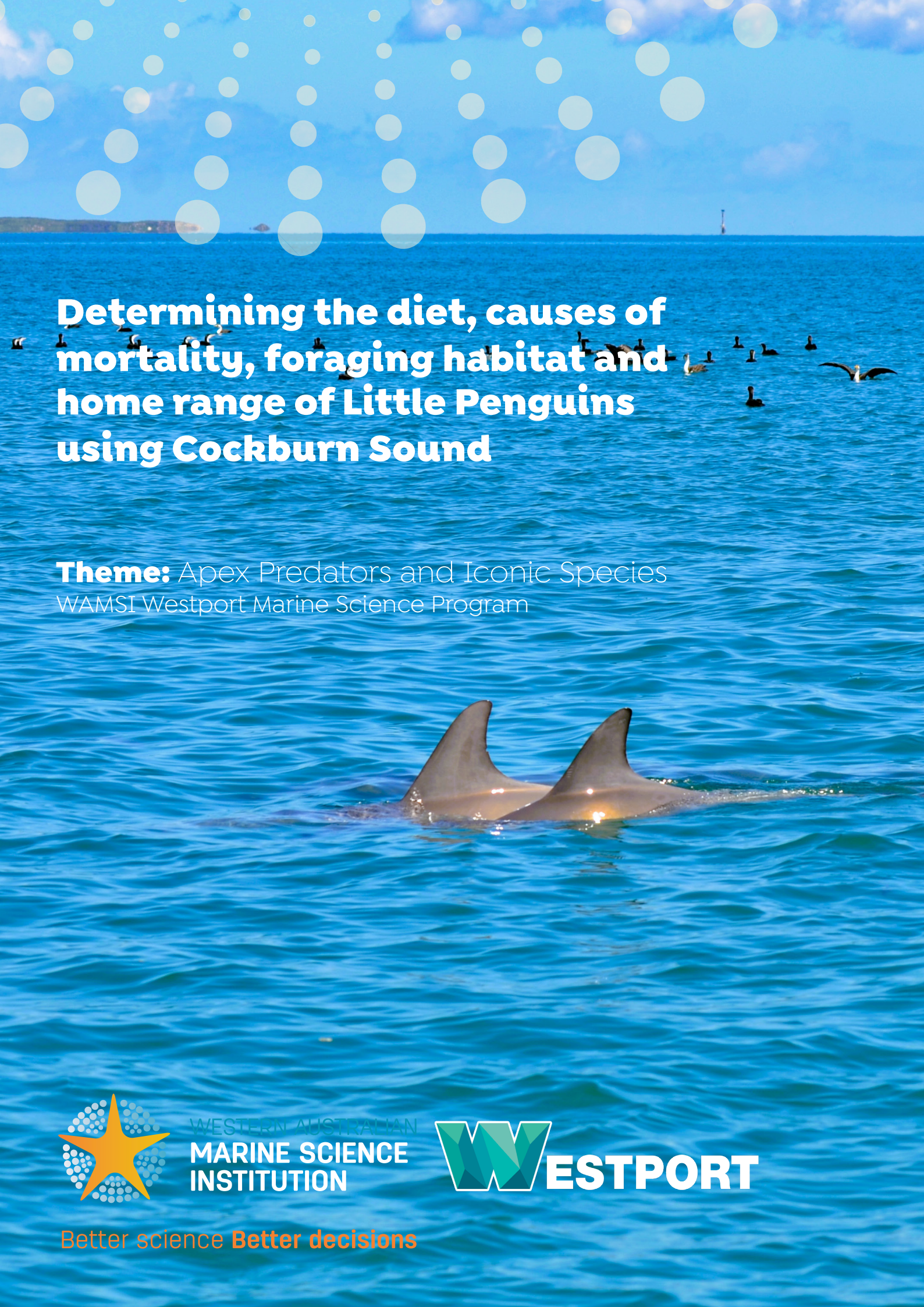
Determining the diet, causes of mortality, foraging habitat and home range of Little Penguins using Cockburn Sound

Theme: Apex Predators and Iconic Species
WAMSI Westport Marine Science Program



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ABOUT THE MARINE SCIENCE PROGRAM

The WAMSI Westport Marine Science Program (WWMSP) is a $13.5 million body of marine research funded by the WA Government. The aims of the WWMSP are to increase knowledge of Cockburn Sound in areas that will inform the environmental impact assessment of the proposed Westport development and help to manage this important and heavily used marine area into the future. Westport is the State Government's program to move container trade from Fremantle to Kwinana, and includes a new container port and associated freight, road and rail, and logistics. The WWMSP comprises more than 30 research projects in the biological, physical and social sciences that are focused on the Cockburn Sound area. They are being delivered by more than 100 scientists from the WAMSI partnership and other organisations.

