (*Eudyptula minor*) Dissertation (MSc) University of Pretoria.
Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. (CR Special 18).
Spahn, S., Sherry, T., 1999. Cadmium and lead exposure associated with reduced growth rates, poorer fledging success of little blue heron chicks (*Egretta caerulea*) in south Louisiana wetlands. Arch. Environ. Contam. Toxicol. 37, 377–384.
Spalding, M.G., Frederick, P.C., McGill, H.C., Bouton, S.N., McDowell, L.R., 2000. Methylmercury accumulation in tissues and its effects on growth and appetite in captive great egrets. J. Wildl. Dis. 36, 411–422.
Sutherland, D.R., Dann, P., 2012. Improving the accuracy of population size estimates for burrow-nesting seabirds. Ibis 154 (3), 488–498. http://dx.doi.org/10.1111/j.1474-919X.2012.01234.x.
Tartu, S., Angelier, F., Wingfield, J.C., Bustamante, P., Labadie, P., Budzinski, H., Weimerskirch, H., Bustnes, J.O., Chastel, O., 2015. Corticosterone, prolactin and egg neglect behavior in relation to mercury and legacy POPs in a long-lived Antarctic bird. Sci. Total Environ. 505, 180–188.
Tartu, S., Goutte, A., Bustamante, P., Angelier, F., Moe, B., Clément-Chastel, C., et al., 2013. To breed or not to breed: endocrine response to mercury contamination by an Arctic seabird. Biol. Lett. 9 (4). http://dx.doi.org/10.1098/rsbl.2013.0317.
Walker, T.I., 1982. Effects of length and locality on the mercury content of blacklip abalone, *Notohaliotis ruber* (Leach), blue mussel, *Mytilus edulis planulatus* (Lamarck), sand flathead, *Platycephalus bassensis* Cuvier & Valenciennes, and long-nosed flathead, *Platycephalus caeruleopunctatus* (McCulloch), from Port Phillip Bay, Victoria. Mar. Freshw. Res. 33 (3), 553–560.
Walker, T.I., 1988. Mercury concentrations in edible tissues of elasmobranchs, teleosts, crustaceans and molluscs from south-eastern Australian waters. Mar. Freshw. Res. 39, 39–49.
Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer Science & Business Media.
Yamashita, Y., Amlund, H., Suzuki, T., Hara, T., Hossain, M.A., Yabu, T., Touhata, K., Yamashita, M., 2011. Selenoneine, total selenium, and total mercury content in the muscle of fishes. Fish. Sci. 77, 679–686.