Environmental Advances 7 (2022) 100177

Contents lists available at ScienceDirect

Environmental Advances

journal homepage: www.sciencedirect.com/journal/environmental-advances

Links between elevated zinc levels, diet and muscle melanisation in sand flathead (*Platycephalus bassensis*) from polluted estuaries

Chun Kit Ooi [a,*], James Haddy [b], Barbara Nowak [b], Jeremy Lyle [c], Yonglin Mai [a], Hamish McLean [a], Trevor Lewis [a]

[a] School of Natural Sciences (Chemistry), University of Tasmania, Locked Bag 1371, Launceston, Tasmania 7250, Australia
[b] Institute for Marine and Antarctic Studies - Launceston, University of Tasmania, Private Bag 1370, Launceston, Tasmania 7250, Australia
[c] Institute for Marine and Antarctic Studies - Taroona, University of Tasmania, Private Bag 49, Hobart, Tasmania 7001, Australia

ARTICLE INFO

Keywords:
Melanisation
Black spot
Fish muscle
Sand flathead
Whole crab
Zinc

ABSTRACT

Muscle melanisation in sand flathead is defined as the presence of abnormal black spots in the fish's flesh and the phenomenon has been associated with Zn pollution in the environment. In this study, Zn levels in the fish muscle and crabs were analysed to investigate the role of Zn in causing muscle melanisation and the fish's uptake of Zn via its diet, crabs. An improved technique for extracting melanised fish muscle from fillets with black spots was developed. It enabled Zn levels in melanised muscle to be more accurately determined when compared to previous study and showed Zn levels were 2.1–3.5 times higher in melanised regions of muscle than non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation. This indicated elevated levels of Zn were localised in the melanised muscle. Muscle melanisation was potentially caused by Zn ions binding to the active site of tyrosinase, an enzyme involved in melanin production, likely facilitated by the presence of higher Zn than Cu in the polluted estuaries. A digestion reagent mixture suitable for microwave assisted acid digestion and analysis of Zn in the whole crab was developed using a mixture of HNO_3 and H_2O_2. Zn levels in the whole crab were 1.5 times higher in 50% of the crab species studied from a polluted estuary compared to the same species from an unpolluted estuary. As sand flathead primarily feed on crabs, the fish's diet was a likely source of elevated Zn for the fish. Overall, this study has described new techniques useful for the study of melanised fish and heavy metal levels in the whole crab. The findings from this study also provided insights that will inform and guide the direction of future research on muscle melanisation in sand flathead.

1. Introduction

Muscle melanisation in fish is defined as abnormal black spots in the flesh, which occurs as sporadic spots or more severely as intensely darkened flesh (Ooi et al., 2022; Ramos et al., 2019). It is a complex phenomenon that affects various fish species across the globe and it can be caused by various factors such as parasitic infection (Esteves et al., 2009; Ramos et al., 2019), viral infection (Bjorgen et al., 2019; Bjorgen et al., 2015; Malik et al., 2021), vaccination (Koppang et al., 2005) and higher levels of Cu in the feed (Cooper and Midling, 2006; Cooper et al., 2011). At the island state of Tasmania, Australia, recreational fishers have been reporting muscle melanisation in the wild sand flathead (*Platycephalus bassensis*) (Stocker et al., 2019). This is a worrying development for the local recreational fishing community as sand flathead represent the most commonly caught finfish species (Lyle et al., 2019; Stocker et al., 2019) and have high recreational value in the state (Frijlink and Lyle, 2013; Jordan, 2001; Yamazaki et al., 2013). Previous study reported that no parasites were found in the melanised muscle (Ooi et al., 2019) and as a wild fish species, sand flathead are not subjected to vaccination. On the other hand, melanised muscle in sand flathead contained higher levels of many heavy metals, with Zn being the only metal that was consistently significantly higher (Ooi, 2016; Ooi et al., 2019). The cause of muscle melanisation in sand flathead is not well understood, but it has been suggested to be associated with environmental pollution (Ooi et al., 2019). This is because sand flathead affected by muscle melanisation are commonly found in Tasmanian estuaries polluted by heavy metals, notably Zn (Ooi et al., 2022; Ooi et al., 2019). This includes the Tamar Estuary (Aquenal Pty Ltd and

* Corresponding author.
E-mail addresses: ck.ooi@utas.edu.au (C.K. Ooi), james.haddy@utas.edu.au (J. Haddy), b.nowak@utas.edu.au (B. Nowak), jeremy.lyle@utas.edu.au (J. Lyle), yonglin.mai@utas.edu.au (Y. Mai), hamish.mclean@utas.edu.au (H. McLean), trevor.lewis@utas.edu.au (T. Lewis).

https://doi.org/10.1016/j.envadv.2022.100177
Received 12 January 2022; Received in revised form 18 January 2022; Accepted 20 January 2022
Available online 21 January 2022

DEPHA, 2008) and one of the most heavily polluted estuaries in the world, the Derwent Estuary (Bloom and Ayling, 1977; Coughanowr et al., 2015). Despite being highly contaminated, the Derwent Estuary has not been included in previous muscle melanisation studies.

A standardised muscle melanisation scoring system was developed to rank the severity of melanisation in sand flathead fillets based on scores 0, 1, 2 and 3 to indicate increasing severity of muscle melanisation (Ooi et al., 2022). In general, it is feasible to extract sufficient melanised muscle from fish with more severe muscle melanisation (melanisation scores 2 and 3) for heavy metal analysis, but there is difficulty in ensuring melanised regions of muscle sampled for analysis accurately represented the black spots on fish muscle (Ooi, 2016). This is because the melanised muscle sampled usually have some non-melanised muscle included, thus affecting sample homogeneity and potentially lowering the accuracy of heavy metal analysis results (Ooi, 2016). On the other hand, the majority of sand flathead affected by muscle melanisation have a minor level of melanisation (melanisation score 1) (Stocker et al., 2019) and it has been difficult to extract sufficient melanised muscle from these fish for analysis. As a result, studies on melanised sand flathead muscle are affected by the use of an inadequate extraction technique and it highlighted the need to develop an improved technique for extracting melanised muscle. To develop an improved extraction technique, the utilisation of magnifying and cutting tools that would provide better accuracy and precision were proposed (Ooi 2016) and will be examined in this study.

Fish living in polluted environment can accumulate heavy metals from their surroundings, including from the water, diet and sediment (Chen and Chen, 1999; Jezierska and Witeska, 2006; Nakayama et al., 2010; Rajotte et al., 2003). The non-migratory benthic feeding sand flathead are usually in contact with the benthic waters and surface sediments (Ayling et al., 1975). In the past, high levels of Zn were reported in the waters and sediments of the Tamar and the Derwent Estuary. However, Zn levels in the benthic waters have declined to levels below the 2000 Australian and New Zealand Environment and Conservation Council (ANZECC) guideline, which is 15 μg/L of Zn in waters at 95% protection level (Aquenal Pty Ltd and DEPHA, 2008; Taylor et al., 2020). Although Zn levels in the surface sediments also declined over the years, the levels have remained higher than the 2000 ANZECC's Interim Sediment Quality Guideline high trigger value of 410 mg/kg for Zn, which indicates high probability of biological effects (Aquenal Pty Ltd and DEPHA, 2008; Taylor et al., 2020). Still, there was limited evidence of a direct relationship between Zn in the sediments and the sand flathead (Hunt, 2008). Alternatively, the accumulation of heavy metals from diet is significant for fish (Spry et al., 1998) and this has also been reported for the sand flathead (Hunt, 2008). Sand flathead are opportunistic feeder (Verdouw et al., 2010) that have a diet that include fish and crustaceans (Ayling et al., 1975), especially crabs (Hunt, 2008). Crabs are known to accumulate heavy metals such as Zn from sediments (Weimin et al., 1994) and the accumulated levels can be influenced by their surrounding environment (Al-Mohanna and Subrahmanyam, 2001; Rainbow et al., 1990). Potential links between sediments polluted by Zn, crabs that accumulate Zn and sand flathead that primarily feed on crabs, may explain the elevated levels of Zn found in the sand flathead. However, heavy metal levels in crabs are usually determined in the muscle, which is dissected prior to digestion and metal analysis (Genc and Yilmaz, 2017). This is a useful method to study crabs in the context of human health but it is not representative of the heavy metal levels consumed by fish since the levels of metal can differ between different crab organs (Chan and Rainbow, 1993; Reinecke et al., 2003; Snyman et al., 2015; Turoczy et al., 2001) and sand flathead eat crabs whole. As there is limited literature on digesting the whole crab for analysis, there is a need to develop an appropriate digestion reagent mixture and method for this purpose.

The main aim of this study was to investigate muscle melanisation in sand flathead and the uptake of Zn via the fish's diet. To study muscle melanisation in sand flathead, Zn levels in melanised and non-melanised regions of muscle were determined and an improved technique for extracting melanised muscle was developed. Zn levels in muscle of sand flathead unaffected by melanisation were also determined to establish the baseline level of Zn in the fish. Zn levels in crabs were studied as they are a potential source of Zn for the fish, thus a reagent mixture and method suitable for digesting the whole crab for analysis of Zn was developed. Based on the results, the advantages of the newly developed techniques, cause of muscle melanisation and role of diet (crabs) in the accumulation of Zn in sand flathead were examined and discussed.

2. Materials and methods

2.1. Sample collection

Sand flathead were collected using hook and line from two northern Tasmania estuaries, the Tamar Estuary (9 sites) and Port Sorell (1 site) (Fig. 1A). Each fish was euthanised immediately upon capture by blunt force trauma to the head as per University of Tasmania (UTAS) Animal Ethics Committee approval (A0017197) and placed into a saltwater ice slurry until return to the laboratory. Sand flathead from south-eastern and eastern Tasmania estuaries were supplied by Nyrstar Hobart and the Institute for Marine and Antarctic Studies, UTAS. This included sand flathead from the Derwent Estuary (8 sites), Norfolk-Frederick Henry Bay, D'Entrecasteaux Channel, and Great Oyster Bay (Fig. 1A). Similar to previous study, Great Oyster Bay was selected as the control site as it is a relatively clean and unpolluted area (Ooi et al. 2019). The collection period and number of samples from each site are provided in Table S1.