FUNDING SOURCES
The $13.5 million WAMSI Westport Marine Science Program was funded by the Western Australian Government, Department of Transport. WAMSI partners provided significant in-kind funding to the program to increase the value to >$22 million.

DATA
Finalised datasets will be released as open data, and data and/or metadata will be discoverable through Data WA and the Shared Land Information Platform (SLIP).

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This report is for the project: Determining the diet, causes of mortality, foraging habitat and home range of little penguins using Cockburn Sound.

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FRONT COVER IMAGE
Theme: Apex predators and iconic species
Front cover image: A pod of dolphins in Cockburn Sound.
Photo courtesy of: Delphine Chabanne (Murdoch University).

Contents

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The WAMSI Westport Marine Science Program is a $13.5 million body of research that is designed to fill knowledge gaps relating to the Cockburn Sound region. It was developed with the objectives of improving the capacity to avoid, mitigate and offset environmental impacts of the proposed Westport container port development and increase the WA Government's ability to manage other pressures acting on Cockburn Sound into the future. Funding for the program has been provided by Westport (through the Department of Transport) and the science projects are being delivered by the Western Australian Marine Science Institution.

1 Determining the diet, causes of mortality, foraging habitat and home range of Little Penguins using Cockburn Sound

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Project
Project 8.1 Determining the diet, causes of mortality, foraging habitat and home range of little penguins using Cockburn Sound

Investigators
The molecular analysis of the faeces was conducted at Murdoch University by Drs Angus Lawrie and Jennifer Chaplin with samples collected by Dr Belinda Cannell. Modelling of faecal sample prey composition with environmental variables was conducted by Dr Cannell. Discussion of the results was written by Drs Lawrie, Chaplin and Cannell.

All other components were conducted at UWA by Dr Belinda Cannell, with technical assistance from Sandra McNeill.

Executive Summary

Little penguins are a listed marine species under the *Commonwealth Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act*). The species is not listed as Threatened and Priority Fauna under the Western Australian *Biodiversity Conservation Act 2016*. However, the conservation of little penguins under the *Act* is provided for, given that the objectives of the *Act* are "to conserve and protect biodiversity and biodiversity components in the State; and to promote the ecologically sustainable use of the biodiversity components in the State (s3)." Furthermore, "native species, habitats, ecological communities, genes, ecosystems and ecological processes" are included as components of biodiversity. Additionally little penguins in the Perth metropolitan region were assessed as having the highest relative threat and the highest conservation value of all marine fauna in the same region (Department of Conservation and Land Management 2003). They are also listed as a key performance indicator for the Shoalwater Islands Marine Park (Department of Environment and Conservation 2007).

This report presents:

1) Data on the most common prey taxa and the total number of prey taxa, focussing on fishes, detected in little penguin faecal samples from Garden Island collected from 2020 - 2023.
2) Stable isotopes (SI) values of adult feathers collected from summer 2020 - mid 2023, and chick down collected from 2020 - 2023.
3) The results from surveys for beach-washed penguins on the shores of Cockburn Sound, and the results of necropsies from any dead penguins found during those surveys or on Garden Island from 2021 - 2023.

Little penguin faecal samples were collected from June 2020 to July 2023 from chicks and adults. A total of 107 faecal samples were analysed. DNA was extracted from two subsamples per sample and each subsample was sequenced twice, giving four assays per sample. Short fragments of the mitochondrial 16S were sequenced to assess the fish diet of little penguins. The 16S SHORT primers were chosen to amplify a portion of this gene because these primers have been shown to be able to identify a variety of different fish to genus or species level. We also used a short fragment nuclear 18S gene to test for the presence of invertebrate prey in a subset of 26 samples. The 18S primers amplify broadly across a range of eukaryotes with taxonomic resolution to above order or family level depending on the taxa.

