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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.

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,

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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 1300 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., 2014) 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.

2. Materials and methods

2.1. Study site and sample collection

All samples were collected within Port Phillip Bay, south-eastern Australia (Fig. 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 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., 2014, 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 (Fig. 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 min. 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 h of sampling.

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 60 °C and then digested in 65% nitric acid (SUPRAPUR, trace metal grade, Merck) and 37% hydrochloric acid (EMSURE, trace metal grade, Merck) at 95 °C. 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%).

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

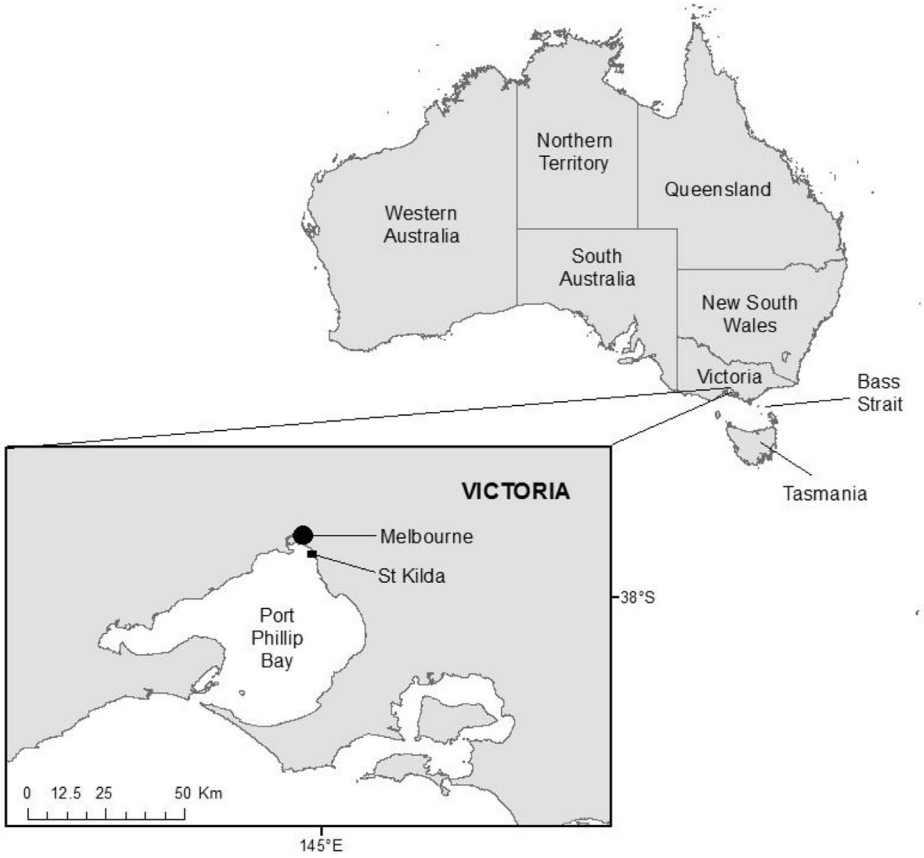

Fig. 1. Little Penguins were sampled at St Kilda from 2011 to 2013.

Table 1
Details of fish, Little Penguin blood and faeces samples collected in Port Philip Bay during 2011–2013.

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	10	31/12/2013	12/12/2013	6	4
		2. Sandy sprat	10				
		3. Pilchard-2 (young adult)	10				

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).

2.3. Stable isotope data

To adjust blood Hg concentrations for dietary shifts, $\partial^{15}N$ 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, see Kowalczyk et al., 2014). Blood Hg concentrations were corrected for any dietary (trophic) influence (Braune, 2007):

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

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.

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.

3. Results

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 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 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 2), these were pooled in the nMDS plots (Fig. 2), as their metal(loid) concentrations within the same species between events were not distinctly different (Fig. 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 2). Average Se concentrations in whole fish were two to three-fold higher in sandy sprat and pilchard than in anchovy (Table 2). These differences are reflected in the NMDS analysis (Fig. 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 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 3).

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

Table 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 Fig. 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 ($r_t = 0.43$, $t_{39} = 3.84$, $p < 0.001$, Fig. 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).

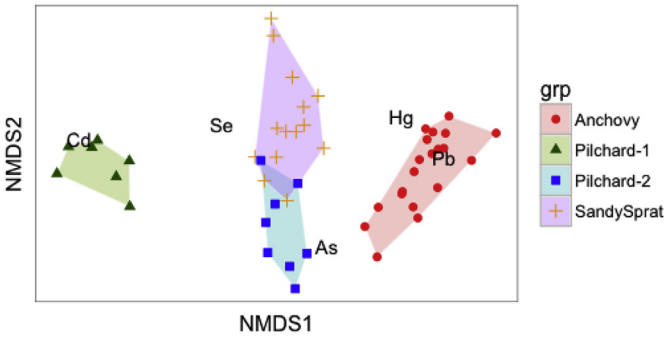

Fig. 2. Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin fish prey items (stress = 0.09). Polygon ellipse lines are drawn for each fish prey species: Anchovy (red full circle), Pilchard-1 (juvenile, green full triangle), Pilchard-2 (young adult, blue full square), Sandy Sprat (orange cross). Trace elements are displayed by their periodic symbols: As, Cd, Hg, Pb and Se. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 (Fig. 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 Fig. 5. After adjusting for trophic shifts, median Hg concentrations were lower in January 2012 and unchanged in

Table 2
Morphometric data and metal(loid) concentrations in whole fish [mg/kg dry weight] collected from Port Philip Bay during 2011–2013, 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 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). Significance ($p < 0.05$) is indicated by bold script.