To confirm the high abundance of crabs in the fish's food intake, 60 sand flathead collected from the two northern Tasmania estuaries were chosen using random selection procedure for gut analysis, where 31 of them had content in their stomach and showed crabs were most commonly found (~38%) when compared to other prey such as shrimp (~8%), small fish (~5%) and isopod (~2%). The Deceitful Cove, located within the Tamar Estuary, is heavily polluted by Zn in the sediments due to effluents from nearby industries in the past, whereas Port Sorell is relatively unpolluted with low population density and limited industrial activity (Aquenal Pty Ltd and DEPHA, 2008; Mondon, 2000; Mondon et al., 2000; Mondon et al., 2001; Ooi et al., 2022). The two sites were chosen for crabs sampling as they are situated close enough to each other (Fig. 1A) to contain the same species of crabs, but with dissimilar levels of environmental pollution. The crabs were collected in a plastic bucket during low tide, then transferred to plastic zip lock bags and placed on ice in an esky, where the crabs were euthanised by chilling. The collection period is provided in Table S2.

2.2. Sample preparation

At the laboratory, the total length (mm), weight (g) and sex were determined for each fish. Fish were then filleted, muscles visually checked for the presence and extent of melanisation, and a melanisation score (i.e., 0, 1, 2 or 3) was assigned to each pair of fillets based on the muscle melanisation scoring system for sand flathead (Ooi et al., 2022; Stocker et al., 2020) (Fig. S1). The measured variables of sand flathead are listed in Table S1. The fillets were kept in individual zip lock bags and frozen at –20 °C for storage.

To prepare the fish samples for heavy metal analysis, each pair of fillets was taken out of the freezer and placed on a white plastic chopping board to thaw. For each pair of fillets with black spots, the melanised muscle was extracted using an improved technique. First, a magnifying glass with stand along with a disposable plastic knife was used to remove a small portion of muscle from the fillet, made up of mostly melanised muscle that included bits of non-melanised muscle. Once removed, the melanised muscle sample was carefully trimmed of non-melanised muscle by using a cover glass (20 × 26 × 0.4 mm) (Paul Marienfeld, Lauda-Königshofen, Germany). To ensure only melanised muscle was included, at least two hours were spent carefully processing

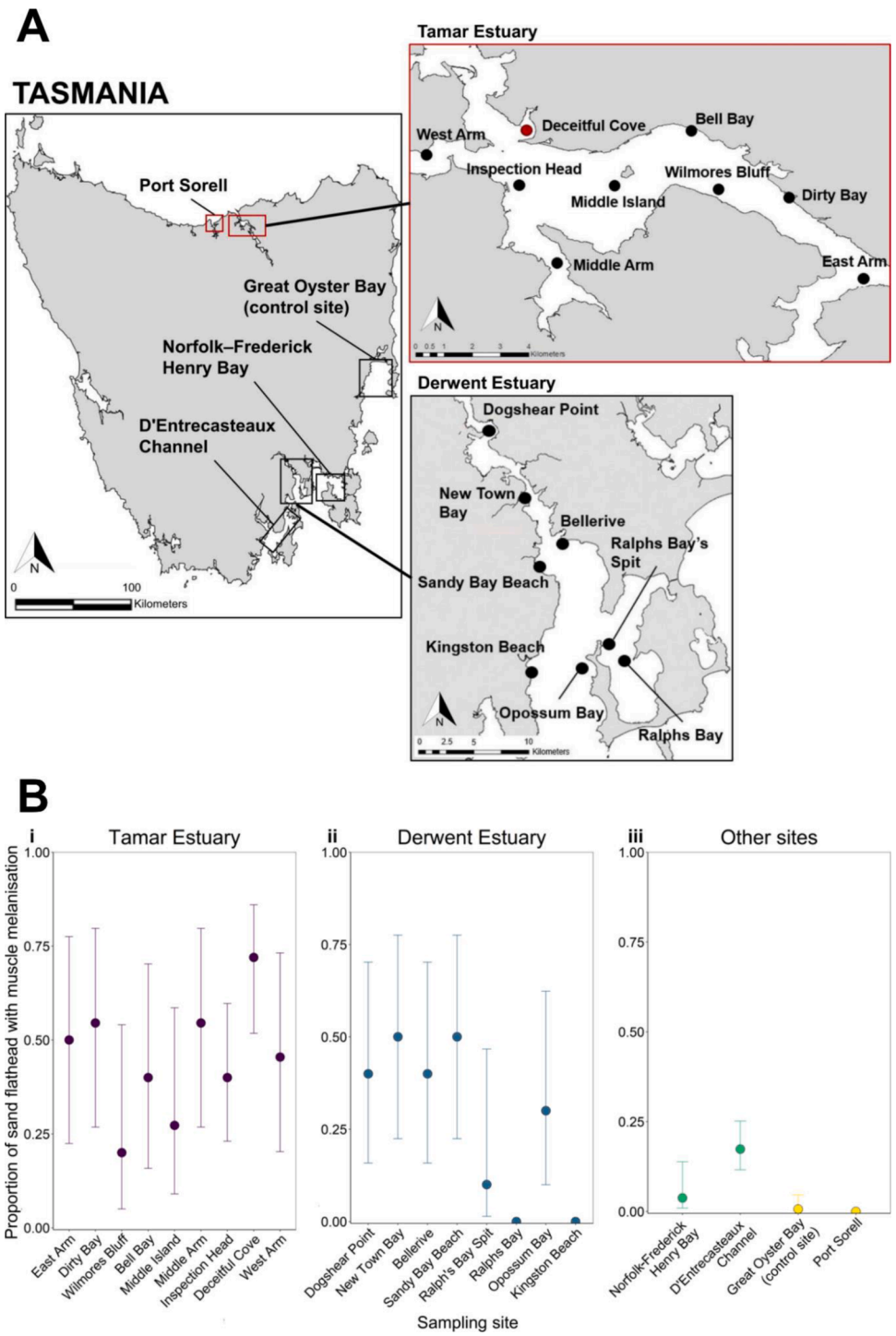

Fig. 1. The sampling sites and proportion of sand flathead with muscle melanisation. (A) The sampling sites for sand flathead at Tasmania, Australia. The estuary systems, the Tamar Estuary and the Derwent Estuary, are enlarged to show the sampling sites within each estuary. Sampling sites where only sand flathead were collected are depicted by black boxes or dots, and sampling sites where both sand flathead and crabs were collected are coloured red. (B) The proportion of sand flathead with muscle melanisation from various sampling sites. The round dot points represent the proportions and include the 95% confidence intervals shown as error bars. Confidence intervals for sampling sites where sand flathead had no muscle melanisation are not plotted.

each pair of melanised fillets and the samples of melanised regions from the same fish were pooled. Melanised muscle samples generally ranged from 0.2–0.7 g (wet weight). For non-melanised regions of muscle in fish with melanisation and fish unaffected by melanisation, around 1.0 g (wet weight) muscle sample was cut from each pair of fillets according to the method described in Ooi et al., (2019) by using a disposable plastic knife.

For the crab samples, the weight (g) was recorded and the species of each crab was identified. The crab species investigated in this study included soldier crab (*Mictyris platycheles*), four-toothed shore crab (*Paragrapsus quadridentatus*), red-spotted shore crab (*Paragrapsus gaimardii*) and mottled shore crab (*Paragrapsus laevis*). Other crab species with insufficient numbers sampled were omitted from the study. The collected data are provided in Table S2. The crabs were placed into their respective species zip lock bags and frozen at –20 °C for storage. To prepare for heavy metal analysis, the crabs were taken out of the freezer, thawed and rinsed with distilled and Milli-Q water to remove any attached sediments.

The fish muscle and whole crab samples were dried at 60°C in an air oven until a constant weight was achieved and ground to powder using a glass pestle and mortar. For each individual fish, around 0.1–0.3 g (dry weight) of ground muscle sample was transferred into individual MARSXpress digestion vessels (CEM, Matthews, NC, USA). For each whole crab, the ground sample was transferred into individual

MARSXpress vessels in full if the total weight was less than 0.5 g (dry weight). Each whole crab with a total weight greater than 0.5 g (dry weight) was thoroughly homogenised via grinding and mixing with the glass pestle and mortar and a plastic spoon, then three technical replicates were prepared. Each replicate consisted of no more than 0.5 g of ground whole crab sample to ensure a clear digest. To check for precision, Zn levels in the technical replicates of 45 ground whole crab samples were determined and the average relative standard deviation (RSD) = 4.23%.

Digestion of samples for heavy metal analysis was largely based on United States Environmental Protection Authority Method 3052 (US EPA 1996) using a microwave assisted acid digestion process in a MARS One (CEM, Matthews, NC, USA). Nitric acid 65% (HNO_3 65%, Suprapur, Merck, Darmstadt, Germany), 10 ± 0.05 mL, was added to individual MARSXpress vessels containing the ground fish muscle samples using an analog-adjustable bottle-top dispenser (Dispensette S Organic, Brand, Wertheim, Germany). The ground whole crab samples were dissolved by microwave assisted acid digestion using different proportions of HNO_3 65% and hydrogen peroxide 30% w/w (H_2O_2 30% w/w, AR grade, Chem-Supply, Gillman, Australia) to test for a mixture that would produce a clear digested sample and utilised in this study (Table 1 and Fig. S2). Results of this indicated that 10 ± 0.1 mL H_2O_2 30% w/w should be added in addition to 10 ± 0.05 mL HNO_3 65% to the ground whole crab samples.