We filtered the data and developed two datasets. Dataset A (10% contribution threshold) provides a conservative assessment of the little penguin diet and given the high stringency of the filtering likely includes some false negatives (e.g., excludes rare but present species). Dataset B (1% contribution threshold) may be more sensitive to false positives than Dataset A however a 1% threshold has been suggested as a suitable cut-off presence/absence point in dietary faecal studies. For the 16S analysis, a total of 14 prey taxa from 10 different families were detected in the samples across the four years for Dataset A, and 19 prey species from 13 different families for Dataset B. Across all four years, anchovy (*Engraulis australis*), pilchard (*Sardinops sagax*) and sandy sprat (*Hyperlophus vittatus*) were the most common prey species, found in more than half of the samples. while *Sardinella* sp. (likely *S. lemuru*, hereafter referred to as sardine) and garfish *(Hyporhamphus melanochir*) were found in approximately 20% of samples. However, the frequency of occurrence, and dominant species, varied between years. The 18S dataset is limited for several reasons but supports the idea that the little penguins on Garden Island mainly consumed fish (rather than invertebrates) during the study period, but also identified the presence, albeit uncommon, of jellyfish and squid.

Diversity analysis of the fish prey identified in each faecal sample showed that the dietary diversity profile for the little penguins in 2020 – 2022 were similar, but their diet in 2023 consisted of significantly more typical and dominant species.

Moult feathers were collected from 66 adults from the summers of 2018/19 - 2023/24. Chick down was collected from 36 chicks during the breeding season in the years 2020 - 2023. Additionally, contour feathers were collected from four deceased chicks in 2023. Samples were analysed for stable isotopes (SI) of $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$, and the SI in the adult feathers represent the foraging of the non-breeding/pre-moult penguins the summer prior to collection. Isotopes in the chick down and contour feathers represent the foraging of the adult penguins a few weeks prior to collection. We investigated potential differences in $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values and the isotopic niche width in:

- Adult feathers between 2018/19, 2019/20, 2020/21, 2021/22 and 2022/23.
- Between chick down/contour feathers collected during the breeding season in 2020, 2021 and 2022 with the adult feathers that were synthesized following that breeding season, i.e. between the breeding and non-breeding/pre-moult penguins.
- The chick down/contour feathers collected in the breeding seasons of 2020, 2021, 2022 and 2023.

The non-breeding/pre-moult penguins foraging in 2019/20 and 2022/23 were feeding more inshore and/or on prey that have a greater access to benthic carbon sources, i.e. seagrasses, comparative to 2018/19, 2020/21 and 2021/22 (higher average $\delta^{13}C$). From the trophic niche analyses, the non-breeding/pre-moult penguins in 2020/21 foraged on the smallest range of prey, whilst those in 2022/23 foraged on the widest range of prey. However, in 2022/23, they generally foraged on prey that were of a lower trophic level (lower $\delta^{15}N$) than in other years.

Comparing the breeding and non-breeding/pre-moult penguins, the trophic niche of the latter was much greater than that of the breeding penguins in the same year, indicating that the penguins were feeding on a wider range of prey when not breeding. In 2020 and 2021, the isotopic niche of the

breeding penguins was completely distinct to that of the non-breeding/pre-moult penguins, whereas the breeding penguins in 2022 completely shared the isotopic niche of non-breeding/pre-moult penguins that year.

We found differences in the SI values between the breeding seasons, but this differed between isotope and year. The most enriched value of $\delta^{13}C$ was in 2021. It is likely that the penguins were feeding on fish that have spawned in the river and moved into Cockburn Sound or were preying on more benthic fish species in this year. The widest trophic niche for all isotopic pair plots occurred in 2023. The wider plot of $\delta^{34}S$ and $\delta^{13}C$ prey in 2023 indicates that the prey came from a mixture of benthic and pelagic sources.

Bayesian mixed models were run to determine the dietary composition of breeding penguins in 2023. The greatest proportion of the diet was from a group of fish that included anchovy, sandy sprat, sardine, garfish and skipjack trevally (*Pseudocaranx wrighti*). Jellyfish were the second most important dietary component.