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 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]

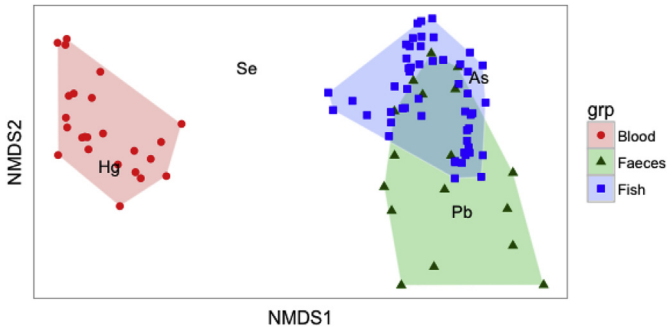

Fig. 3. Two-dimensional NMDS plots with Bray-Curtis distance for Little Penguin blood, faeces and fish prey items (all fish species pooled) for the four events (stress = 0.04). Polygon ellipse lines are drawn for each matrix: Blood (red full circle), Faeces (green full triangle), Fish (blue full square). 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

September and November 2012, however, variation in November 2012 increased (Fig. 5 right panel).

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 Tweeds Head, New South Wales and Oyster Harbour, Western Australia, Australia, were

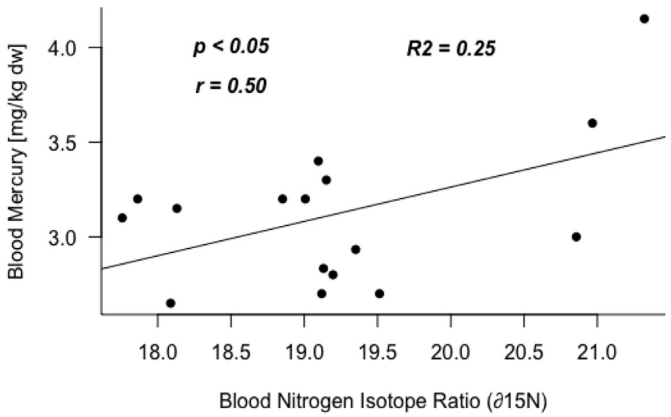

Fig. 4. Relationship between Little Penguin blood mercury concentrations and blood $\partial^{15}N$ values (paired samples, n = 16), collected in January, September and November of 2012 at St Kilda.

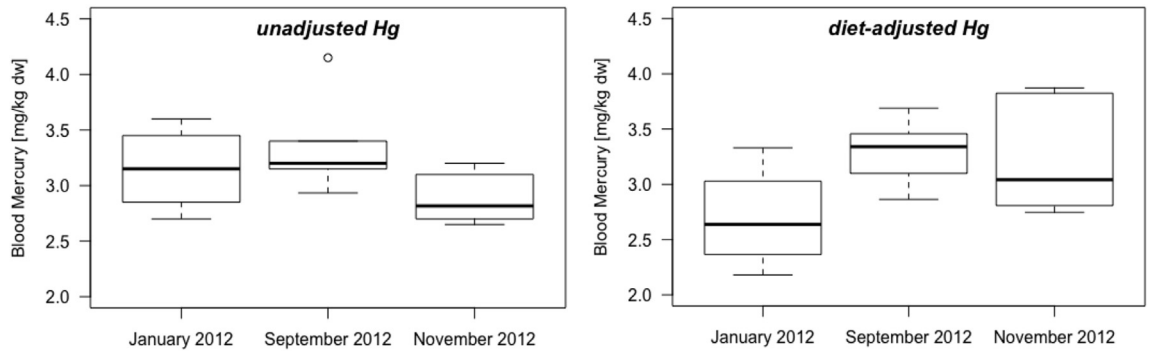

Fig. 5. Little Penguin blood mercury concentrations; unadjusted (left) and adjusted (right) for diet. Samples were collected at St Kilda in January 2012 (n = 4), September 2012 (n = 6) and November 2012 (n = 6).

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 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 (Fig. 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., 2014).

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., 2014, 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 (Fig. 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 (Fig. 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 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 (2011–2013) 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 $\partial^{15}N$ 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 ($\partial^{15}N$) 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. 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 resuspension of contaminated sediments (PoMC, 2010), this and previous studies (Finger et al., 2015, 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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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.01.059.

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