Table 1
Different proportions of nitric acid (HNO_3 65%) and hydrogen peroxide (H_2O_2 30%) added to ground whole crab samples for microwave assisted acid digestion.

Reagent mixture tested	Ref. A	Ref. B	Condition of digest
3 mL HNO_3	Annabi et al. (2018)	-	N/A
10 mL HNO_3	Hwang et al. (2017)	Nedzarek et al. (2019)	Cloudy
1 mL HNO_3 + 1 mL H_2O_2	Lima et al. (2018)	-	N/A
3 mL HNO_3 + 1 mL H_2O_2	Silva et al. (2018)	-	N/A
4 mL HNO_3 + 2 mL H_2O_2	Hamilton et al. (2008)	-	N/A
5 mL HNO_3 + 2 mL H_2O_2	Ariano et al. (2015); Olgunoglu and Olgunoglu (2016)	-	N/A
5 mL HNO_3 + 5 mL H_2O_2	-	-	Cloudy
6 mL HNO_3 + 4 mL H_2O_2	-	-	Cloudy
7 mL HNO_3 + 3 mL H_2O_2	Genc and Yilmaz (2017)	-	Cloudy
8 mL HNO_3 + 2 mL H_2O_2	-	-	Cloudy
10 mL HNO_3 + 2 mL H_2O_2	-	-	Almost clear/ cloudy
4 mL HNO_3 + 4 mL H_2O_2 + 4 mL H_2O	Lemos et al. (2018)	-	Almost clear/ cloudy
5 mL HNO_3 + 5 mL H_2O_2 + 5 mL H_2O	-	-	Almost clear
6 mL HNO_3 + 6 mL H_2O_2 + 6 mL H_2O	-	-	Almost clear
10 mL HNO_3 + 10 mL H_2O_2	-	-	**Clear/ almost clear**

Each reagent mixture tested on n = 2–4 samples of individual whole crab. Condition of digest, 'Cloudy', 'Almost clear' and 'Clear' represent not fully digested + cloudy, almost fully digested + leftover sediments and fully digested + clear digest, respectively. Illustrations for the condition of digest are available at Fig. S2. Bold condition of digest = reagent mixture that produced the clearest digest in this study, though slight leftover sediments may remain for crab species that ingest sand. The reagent mixtures tested were selected and modified based on published work that utilised microwave assisted acid digestion to study crabs. Ref. A includes published work that analysed certain parts of, for example, muscle in crabs. Ref. B includes published work that analysed the whole crab (≤ 20 mm small species). N/A represents untested reagent mixture in this study due to the low volume of reagent used in the published work.

All samples were left to stand in the open for at least 15 min in a fume cupboard to undergo pre-digestion. The vessels were then loaded onto the MARS One and acid digestion was conducted based on a preloaded method, the CEM Methods for Animal Tissue: Ramp time 20 min; Hold time 15 min; Temperature 200 °C; Power 500–800 W. Once completed and cooled, the digested samples were quantitatively transferred into individual 120 mL high density polyethylene storage containers (Sarstedt, Nümbrecht, Germany). The samples were made up to approximately 50 mL with Milli-Q water and stored at 3 °C in a refrigerator until analysis.

2.3. Instrumental analysis

A XplorAA Atomic Absorption Spectrometer (GBC Scientific Equipment, Melbourne, Australia) was used with an air-acetylene flame throughout the study to perform routine flame atomic absorption spectroscopy (FAAS) analysis of Zn in all the fish muscle and whole crab samples. Standards for FAAS analysis of Zn were prepared weekly from Scharlau Zn standard solution 1000 mg/L for AA (Scharlab, Barcelona, Spain). All standards were stabilised with the addition of HNO_3 65% (1% v/v). European Reference Material - BB422 fish muscle (certified value = 16.0 mg/kg, certified uncertainty = 1.1 mg/kg; European Commission – Joint Research Centre, Geel, Belgium) was analysed and the average measured value for Zn was 16.5 mg/kg, SD = 0.8 mg/kg, [95% CI: 15.6, 17.5], n = 5. The average recovery for Zn was 103%.

2.4. Data and statistical analysis

Statistical analyses in this study were carried out using RStudio Version 1.3.959 (RStudio, Boston, MA, USA) and SPSS Statistics Version 25 (IBM, Armonk, NY, USA). The significance threshold was set at $p \leq 0.05$. Data were expressed as mean, standard deviation (SD), [95% confidence interval (CI)] and number of samples (n).

To determine if muscle melanisation in sand flathead was related to multiple predictors (condition factor, sampling sites, sex, and the interaction between condition factor and sex), a logistic regression model was fitted to the data using maximum likelihood. The interaction between condition factor and sex was analysed as prior literature indicated the likelihood of muscle melanisation was not related to condition factor and sex, but their interaction was not studied (Stocker et al., 2020). Due to multicollinearity, fish total weight and length were converted to a condition factor to avoid ambiguity in the interpretations. The condition factor was calculated using the Fulton's condition factor (K):

$$K = 100\ \left(W / L^3\right)$$

where W = fish body weight (g), L = fish total length (cm) and a scaling factor of 100 to bring K close to a value of one (Froese, 2006; Nash et al., 2006; Ricker, 1975). A set of possible models describing the relationship between the predictors and presence of melanisation in fish muscle were compared and selected based on the Akaike information criterion (AIC). The difference in AIC values were compared across the fitted models to determine the most parsimonious model (Xu et al., 2010). Due to separation, a penalised likelihood was applied using the package 'logistf' (Heinze et al., 2020) to ensure the estimates were unbiased (Cole et al., 2014; Heinze and Schemper, 2002).

One-sample t-test was used to determine if mean Zn concentrations in fish muscle obtained in this study were different to previously published data (Ooi et al., 2019) and to determine if mean Zn concentrations in different species of crabs were different to the minimum metabolic Zn concentrations required by crustaceans (White and Rainbow, 1985).

Welch's t-test with unequal variances assumed was used to study if mean Zn concentrations in each crab species differed between polluted and unpolluted estuaries.

One-way ANOVA with unequal variances assumed using the Welch test and the Games-Howell post hoc test were used to investigate the difference in mean Zn concentrations between different types of fish muscle sample from different areas in Tasmania. In this analysis, the sampling sites were categorised into four major areas, namely the Tamar Estuary, the Derwent Estuary, D'Entrecasteaux Channel and Great Oyster Bay (control site). Other sampling sites with insufficient numbers collected were omitted from this analysis. Samples with insufficient mass extracted from the fillets were also omitted (See Section 3.2 for more details).

Statistical analyses in this study were carried out using Zn concentrations in dry weight to facilitate comparisons with previously published data. The data were also converted to wet weight to allow for comparisons with other published data and guidelines for Zn levels in fish. A conversion factor (*CF*) was used to convert the measured Zn concentrations in dry weight to wet weight (Yap et al. 2020):

$$CF = \textit{fish muscle sample dry weight } (g) / \textit{fish muscle sample wet weight } (g)$$

$$\textit{Concentration } (\textit{wet weight mg} / kg) = CF \times \textit{concentration } (\textit{dry weight mg} / kg)$$

All reported concentrations in this study are in dry weight unless otherwise stated.

3. Results and discussion

3.1. Likelihood of muscle melanisation

Model fitted to the data did not suggest there was an interaction between condition factor and sex (LRT, $G_1 = 5.64$, $p = 0.131$), thus the model was refitted with the interaction removed. There was limited evidence that sex (LRT, $G_1 = 1.72$, $p = 0.788$) and condition factor (LRT, $G_1 = 0.454$, $p = 0.500$) influenced the likelihood of muscle melanisation in this study. However, there was statistical evidence that presence of muscle melanisation in sand flathead was influenced by the sampling site (LRT, $G_1 = 152.66$, $p < 0.001$). The model with only sampling site included was the best AIC model (Table 2), suggesting that among the predictors tested, presence of muscle melanisation was primarily related to the sampling site. As the sampling site is categorical, the logistic regression fitted the proportion of 'successes', defined as the presence of muscle melanisation, within each site (Fig. 1B).

Our results showed muscle melanisation was more commonly found at most of the sampling sites located within estuaries impacted by anthropogenic activities and Zn pollution, namely the Tamar and the Derwent Estuary. For example, muscle melanisation was commonly observed in sand flathead from Deceitful Cove and Middle Arm in the Tamar Estuary, and New Town Bay in the Derwent Estuary (Fig. 1B(i) and (ii)). Monitoring reports stated these sites have elevated Zn in the sediments and the levels are usually higher than other sites within the estuary (Aquenal Pty Ltd and DEPHA, 2008; Coughanowr et al., 2015; Whitehead et al., 2010). At D'Entrecasteaux Channel, the proportion of sand flathead with muscle melanisation was lower than many of the sites in the adjacent Derwent Estuary (Fig. 1B(ii) and (iii)). Monitoring report stated D'Entrecasteaux Channel potentially also has elevated Zn in the sediments, but the levels and severity of pollution are lower when compared to the Derwent Estuary (Parsons 2012). By contrast, sites in the Derwent Estuary reported to have low Zn levels in the sediments, for example, Ralphs Bay (Whitehead et al., 2010), recorded little to no fish with muscle melanisation (Fig. 1B(ii)). In addition, sand flathead from sampling sites such as Great Oyster Bay and Port Sorell located in estuaries unpolluted by Zn (Mondon, 2000; Mondon et al., 2000; Ooi et al., 2019) also recorded little to no muscle melanisation (Fig.1B(iii).

Table 2
Summary of the AIC analysis.