A program was established to regularly check the beaches on the eastern margin of Cockburn Sound and was composed of community volunteers and employees of industries adjacent to the foreshore. The eastern foreshore was divided into 18 segments, approximately 1 km long. A total of 865 surveys were conducted from February 2022 to the end of January 2024. No penguins were found dead during the surveys, but one penguin was found deceased on Garden Island in August 2022. A basic necropsy was performed on it at the DPIRD Diagnostics and Laboratory Services, the penguin did not die from starvation. It had no external or internal injuries. No definitive cause of death was identified. An injured penguin was found on Garden Island on 23/1/23. More than half of its left flipper had been amputated and it had a laceration to its left foot. These injuries were consistent with a boat strike. The penguin was euthanised.

As part of another project for the Department of Defence (DoD) (by Cannell), the foraging habit and home range of breeding penguins from Garden Island was determined in 2022 and 2023. The maps have been supplied for this report with permission from DoD.

In 2022 and 2023, data were obtained from satellite tags deployed on eight penguins during the incubation stage of breeding, and from GPS tags deployed on four penguins during the chick guard-stage. The penguins conducted trips that ranged from 1 – 9 days during the incubation period, but only single day trips occurred during the chick-guard phase. During the incubation period the penguins remained within Cockburn Sound, using the eastern margin, central basin, western margin and Kwinana Shelf, with some penguin overlapping with the Stage 3 Preferred Option for the Port. During the chick-rearing period, the penguins core foraging habitat was located in the southern half of Cockburn Sound (2022 - data from three penguins) and in the north-west of Cockburn Sound (2023 - data from one penguin).

Acknowledgements

This project was made possible by the generous donation of time by many volunteers, who conducted the surveys along the beaches on the Eastern margins of the Cockburn Sound foreshore. Collection of faecal and feather samples, and tagging of penguins to determine foraging habitat, were permissible with multiple permits: UWA animal ethics permits- 2019/RA/3/100/1578, 2022/ET001021; Department of Biodiversity, Conservation and Attractions Permits F025000163-5 and F025000163-6; Department of Agriculture, Water and the Environment Permit E2020-0177, and Department of Climate Change, Energy, the Environment and Water Permit E2023-0225.

We thank WWMSP Project *Spatial distribution and temporal variability in life stages of key fish species in Cockburn Sound* for the collection of fish samples used in the Stable Isotope Analysis (SIA). We also thank WWMSP Project *Trophic pathways and food web structure of Cockburn Sound and Owen Anchorage* for conducting the SIA of $\delta^{15}N$, $\delta^{13}C$ (lipid not removed) in likely penguin prey samples and $\delta^{34}S$ in a subset of likely prey samples, in November 2021 and May 2022.

2 Introduction

In WA, little penguins are found on offshore islands, and their distribution extends from Carnac Island, 10 km south-west of Fremantle, through to the Recherché Archipelago, i.e. within the South-west Marine Bioregion. Within this region, little penguins were identified as a regional priority for conservation (Department of Sustainability, Environment, Water, Population and Communities 2012). The northern-most colonies are located in the Perth metropolitan region, on Penguin, Garden and Carnac islands. Little penguins are a listed marine species under the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act*). The species is not listed as Threatened and Priority Fauna under the Western Australian *Biodiversity Conservation Act 2016*. However, the conservation of little penguins under the *Act* is provided for, given that the objectives of the *Act* are "to conserve and protect biodiversity and biodiversity components in the State; and to promote the ecologically sustainable use of the biodiversity components in the State (s3)." Furthermore, "native species, habitats, ecological communities, genes, ecosystems and ecological processes" are included as components of biodiversity. Furthermore, little penguins in the Perth metropolitan region were assessed as having the highest relative threat and the highest conservation value of all marine fauna in the same region (Department of Conservation and Land Management 2003). They are also listed as a key performance indicator for the Shoalwater Islands Marine Park (Department of Environment and Conservation 2007).

On both Penguin and Garden islands, the penguins have a protracted breeding season, with eggs laid any time from April-December (Wooller et al. 1991, Cannell 2004, Cannell et al. 2012, Cannell et al. 2024). A clutch of two eggs is generally laid, and two clutches can be laid in a season. Both parents share incubation and chick rearing. The eggs are incubated for approximately five weeks with the parents swapping every 3 - 12 (or more) days (Chiaradia & Kerry 1999, Cannell 2016, 2019). Once hatched, the parents take turns guarding the chick/s, swapping every 1-2 days (Chiaradia & Kerry 1999, Cannell 2018, 2019). The guard phase extends for 2 - 3 weeks, after which both parents forage during the day. However, the parents alternate between long trips that last for several days, and short trips, lasting for one day (Saraux et al. 2011) The number of long trips they take is related to the mass of the adult (Saraux et al. 2011). Regardless of whether they conduct a long or short trip. they return in the evening to feed the chicks. The chicks leave the nest when they are fully fledged, i.e. have the "adult" contour feathers, which occurs when the chicks are approximately eight weeks old. Little penguins have a high site fidelity, returning to the same nest, or one close by, each year. They also return to their natal colony, i.e. the one where they hatched.

Breeding penguins from Garden Island have been found to forage within Cockburn Sound, and the penguins that inhabit the north-east side of Penguin Island typically foraged within Cockburn Sound, but during the incubation period (Cannell 2009, 2016, 2019). Little penguins prey on small baitfish and cephalopods and are regarded as a generalist predator. Previous studies of the little penguins foraging within Cockburn Sound found they predominantly preyed on sardine (*Sardinella lemuru*), anchovy (*Engraulis australis*) and pilchard (*Sardinops sagax*). To a lesser extent they preyed on sandy sprat (*Hyperlophus vittatus*), blue sprat (*Spratelloides robustus*), silverbelly (*Parequula melbournensis*), hardyheads (*Atherinomorus vaigiensis*) and sea mullet (*Mugil cephalus*) (Oliver 2009, Cannell et al. 2011, Cannell et al. 2013a, Murray et al., 2011 and Cannell unpubl. data). These data were generated using both regurgitant samples and molecular studies (DNA analysis) of penguin faeces. DNA analysis employs metabarcoding, which involves PCR-amplification and high throughput sequencing (HTS) of taxonomically informative DNA markers, i.e. barcodes (Pompanon et al. 2012). This method is non-intrusive and can be used to identify prey items from faecal samples to genus or species-level (Ando et al. 2020, Bowser et al. 2013, Liu et al. 2021).

The stable isotope (SI) analysis of feathers is another method to investigate the foraging ecology of seabirds when the feathers were synthesized (Cherel et al. 2008). The synthesis of feathers in adult little penguins occurs just prior to their complete (i.e. catastrophic) moult, which, on Garden Island, is

typically any time between November and February (Cannell unpubl. data). The feather synthesis generally begins when they are at sea (Cherel et al. 1994) and is completed when they are confined to land for 2 - 3 weeks during their catastrophic moult. Thus, the SI signature in the adult feathers represents the foraging ecology of the non-breeding/pre-moult adults in the summer period the year prior to collection, but potentially with some enrichment from the penguin's own tissues. The synthesis of mesoptyle down in chicks occurs at approximately four weeks of age and can be collected once the chicks begin to synthesize their adult contour feathers, from approximately six weeks of age. As chicks are solely fed by their parents until they permanently leave the nest, then the SI signature in the chick down represents the foraging ecology of the adults a few weeks prior to collection of the chick down or contour feathers.

The SI signature of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in the penguin feathers and chick down/ contour feathers details different aspects of the foraging ecology. For example, $\delta^{13}C$ reflects primary production sources and is more enriched in productive inshore areas and benthic food webs, i.e. seagrass dominated areas, compared to offshore and pelagic food webs dominated by phytoplankton-derived material (e.g. Hobson et al. 1994, Cherel & Hobson 2007, Morkūnė et al. 2016). It can also vary between size classes of the same prey species (Kowalczyk et al. 2015). $\delta^{15}N$ reflects the trophic level of a consumer, increasing at each trophic level (e.g. Hobson & Clark 1992, Bearhop et al. 2002), and increasing as size classes of the same prey species get larger (Bearhop et al. 2006). As noted above, $\delta^{15}N$ can be enriched from the penguin's tissues during the catastrophic moult, and the trophic level of prey consumed by the adult penguins must be interpreted cautiously (Cherel et al. 2005, Ceia et al. 2021). Despite this, recent studies on chinstrap (*Pygoscelis antarctica*) and gentoo (*P. papua*) penguins found no difference in the $\delta^{15}N$ values between adults and chicks, suggesting that similar prey was consumed by both the non-breeding and breeding birds (Ceia et al. 2021). $\delta^{34}S$ can be useful to distinguish between pelagic/offshore and benthic/inshore components in food webs (e.g. Connolly et al. 2004, Louis et al. 2014, Morkūnė et al. 2016). It can also indicate if producers are utilising sulfur (S) from seawater (more enriched) or from sediments (less enriched) (Connolly et al. 2004). Additionally, the determination of the isotopic niche width of seabirds (Jackson et al. 2011, Lavoie et al. 2012, Ceia et al. 2014) allows for direct intra- and inter-annual comparison between seabird communities (Bearhop et al. 2004, Jackson et al. 2011, Calado et al. 2018). The isotopic niche is obtained using a bivariate plot of the $\delta^{13}C$ and $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ or $\delta^{34}S$ and $\delta^{15}N$ value of each sample (Bearhop et al. 2004, Newsome et al. 2007, Jackson et al. 2011, Rossman et al. 2016). It is particularly informative for the non-breeding season (Hobson & Bond 2012), when penguins are not only often absent, but can also tend to range further from their breeding/roosting colony.