Model descriptions	Model	*K*	In*L*	ΔAIC
All main effects included	y~1+cf+s+ss	26	-185.4	7.8
Sex and sampling site effect	y~1+s+ss	25	-185.6	6.2
Condition factor and sampling site effect	y~1+cf+ss	22	-186.3	1.5
Sampling site effect	y~1+ss	21	-186.5	**0.0**
Condition factor and sex effect	y~1+cf+s	6	-261.7	120.4
Sex effect	y~1+s	5	-262.2	119.4
Condition factor effect	y~1+cf	2	-264.9	118.7
Null effect	y~1	1	-265.4	117.6

The main effects: condition factor, sampling site and sex, are indicated by cf, ss and s, respectively. K = number of estimated parameters and InL = maximum log-likelihood. Bold ΔAIC values = best model set.

While sampling in previous muscle melanisation studies were limited to smaller sample size and sites located in northern Tasmania (Ooi et al., 2022; Stocker et al., 2020), or limited to only two sites from south-eastern Tasmania (Ooi et al., 2019), we were able to greatly expand this and sampled in a broader scale. In total, we sampled from ten sites in northern Tasmania, ten sites in south-eastern Tasmania and one control site in eastern Tasmania. The heavily polluted Derwent Estuary that was not previously studied for muscle melanisation was also sampled for the first time. Our results are in agreement with published literature that stated muscle melanisation was likely caused by site-specific factors (Stocker et al., 2020), and one of the major factors was hypothesised to be heavy metal, notably Zn, pollution in the sediments (Ooi et al., 2022; Ooi et al., 2019). Indeed, sand flathead with muscle melanisation were more commonly found in polluted areas in this study, with higher incidence typically recorded at contaminated sites located within the Tamar and the Derwent Estuary.

3.2. Improved extraction technique for melanised muscle

Zn levels in sand flathead from this study were compared to the levels determined in sand flathead collected during 2015 and analysed in a previous study (Ooi et al., 2019). Comparisons were made between muscle samples of sand flathead caught from the same sampling sites in this study and the previous study. For melanised regions of muscle, there were significant differences in mean concentrations of Zn between sand flathead in this study and the previous study (Ooi et al. 2019). At Deceitful Cove, the mean concentration of Zn in the melanised regions of muscle in this study (37.8 mg/kg, SD = 8.6 mg/kg, [95% CI: 32.5, 43.2], $n = 10$) was significantly higher when compared to the result reported in the previous study (Ooi et al., 2019) (29.6 mg/kg, $t(9) = 3.016$, $p = 0.015$). Similarly, the mean concentration of Zn in melanised regions of muscle of fish from Norfolk-Frederick Henry Bay and D'Entrecasteaux Channel in the current study (56.7 mg/kg, SD = 10.5 mg/kg, [95% CI: 48.9, 64.5], $n = 7$) was significantly higher than reported previously (Ooi et al., 2019) (37.7 mg/kg, $t(6) = 4.771$, $p = 0.003$).

On the other hand, there were no significant differences in mean concentrations of Zn between sand flathead in this study and the previous study (Ooi et al., 2019) for non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation. At Deceitful Cove, the mean concentration of Zn in the non-melanised regions of muscle from fish with melanisation in the current study (19.6 mg/kg, SD = 4.0 mg/kg, [95% CI: 17.3, 21.8], $n = 12$) was not significantly different to the result reported in the previous study (Ooi et al., 2019) (17.6 mg/kg, $t(11) = 1.708$, $p = 0.116$). Similarly, the mean concentration of Zn in the non-melanised regions of muscle from fish with melanisation from Norfolk-Frederick Henry Bay and D'Entrecasteaux Channel (19.5 mg/kg, SD = 5.1 mg/kg, [95% CI: 15.8, 23.3], $n = 7$) was also not significantly different to those reported previously (Ooi et al., 2019) (23.0 mg/kg, $t(6) = 1.797$, $p = 0.122$). In addition, mean concentration of Zn in the muscle of fish unaffected by melanisation from Great Oyster Bay in this study (16.4 mg/kg, SD = 4.2 mg/kg, [95% CI: 15.3, 17.6], $n = 52$) was not significantly different to those reported

previously (Ooi et al., 2019) (17.5 mg/kg, $t(51) = 1.869$, $p = 0.067$).

Significantly higher levels of Zn were recorded in the current study most likely due to the improved technique for extracting melanised muscle. The improved technique ensured melanised muscle sampled included very minimal non-melanised muscle, thus enhancing sample homogeneity and giving more accurate results for Zn in the melanised regions of muscle. Sufficient mass of melanised muscle sample was successfully extracted from severely or moderately (melanisation score 3 or 2, respectively) melanised fillets in the majority of cases (~80%), but limitations associated with the extraction process persisted. This is because many fish had only a minor level of melanisation (melanisation score 1) and extracting sufficient mass for analysis was unachievable. Despite that melanised muscle was extracted from fish with melanisation score 1 using the improved technique, most of the results for these were omitted due to low mass sampled from the melanised region resulting in low accuracy of the analytical result.

For the non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation, the extraction technique used was the same as the previous study (Ooi et al., 2019) and the Zn levels have remained unchanged. This trend is in close agreement with published literature, where Zn levels in white sand flathead muscle from Port Phillip Bay, Victoria, Australia have remained comparatively unchanged for 20 years from 1995 (3.8–6.8 mg/kg wet weight) to 2015 (5.93–7.16 mg/kg wet weight) (Gagnon et al., 2016). Similarly, Zn levels in non-melanised regions of muscle, or white fish muscle, in the present study were similar to the levels obtained in 2015 (Ooi et al., 2019). The levels of Zn in white sand flathead muscle in the present study (3.6–4.9 mg/kg wet weight), along with the results from previous studies (Gagnon et al., 2016; Ooi et al., 2019), show that the baseline level for Zn in sand flathead white muscle is lower than or around 5 mg/kg wet weight, which conforms to the Australia New Zealand Food Authority generally expected levels (GELs) of 5 mg/kg wet weight for Zn in fish (FSANZ, 2001). For melanised regions of muscle, Zn levels (12.4–15.7 mg/kg wet weight) exceed the GELs (FSANZ, 2001).

3.3. Zn levels and muscle melanisation in sand flathead

There was a significant difference in mean Zn levels when comparing between the different types of muscle in sand flathead from different sampling areas ($F(9,43) = 22.148$, $p < 0.001$). A post hoc test revealed the difference in Zn levels were mainly observed when comparing the melanised regions of muscle to non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation.

The mean Zn concentration in fish from the Tamar Estuary was about 2.1 times higher in melanised regions of muscle (39.2 mg/kg, SD = 9.4 mg/kg, [95% CI: 33.5, 44.9], $n = 13$) compared to non-melanised regions of muscle from fish with melanisation (19.1 mg/kg, SD = 4.1 mg/kg, [95% CI: 17.9, 20.3], $n = 49$, $p < 0.001$) and fish unaffected by melanisation (18.9 mg/kg, SD = 3.6 mg/kg, [95% CI: 18.1, 19.8], $n = 78$, $p < 0.001$) (Fig. 2). Compared to muscle of fish unaffected by melanisation from the control site (16.4 mg/kg, SD = 4.2 mg/kg, [95% CI: 15.3, 17.6], $n = 52$), the mean concentration was about 2.4 times higher ($p < 0.001$) (Fig. 2). There was no significant difference in mean Zn concentration between the non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation ($p = 1.000$) from the Tamar Estuary (Fig. 2). However, higher mean Zn concentrations were observed when the non-melanised regions of muscle from fish with melanisation ($p = 0.044$) and fish unaffected by melanisation ($p = 0.020$) from the Tamar Estuary were compared to muscle of fish unaffected by melanisation from the control site (Fig. 2).

In fish from the Derwent Estuary, the mean Zn concentration was about 3.1 times higher in melanised regions of muscle (52.0 mg/kg, SD = 15.2 mg/kg, [95% CI: 40.5, 64.0], $n = 7$) compared to non-melanised regions of muscle from fish with melanisation (16.7 mg/kg, SD = 2.2 mg/kg, [95% CI: 15.7, 17.7], $n = 20$, $p = 0.012$) and fish unaffected by melanisation (17.0 mg/kg, SD = 3.0 mg/kg, [95% CI: 15.9, 17.9], $n = 26$, $p = 0.012$) (Fig. 2). Compared to muscle of fish unaffected by melanisation from the control site, the mean concentration was about 3.2 times higher ($p = 0.011$) (Fig. 2). There were no significant differences in mean Zn concentrations between the non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation from the Derwent Estuary ($p = 1.000$), and muscle of fish unaffected by

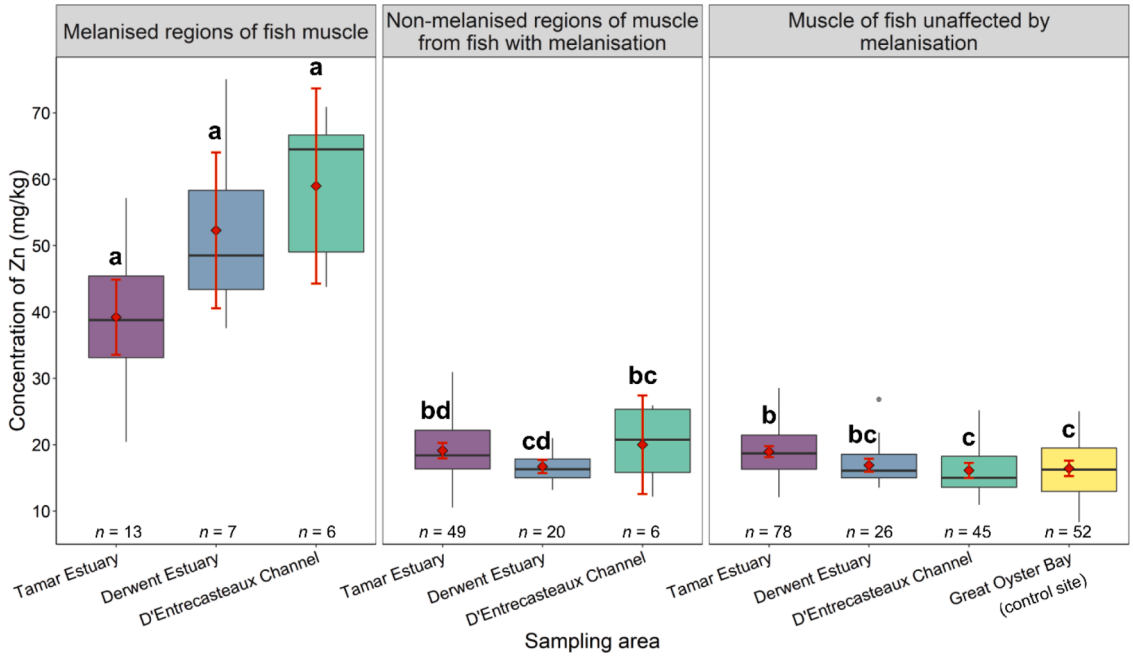

Fig. 2. The concentration of Zn (mg/kg dry weight) in the muscle of sand flathead from various sampling areas in Tasmania. Red diamond points indicate the mean concentrations of Zn and error bars depict the 95% confidence intervals. Different lowercase letter indicates significant difference at $p < 0.05$. The boxplots show the distribution of data based on the five-number summary including the minimum (lower whisker), first quartile (lower box outline), median (horizontal black line inside box), third quartile (upper box outline) and maximum (upper whisker). Black dot point indicate outlier.

melanisation from the control site ($p = 1.000$ and $p = 0.999$) (Fig. 2).

The mean Zn concentration in fish from D'Entrecasteaux Channel was about 3.3 times higher in melanised regions of muscle (58.1 mg/kg, SD = 10.8 mg/kg, [95% CI: 44.3, 73.7], $n = 6$) compared to non-melanised regions of muscle from fish with melanisation (19.4 mg/kg, SD = 5.5 mg/kg, [95% CI: 12.6, 27.4], $n = 6$, $p = 0.001$) and fish unaffected by melanisation (16.2 mg/kg, SD = 3.3 mg/kg, [95% CI: 15.0, 17.3], $n = 45$, $p = 0.003$) (Fig. 2). Compared to muscle of fish unaffected by melanisation from the control site, the mean concentration was about 3.5 times higher ($p = 0.003$) (Fig. 2). There were no significant differences in mean Zn concentrations between the non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation from D'Entrecasteaux Channel ($p = 0.895$), and muscle of fish unaffected by melanisation from the control site ($p = 0.930$ and $p = 1.000$) (Fig. 2).

The mean Zn concentrations in melanised regions of muscle did not differ significantly between sand flathead from the Tamar Estuary, the Derwent Estuary ($p = 0.598$) and D'Entrecasteaux Channel ($p = 0.084$), nor between those from the Derwent Estuary compared to D'Entrecasteaux Channel ($p = 0.995$) (Fig. 2). Similarly, there were no significant differences between the mean Zn concentrations in non-melanised regions of muscle from fish with melanisation from the Tamar Estuary compared to the Derwent Estuary ($p = 0.080$) and to D'Entrecasteaux Channel ($p = 1.000$), nor between those from the Derwent Estuary compared to D'Entrecasteaux Channel ($p = 0.958$) (Fig. 2).Therefore, Zn concentrations can be divided into two main groups depending on whether melanisation was present in the fish muscle. Within the melanised regions of muscle, Zn concentrations were greatly elevated, about 2.1–3.5 times higher than in non-melanised regions. In non-melanised regions or unaffected muscle, Zn concentrations were around the baseline level and similar to the levels observed in fish muscle from the control site. Even though some of the sampling areas are situated as far as 250 km apart, this trend was consistent for all the sand flathead investigated in this study.

Presence of muscle melanisation in fish has been associated with elevated levels of heavy metals (Cooper and Midling, 2006; Cooper et al., 2011; Ooi et al., 2019). It is shown that melanin, normally found in melanised or pigmented tissues in, for example, muscles, eyes and feathers, is usually enriched with elements such as Zn, Cu, Mg, Ca or Fe (Bowness et al., 1952; Chatelain et al., 2014; Cooper and Midling, 2006; Edwards et al., 2016; Hong and Simon, 2007; McGraw, 2003; Niecke et al., 1999; Wogelius et al., 2011) as it can function as a reservoir or sink for heavy metals and other toxic substances (Chatelain et al., 2014; Cooper and Midling, 2006; Edwards et al., 2016; Hong and Simon, 2007; McGraw, 2003). As a result, melanin pigments in the melanised regions of muscle in sand flathead was suggested to be a sink for sequestering excessive heavy metals in the fish, especially Zn (Ooi et al., 2019).

The occurrence of muscle melanisation has been observed in both wild and farmed Atlantic cod from Norway, but the presence and severity were much lower in the wild fish than in the farmed fish (Cooper and Midling, 2006; Cooper et al., 2011). Increased occurrence of muscle melanisation in farmed Atlantic cod was linked to higher levels of Cu in the feed (Cooper et al., 2011) and reported as arising due to an increase in the activity of tyrosinase, a copper-dependent enzyme that catalyses melanin production (Cooper and Midling, 2006). The resultant melanin was associated with blood vessels and muscle, and higher levels of Cu were found in these melanised tissues compared to non-melanised tissues (Cooper and Midling, 2006). In the wild sand flathead considered in the current study, the melanin has also been associated with blood vessels and muscle (Ooi et al., 2019; Stocker et al., 2020). However, in this case the melanised tissues were found to contain higher levels of Zn rather than Cu.

While Cu ions are traditionally expected to bind to the active site of the tyrosinase family, recent studies in human tyrosinase have contradicted that assumption and shown the presence of Zn ions in the active site involved in melanogenesis (Lai et al., 2017; Solano, 2018). The process is facilitated under certain conditions, most importantly the presence of Zn ions along with low availability of Cu (Solano, 2018). This has in turn presented an interesting premise for the cause of muscle melanisation in sand flathead, since the three enzymes associated with the biosynthesis of melanin in humans, namely tyrosinase, tyrosinase related-protein 1 and tyrosinase related-protein 2, are also present in teleost fishes (Lai et al., 2017; Sato et al., 1999; Solano, 2018) such as the sand flathead. Furthermore, the polluted estuaries investigated in this study also demonstrated higher Zn levels when compared to Cu. Monitoring reports indicated Zn levels (34–1030 mg/kg) in the surface sediments throughout the Tamar Estuary were about 2–10 times higher than Cu (4–472 mg/kg), and certain sites such as the Deceitful Cove recorded Zn (6050 mg/kg) levels that were up to 47 times higher than Cu (130 mg/kg) (Aquenal Pty Ltd and DEPHA, 2008). For the Derwent Estuary, Zn levels (2103–4228 mg/kg) in the surface sediments were on average 20–26 times higher than Cu (106–164 mg/kg) (Coughanowr et al., 2015). As a result, the cause of muscle melanisation in sand flathead is possibly related to the presence of higher Zn than Cu in the estuaries and the Zn ions may bind to the active site of tyrosinase enzyme, thereby inducing melanin production.

3.4. Zn levels in sand flathead's diet (crabs)

The mean Zn concentrations recorded in four-toothed shore crabs from Port Sorell (52.4 mg/kg, SD = 3.7 mg/kg, [95% CI: 49.8, 55.0], $n = 10$, $t(9) = 2.074$, $p = 0.068$), red-spotted shore crabs from Deceitful Cove (51.5 mg/kg, SD = 4.1 mg/kg, [95% CI: 47.2, 55.7], $n = 6$, $t(5) = 0.872$, $p = 0.423$) and Port Sorell (49.8 mg/kg, SD = 5.8, [95% CI: 46.1, 53.5], $n = 12$, $t(11) = 0.124$, $p = 0.903$), and mottled shore crabs from Deceitful Cove (51.8 mg/kg, SD = 5.2 mg/kg, [95% CI: 52.8, 66.0], $n = 10$, $t(9) = 1.103$, $p = 0.299$) were not significantly different to the literature value for minimum metabolic requirement in crabs, 50.0 mg/kg dry weight (Wang and Rainbow, 2020; White and Rainbow, 1985) (Fig. 3). However, mean Zn concentrations significantly higher than this base level were observed in other crabs in these estuaries, namely soldier crabs from Deceitful Cove (130.1 mg/kg, SD = 14.8 mg/kg, [95% CI: 119.5, 140.6], $n = 10$, $t(9) = 17.121$, $p < 0.001$) and Port Sorell (86.1 mg/kg, SD = 8.4 mg/kg, [95% CI: 80.0, 92.1], $n = 10$, $t(9) = 13.5$, $p < 0.001$), four-toothed shore crabs from Deceitful Cove (76.9 mg/kg, SD = 13.2 mg/kg, [95% CI: 60.5, 93.3], $n = 5$, $t(4) = 4.555$, $p = 0.010$), and mottled shore crabs from Port Sorell (59.4 mg/kg, SD = 5.3 mg/kg, [95% CI: 52.8, 66.0], $n = 5$, $t(4) = 3.974$, $p = 0.016$) (Fig. 3).

The minimum metabolic Zn concentration required by crustaceans, including crabs, is estimated to be 50.0 mg/kg dry weight (Wang and Rainbow, 2020; White and Rainbow, 1985). Zn levels in most crabs investigated in this study were in close agreement with this value and no individual crab species recorded Zn concentrations that were significantly lower than 50.0 mg/kg. Levels of Zn varied somewhat between crab species, but this was anticipated as the accumulation of heavy metal in crabs can vary between taxa, feeding habit and behavioural strategy (Rainbow et al., 1990; Rainbow and White, 1989).

For Deceitful Cove soldier crabs, the mean Zn concentration was about 1.5 times higher than those from the comparatively unpolluted Port Sorell ($t(14) = 8.171$, $p < 0.001$) (Fig. 3). Similarly, the Deceitful Cove four-toothed shore crabs had mean Zn concentration that was about 1.5 times higher than individuals from Port Sorell ($t(4) = 4.070$, $p = 0.013$) (Fig. 3). In contrast, mean Zn concentration in mottled shore crabs from Port Sorell was slightly higher, about 1.1 times, than individuals from Deceitful Cove ($t(8) = 2.628$, $p = 0.030$) (Fig. 3).

There is evidence for crabs being able to regulate Zn levels in their body (Rainbow et al., 1990). This has been reported in decapod crustaceans, where essential metals including Zn are regulated by changing the Zn excretion rate to match the Zn uptake rate (Rainbow et al., 1990). However, there is generally a threshold above which net accumulation of metals will occur (Al-Mohanna and Subrahmanyam, 2001; Rainbow and White, 1989). This appears to have occurred for soldier crabs and

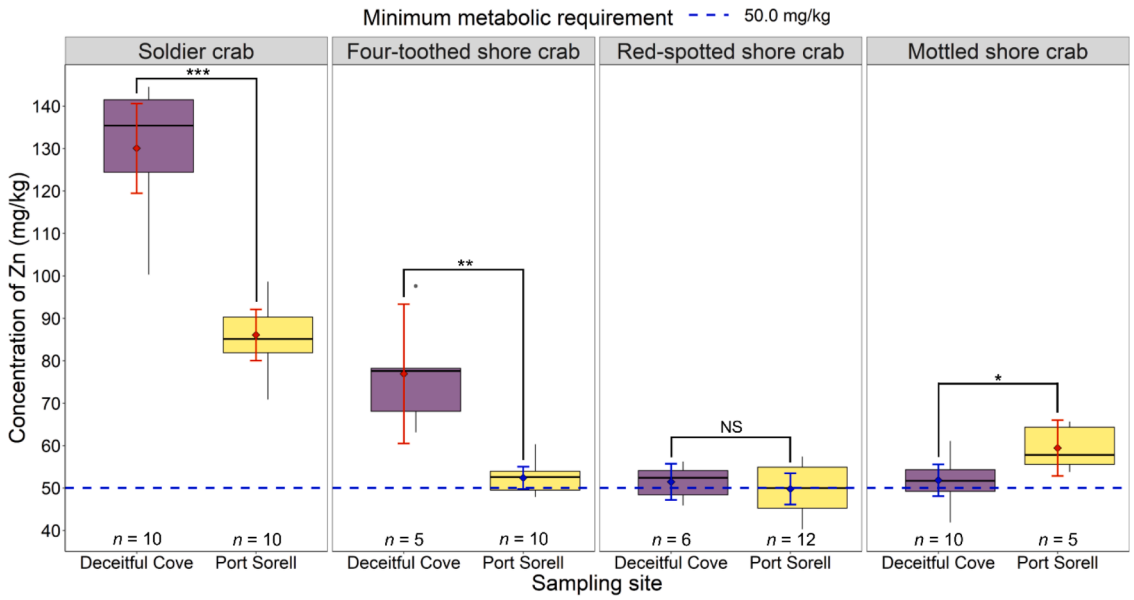

Fig. 3. The concentration of Zn (mg/kg dry weight) in different crab species from Deceitful Cove and Port Sorell. Diamond points indicate the mean concentrations of Zn and error bars depict the 95% confidence intervals. Asterisks indicate significant difference in mean Zn concentrations when comparing between the same species of crab from different sampling site, where *, ** and *** indicate $p = 0.030$, $p = 0.013$ and $p < 0.001$, respectively. NS indicates no significant difference. Blue dashed line represents the base value from published data (Wang and Rainbow 2020; White and Rainbow 1985). A difference in diamond point colour (i.e., red) to the dashed line colour (i.e., blue) indicates significant difference ($p < 0.05$) in mean Zn concentration to the base value. The boxplots show the distribution of data based on the five-number summary including the minimum (lower whisker), first quartile (lower box outline), median (horizontal black line inside box), third quartile (upper box outline) and maximum (upper whisker). Black dot point indicate outlier.

four-toothed shore crabs from Deceitful Cove, which is known to contain sediments highly polluted by Zn (Aquenal Pty Ltd and DEPHA, 2008). For mottled shore crabs, individuals sampled from Port Sorell were likely older and thus accumulated more Zn as they were on average 6 times heavier than individuals sampled from Deceitful Cove (Table S2). The higher levels of Zn in the crabs likely resulted from a Zn uptake rate that exceeded the excretion rate, thus resulting in net accumulation of Zn in the crab's body (Rainbow et al., 1990). As soldier crabs ingest sand to filter for food (Edgar, 1997), they have accumulated significantly higher levels of Zn in their body than other crab species. For example, light-blue soldier crabs (*Mictyris longicarpus*) are shown to be capable of accumulating more heavy metals from the sediments than from water (Weimin et al., 1994). Elevated Zn levels were also observed in four-toothed shore crabs from Deceitful Cove, but the elevation was less than for soldier crabs. This is likely due to the four-toothed shore crabs being scavengers and not consuming sand (Taylor and Poore, 2020b). Similarly, mottled shore crabs are carnivorous and do not ingest sand (Taylor and Poore, 2020a).

There was no significant difference in the mean Zn level between red-spotted shore crabs ($t(14) = 0.704$, $p = 0.493$) from Deceitful Cove and Port Sorell (Fig. 3). The Zn concentrations were maintained around the minimum metabolic requirement of 50.0 mg/kg, suggesting Zn levels are regulated more effectively in this crab species.

For each crab species, the whole crab was digested and analysed for Zn, and 50% of the crab species investigated in this study exhibited higher Zn concentrations when residing in a polluted estuary. As heavy metal accumulation in sand flathead was reported to be greatly influenced by their diet (Hunt, 2008), this study shows crabs that accumulated Zn may serve as the intermediate link between elevated Zn in the sediments and the fish. Since sand flathead are non-migratory (Ayling et al., 1975) and primarily consume crabs (Hunt, 2008), it is possible that the constant consumption of crabs residing in Zn contaminated sites resulted in overabundance of Zn in the sand flathead's body. As melanisation in sand flathead has been associated with the muscle fibres and blood vessels, the excess Zn could be transported via blood circulation and localised in the melanised regions of muscle (Ooi et al., 2019). In this case, melanin in the melanised regions of muscle would act as a sink to sequester the excess Zn (Ooi et al., 2019).

4. Conclusions

This study established that occurrence of muscle melanisation in sand flathead was influenced by the levels of Zn pollution in the fish's surrounding environment. An improved technique for extracting melanised muscle was successfully developed and led to more accurate results of Zn being determined in the melanised muscle. It showed that Zn levels in the melanised regions of muscle were significantly higher (about 2.1–3.5 times) than non-melanised regions of muscle from fish with melanisation and fish unaffected by melanisation, where Zn levels were near the baseline level. The findings indicated elevated levels of Zn were localised in the melanised regions of muscle and the possible cause of muscle melanisation in sand flathead was explored. The newly developed reagent mixture and method were suitable for the digestion and analysis of Zn in the whole crab. Zn levels were significantly higher (about 1.5 times) in 50% of the crab species studied from a polluted estuary compared to an unpolluted estuary. As sand flathead primarily feed on crabs, the diet was a likely source of elevated Zn for the fish. Overall, findings from this study will inform and shape the direction of future work on muscle melanisation in sand flathead. Aside from the sand flathead, the newly developed techniques are applicable to other fish and crab species and will be useful for muscle melanisation and environmental pollution research in general.

CRediT authorship contribution statement

Chun Kit Ooi: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **James Haddy:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Barbara Nowak:** Conceptualization, Resources, Writing – review & editing, Supervision, Project

administration, Funding acquisition. **Jeremy Lyle:** Conceptualization, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Yonglin Mai:** Methodology, Validation, Investigation, Writing – review & editing, Funding acquisition. **Hamish McLean:** Methodology, Validation, Investigation, Writing – review & editing, Funding acquisition. **Trevor Lewis:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding sources

This work was supported by the Institute for Marine and Antarctic Studies and the University of Tasmania [Tasmania Graduate Research Scholarship for Chun Kit Ooi; and Dean's Summer Research Scholarship for Yonglin Mai and Hamish McLean].

Acknowledgment

The authors wish to thank Nyrstar Hobart and the Institute for Marine and Antarctic Studies, University of Tasmania for sharing and contributing sand flathead muscle samples from south-eastern and eastern Tasmania. Additional thanks are extended to Clayton Stocker, Mike Williams and Graeme Ewing for their assistance with sand flathead collection and processing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2022.100177.

References

Al-Mohanna, S.Y., Subrahmanyam, M.N.V., 2001. Flux of heavy metal accumulation in various organs of the intertidal marine blue crab, *Portunus pelagicus* (L.) from the Kuwait coast after the Gulf War. Environ. Int. 27, 321–326. https://doi.org/10.1016/S0160-4120(01)00063-0.

Annabi, A., Bardelli, R., Vizzini, S., Mancinelli, G., 2018. Baseline assessment of heavy metals content and trophic position of the invasive blue swimming crab *Portunus segnis* (Forskål, 1775) in the Gulf of Gabès (Tunisia). Mar. Pollut. Bull. 136, 454–463. https://doi.org/10.1016/j.marpolbul.2018.09.037.

Aquenal Pty Ltd and DEPHA, 2008. State of the Tamar Estuary. Department of Environment, Parks, Heritage and the Arts, Hobart.

Ariano, A., Voi, A.L., D'Ambola, M., Marrone, R., Cacace, D., Severino, L., 2015. Levels of cadmium in white and brown meat of warty crab (*Eriphia verrucosa*). J. Food Prot. 78, 2253–2256. https://doi.org/10.4315/0362-028X.JFP-15-214.

Ayling, G.M., Wilson, K.C., Ratkowsky, D.A., 1975. Sand flathead (*Platycephalus bassensis*), an indicator species for mercury pollution in Tasmanian waters. Mar. Pollut. Bull. 6, 142–144.

Bjorgen, H., Haldorsen, R., Oaland, O., Kvellestad, A., Kannimuthu, D., Rimstad, E., Koppang, E.O., 2019. Melanized focal changes in skeletal muscle in farmed Atlantic salmon after natural infection with *Piscine orthoreovirus* (PRV). J. Fish Dis. 42, 935–945. https://doi.org/10.1111/jfd.12995.

Bjorgen, H., et al., 2015. *Piscine orthoreovirus* (PRV) in red and melanised foci in white muscle of Atlantic salmon (*Salmo salar*). Vet. Res. 46, 89. https://doi.org/10.1186/s13567-015-0244-6.

Bloom, H., Ayling, G.M., 1977. Heavy metals in the Derwent Estuary. Environ. Geol. 2, 3–22. https://doi.org/10.1007/BF02430661.

Bowness, J.M., Morton, R.A., Shakir, M.H., Stubbs, A.L., 1952. Distribution of copper and zinc in mammalian eyes. Occurrence of metals in melanin fractions from eye tissues. Biochem. J. 51, 521–530. https://doi.org/10.1042/bj0510521.

Chan, H.M., Rainbow, P.S., 1993. The accumulation of dissolved zinc by the shore crab *Carcinus Maenas* (L.). Ophelia 38, 13–30. https://doi.org/10.1080/00785326.1993.10429921.

Chatelain, M., Gasparini, J., Jacquin, L., Frantz, A., 2014. The adaptive function of melanin-based plumage coloration to trace metals. Biol. Lett. 10, 20140164 https://doi.org/10.1098/rsbl.2014.0164.

Chen, M.H., Chen, C.Y., 1999. Bioaccumulation of sediment-bound heavy metals in grey mullet, *Liza macrolepis*. Mar. Pollut. Bull. 39, 239–244. https://doi.org/10.1016/S0025-326X(99)00027-2.

Cole, S.R., Chu, H., Greenland, S., 2014. Maximum likelihood, profile likelihood, and penalized likelihood: a primer. Am. J. Epidemiol. 179, 252–260. https://doi.org/10.1093/aje/kwt245.

Cooper, M., Midling, K.Ø., 2006. Blood vessel melanosis: a physiological detoxification mechanism in Atlantic cod (*Gadus morhua*). Aquac. Int. 15, 43–54. https://doi.org/10.1007/s10499-006-9067-9.

Cooper, M., Olsen, R.L., Seliussen, J., Gannefors, C., 2011. Dietary trace metal supplements promote blood vessel melanosis in fillets of juvanile farmed Atlantic cod, *Gadus morhua* L. J. World Aquac. Soc. 42, 222–229.

Coughanowr, C., Whitehead, S., Whitehead, J., Einoder, L., Taylor, U., Weeding, B., 2015. State of the Derwent Estuary 2015: a review of environmental data from 2009 to 2014. Derwent Estuary Program, Hobart.

Edgar, G.J., 1997. Australian marine life: the plants and animals of temperate waters. Reed Books, Kew.

Edwards, N.P., et al., 2016. Elemental characterisation of melanin in feathers via synchrotron X-ray imaging and absorption spectroscopy. Sci. Rep. 6, 34002. https://doi.org/10.1038/srep34002.

Esteves, A., Seixas, F., Carvalho, S., Nazario, N., Mendes, M., Martins, C., 2009. *Huffmanela* sp. (Nematoda: Trichosomoididae) muscular parasite from *Trisopterus luscus* captured off the Portuguese coast. Dis. Aquat. Organ. 84, 251–255. https://doi.org/10.3354/dao02048.

Frijlink, S., Lyle, J.M., 2013. Establishing historical baselines for key recreational and commercial fish stocks in Tasmania. Institute for Marine and Antarctic Studies, Hobart.

Froese, R., 2006. Cube law, condition factor and weight-length relationships: history, meta-analysis and recommendations. J. Appl. Ichthyol. 22, 241–253. https://doi.org/10.1111/j.1439-0426.2006.00805.x.

FSANZ, 2001. Generally expected levels (GELs) for metal contaminants. Food Standards Australia New Zealand, Majura Park.

Gagnon, M.M., Baker, J.K., Long, S.M., Hassell, K.L., Pettigrove, V.J., 2016. Contaminant (PAHs, OCs, PCBs and trace metals) concentrations are declining in axial tissue of sand flathead (*Platycephalus bassensis*) collected from an urbanised catchment (Port Phillip Bay, Australia). Mar. Pollut. Bull. 109, 661–666. https://doi.org/10.1016/j.marpolbul.2016.05.003.

Genc, T.O., Yilmaz, F., 2017. Metal accumulations in water, sediment, crab (*Callinectes sapidus*) and two fish species (*Mugil cephalus* and *Anguilla anguilla*) from the Köyceğiz lagoon system–Turkey: an index analysis approach. Bull. Environ. Contam. Toxicol. 99, 173–181. https://doi.org/10.1007/s00128-017-2121-7.

Hamilton, M.A., Rode, P.W., Merchant, M.E., Sneddon, J., 2008. Determination and comparison of heavy metals in selected seafood, water, vegetation and sediments by inductively coupled plasma-optical emission spectrometry from an industrialized and pristine waterway in Southwest Louisiana. Microchem. J. 88, 52–55. https://doi.org/10.1016/j.microc.2007.09.004.

Heinze, G., Ploner, M., Jiricka, L., 2020. In: logistf: Firth's bias-reduced logistic regression, R package version 1.24. Medical University Vienna, Vienna.

Heinze, G., Schemper, M., 2002. A solution to the problem of separation in logistic regression. Stat. Med. 21, 2409–2419. https://doi.org/10.1002/sim.1047.

Hong, L., Simon, J.D., 2007. Current understanding of the binding sites, capacity, affinity, and biological significance of metals in melanin. J. Phys. Chem. B 111, 7938–7947. https://doi.org/10.1021/jp071439h.

Hunt, A.S., 2008. A field study of the relationships between diet and heavy metal concentrations in the sand flathead (*Platycephalus bassensis*). Honours thesis. University of Tasmania, Hobart.

Hwang, D.W., Choi, M., Lee, I.S., Shim, K.B., Kim, T.H., 2017. Concentrations of trace metals in tissues of *Chionoecetes* crabs (*Chionoecetes japonicus* and *Chionoecetes opilio*) caught from the East/Japan Sea waters and potential risk assessment. Environ. Sci. Pollut. Res. Int. 24, 11309–11318. https://doi.org/10.1007/s11356-017-8769-z.

Jezierska, B., Witeska, M., 2006. The metal uptake and accumulation in fish living in polluted waters. Soil and water pollution monitoring, protection and remediation. In: Twardowska, I., Allen, H.E., Häggblom, M.M., Stefaniak, S. (Eds.), In: NATO Science Series, 69. Springer, Dordrecht, pp. 107–114. https://doi.org/10.1007/978-1-4020-4728-2_6.

Jordan, A.R., 2001. Reproductive biology, early life-history and settlement distribution of sand flathead (*Platycephalus bassensis*) in Tasmania. Mar. Freshw. Res. 52, 589–601.

Koppang, E.O., Haugarvoll, E., Hordvik, I., Aune, L., Poppe, T.T., 2005. Vaccine-associated granulomatous inflammation and melanin accumulation in Atlantic salmon, *Salmo salar* L., white muscle. J. Fish Dis. 28, 13–22.

Lai, X., Wichers, H.J., Soler-Lopez, M., Dijkstra, B.W., 2017. Structure of human tyrosinase related protein 1 reveals a binuclear zinc active site important for melanogenesis. Angew. Chem. Int. Ed Engl. 56, 9812–9815. https://doi.org/10.1002/anie.201704616.

Lemos, M.S., Cruz, A.S., Fernandes Dantas, K.G., 2018. Microwave-assisted digestion procedures using diluted oxidant mixture for elemental analysis of crustaceans by MIP OES. Biol. Trace Elem. Res. 191 (1), 224–230. https://doi.org/10.1007/s12011-018-1593-6.

Lima, D., Guedes, W., Costa, V., Amorim, F., 2018. Application of factorial design and desirability function to develop a single analytical procedure for the determination of metals in different tissues of blue crab (*Callinectes danae*). J. Braz. Chem. Soc. 29 (9), 1885–1893. https://doi.org/10.21577/0103-5053.20180064.

Lyle, J.M., Stark, K.E., Ewing, G.P., Tracey, S.R., 2019. 2017-18 Survey of Recreational Fishing in Tasmania. Institute for Marine and Antarctic Studies, Hobart.

Malik, M.S., Bjorgen, H., Nyman, I.B., Wessel, O., Koppang, E.O., Dahle, M.K., Rimstad, E., 2021. PRV-1 infected macrophages in melanized focal changes in white muscle of Atlantic salmon (*Salmo salar*) correlates with a pro-inflammatory environment. Front. Immunol. 12, 664624 https://doi.org/10.3389/fimmu.2021.664624.

McGraw, K.J., 2003. Melanins, metals and mate quality. Oikos 102 (2), 402–406. http://www.jstor.org/stable/3548043.

Mondon, J.A., 2000. Ecotoxicology of contaminated marine sediments in Tasmanian estuaries. PhD thesis. University of Tasmania, Hobart.

Mondon, J.A., Duda, S., Nowak, B.F., 2000. Immune response of greenback flounder *Rhombosolea tapirina* after exposure to contaminated marine sediment and diet. Mar. Environ. Res. 50, 443–450. https://doi.org/10.1016/S0141-1136(00)00253-1.

Mondon, J.A., Nowak, B.F., Sodergren, A., 2001. Persistent organic pollutants in oysters *Crassostrea gigas* and sand flathead *Platycephalus bassensis* from Tasmanian estuarine and coastal waters. Mar. Pollut. Bull. 42, 157–161. https://doi.org/10.1016/S0025-326X(00)00211-3.

Nakayama, S.M., et al., 2010. Heavy metal accumulation in lake sediments, fish (*Oreochromis niloticus* and *Serranochromis thumbergi*), and crayfish (*Cherax quadricarinatus*) in Lake Itezhi-tezhi and Lake Kariba, Zambia. Arch. Environ. Contam. Toxicol. 59, 291–300. https://doi.org/10.1007/s00244-010-9483-8.

Nash, R.D.M., Valencia, A.H., Geffen, A.J., 2006. The origin of Fulton's condition factor - setting the record straight. In: The Origin of Fulton's Condition Factor - Setting the Record Straight, 31. American Fisheries Society, Bethesda. https://folk.uib.no/nfiag/nfiag/reprints/Nashetal%202006FISH.pdf.

Nedzarek, A., Czerniejewski, P., Torz, A., 2019. Microelements and macroelements in the body of the invasive Harris mud crab (*Rhithropanopeus harrisii*, Maitland, 1874) from the central coast of the South Baltic Sea. Environ. Monit. Assess. 191 (8), 499. https://doi.org/10.1007/s10661-019-7564-3.

Niecke, M., Heid, M., Krüger, A., 1999. Correlations between melanin pigmentation and element concentration in feathers of white-tailed Eagles (*Haliaeetus albicilla*). J. Ornithol. 140, 355–362. https://doi.org/10.1007/BF01651032.

Olgunoglu, M., Olgunoglu, İ., 2016. Heavy metal contents in blue swimming crab from the northeastern Mediterranean Sea, Mersin Bay, Turkey. Pol J Environ. Stud. 25 (5), 2233–2237. https://doi.org/10.15244/pjoes/62795.

Ooi, C.K., 2016. A study of the relationship between heavy metal concentrations and muscle melanisation in southern sand flathead (*Platycephalus bassensis*) from Tasmania. Honours thesis. University of Tasmania, Launceston.

Ooi, C.K., Lewis, T., Nowak, B., Lyle, J., Haddy, J., 2022. The use of image analysis techniques for the study of muscle melanisation in sand flathead (*Platycephalus bassensis*). Environ. Pollut. 292, 118360 https://doi.org/10.1016/j.envpol.2021.118360.

Ooi, C.K., Ware, M., Lewis, T., Lyle, J., Nowak, B.F., 2019. Muscle melanisation in sand flathead (*Platycephalus bassensis*) and links to elevated zinc levels. Mar. Pollut. Bull. 149, 110493 https://doi.org/10.1016/j.marpolbul.2019.110493.

Parsons, K.E., 2012. State of the D'Entrecasteaux Channel and the Lower Huon Estuary 2012. Ecomarine Consulting, Kingston.

Rainbow, P.S., Phillips, D.J.H., Depledge, M.H., 1990. The significance of trace metal concentrations in marine invertebrates: a need for laboratory investigation of accumulation strategies. Mar. Pollut. Bull. 21, 321–324. https://doi.org/10.1016/0025-326X(90)90791-6.

Rainbow, P.S., White, S.L., 1989. Comparative strategies of heavy metal accumulation by crustaceans: zinc, copper and cadmium in a decapod, an amphipod and a barnacle. Hydrobiologia 174, 245–262. https://doi.org/10.1007/BF00008164.

Rajotte, J., Pyle, G., Couture, P., 2003. Indicators of chronic metal stress in wild yellow perch from metal-contaminated environments. In: Proceedings of the Paper Presented at the Conference Presentations, Mining and Environment, 28th annual Meeting. Conference Presentations, Mining and Environment, 28th Annual Meeting.

Ramos, P., Carvalho, R., Rosa, F., Alexandre-Pires, G., Seixas, F., Esteves, A., Huffman, D., 2019. *Huffmanela lusitana* sp. n. (Nematoda: Trichosomoididae) infecting pouting, *Trisopterus luscus* (Teleostei: Gadidae) off the Atlantic coast of Portugal. Int. J. Parasitol. Parasites Wildl. 9, 266–273. https://doi.org/10.1016/j.ijppaw.2019.05.010.

Reinecke, A.J., Snyman, R.G., Nel, J.A.J., 2003. Uptake and distribution of lead (Pb) and cadmium (Cd) in the freshwater crab, *Potamonautes perlatus* (crustacea) in the Eerste River, South Africa. Water Air Soil Pollut. 145, 395–408. https://doi.org/10.1023/A:1023602121272.

Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. J. Fish. Res. Board Can. 191, 1–382.

Sato, S., et al., 1999. Structure and developmental expression of the ascidian *TRP* gene: insights into the evolution of pigment cell-specific gene expression. Dev. Dyn. 215, 225–237. https://doi.org/10.1002/(SICI)1097-0177(199907)215, 3<225::AID-AJA5>3.0.CO;2-S.

Silva, B., Morales, G.P., Gutjahr, A.L.N., Freitas Faial, K.D.C., Carneiro, B.S., 2018. Bioacumulation of trace elements in the crab *Ucides cordatus* (Linnaeus, 1763) from the macrotidal mangrove coast region of the Brazilian Amazon. Environ. Monit. Assess. 190, 214. https://doi.org/10.1007/s10661-018-6570-1.

Snyman, R.G., Reinecke, A.J., Nel, J.A.J., 2015. Uptake and distribution of copper in the freshwater crab Potamonautes perlatus (Crustacea) in the Eerste River, South Africa. Afr. Zool. 37, 81–89. https://doi.org/10.1080/15627020.2002.11657158.

Solano, F., 2018. On the metal cofactor in the tyrosinase family. Int. J. Mol. Sci. 19 https://doi.org/10.3390/ijms19020633.

Spry, D.J., Hodson, P.V., Wood, C.M., 1998. Relative contributions of dietary and waterborne zinc in the rainbow trout, *Salmo gairdneri*. Can J Fish Aquat Sci 45, 32–41. https://doi.org/10.1139/f88-005 https://doi.org/.

Stocker, C., Lyle, J.M., Nowak, B., 2019. Fisher survey of muscle melanisation in sand flathead (*Platycephalus bassensis*). Institute for Marine and Antarctic Studies, Hobart.

Stocker, C.W., Haddy, J., Lyle, J., Nowak, B.F., 2020. Muscle melanisation of southern sand flathead (*Platycephalus bassensis*) in the Tamar Estuary, Tasmania, Australia. Environ. Pollut. 256, 113452 https://doi.org/10.1016/j.envpol.2019.113452.

J. Taylor, G. Poore (2020a) *Paragrapsus laevis* mottled shore crab in museums Victoria collections. Museums Victoria. https://collections.museumsvictoria.com.au/species/8671. Accessed 13 June 2021.

J. Taylor, G. Poore (2020b) *Paragrapsus quadridentatus* four-toothed shore crab in museums Victoria collections. Museums Victoria. https://collections.museumsvictoria.com.au/species/8672. Accessed 13 June 2021.

Taylor, U., Whitehead, S., Visby, I., Weller-Wong, A., Proemse, B., 2020. State of the Derwent Estuary - 2020 update. An update and review of environmental data and activities. Derwent Estuary Program, Hobart.

Turoczy, N.J., Mitchell, B.D., Levings, A.H., Rajendram, V.S., 2001. Cadmium, copper, mercury, and zinc concentrations in tissues of the King Crab (*Pseudocarcinus gigas*) from southeast Australian waters. Environ. Int. 27, 321–334. https://doi.org/10.1016/S0160-4120(01)00064-2.

US EPA (1996) Method 3052: Microwave assisted acid digestion of siliceous and organically based matrices. United States Environmental Protection Authority, Washington, D.C.

Verdouw, J.J., Macleod, C.K., Nowak, B.F., Lyle, J.M., 2010. Implications of age, size and region on mercury contamination in estuarine fish species. Water Air Soil Pollut. 214, 297–306. https://doi.org/10.1007/s11270-010-0424-y.

Wang, W.X., Rainbow, P.S., 2020. Environmental pollution of the Pearl River Estuary, China. Estuaries of the World, 1st ed. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-61834-9.

Weimin, Y., Batley, G.E., Ahsanullah, M., 1994. Metal bioavailability to the soldier crab *Mictyris longicarpus*. Sci. Total Environ. 141, 27–44. https://doi.org/10.1016/0048-9697(94)90015-9 https://doi.org/.

White, S.L., Rainbow, P.S., 1985. On the metabolic requirements for copper and zinc in molluscs and crustaceans. Mar. Environ. Res. 16, 215–229. https://doi.org/10.1016/0141-1136(85)90139-4.

Whitehead, J., Coughanowr, C., Agius, J., Chrispijn, J., Taylor, U., Wells, F., 2010. State of the Derwent Estuary 2009: a review of pollution sources, loads, and environmental quality data from 2003 - 2009. Department of Primary Industries, Parks, Water and Environment, Hobart.

Wogelius, R.A., et al., 2011. Trace metals as biomarkers for eumelanin pigment in the fossil record. Science 333, 1622–1626. https://doi.org/10.1126/science.1205748.

Xu, C., Letcher, B.H., Nislow, K.H., 2010. Context-specific influence of water temperature on brook trout growth rates in the field. Freshw. Biol. 55, 2253–2264. https://doi.org/10.1111/j.1365-2427.2010.02430.x.

Yamazaki, S., Rust, S., Jennings, S., Lyle, J., Frijlink, S., 2013. Valuing recreational fishing in Tasmania and assessment of response bias in contingent valuation. Aust. J. Agric. Resour. Econ. 57, 193–213. https://doi.org/10.1111/j.1467-8489.2012.00614.x.

Yap, C.K., et al., 2020. Human health risk assessments of trace metals on the clam *Corbicula javanica* in a tropical river in Peninsular Malaysia. Int J Environ Res Public Health 18. https://doi.org/10.3390/ijerph18010195.