Lipid content in tissues can alter their $\delta^{13}C$ values, and thus variability in these values can potentially be misinterpreted as changes in the diet or habitat (Logan et al. 2008). For example, samples with a high lipid concentration can be 3 - 4‰ more negative than samples in which the lipid has been extracted. In contrast, samples with a low lipid concentration are unlikely to show any difference in the $\delta^{13}C$ values in samples with and without lipid extraction (Post et al. 2007). Variability in $\delta^{13}C$ values can be corrected either by extracting lipids in the samples prior to analysis, or by using mathematical corrections after the analysis (Logan et al. 2008). But there is no consensus as to the best method to account for lipids (Post et al. 2007). Additionally, $\delta^{15}N$ values may alter following the extraction of lipids, due to the loss of some non-lipid compounds. Therefore, it is necessary to analyse both the untreated samples for $\delta^{15}N$ and treated samples for $\delta^{13}C$ (Sweeting et al. 2006). Notably, feathers do not require delipidation before analysis (Cherel et al. 2008).

In a recent study on the causes of mortality of little penguins from Penguin and Garden islands, the most prevalent cause was due to watercraft injury, accounting for 25% of penguin carcases necropsied. The second-most prevalent cause of mortality was starvation (Cannell et al. 2016). High incidence of starvation was observed in carcases found in 2011 (during the severe marine heatwave), in mid-2017 (Cannell et al. 2024), and in mid-2021. More than four times the average number of dead penguins were found in 2011, and most of these were emaciated (Cannell et al. 2019). Twenty-seven dead

penguins were found in July-September 2021 in WA (mostly in the Perth metropolitan region), with at least 12 of these being emaciated. This is 10 times the average number of dead penguins found for these months (excluding the large number found in 2011, see Cannell et al. 2024), and at least double the average number to have died from starvation in these months (it is possibly greater given the cause of mortality for a third of the dead penguins has not yet been identified; Cannell unpubl. data). This event coincided with increased tannins, chlorophyll blooms and turbidity in Cockburn Sound from unprecedented winter rainfall (Pattiaratchi & Thomson 2021). *Toxoplasma* and/or *Haemoproteus* protozoal infections have also been implicated as the cause of mortality of penguins from Penguin Island in 2011 – 2013 (Cannell et al. 2013b, Campbell et al. 2022, Cannell et al. 2024).

2.1 Project Aims

The aims of the project are:

1) Obtain annual diet composition and temporal changes in diet composition of penguins, using:
    a. DNA analysis in penguin faeces, and
    b. Stable isotope (SI) analysis in adult penguin feathers and chick down/contour feathers.
2) Determine causes of mortality of penguins and temporal changes in the numbers of dead penguins found within the Cockburn Sound area.
3) Determine foraging habitat and home range of breeding penguins (externally funded by DoD, habitat maps are included in this final report with permission from DoD)
4) Determine if any plausible and viable mitigation options are available to prevent penguins from potentially starving during the dredging campaign (completed and published in a separate report – Cannell 2023).

This final report presents:

1a) Data on the most common prey taxa and the total number of prey taxa, focussing on fishes, detected in little penguin faecal samples from Garden Island collected from 2020-2023.

1b) SI values of feathers and down collected from 2020-December 2023, and SI values of potential penguin fish prey collected in summer 2021, winter 2022, summer 2022 and winter 2023. These data will be used to inform the proposed dietary components of the penguins where possible. Adult moult feathers collected in summer 2022/23 were grown in summer 2021/22, i.e. aligns with fish collected in summer 2021, and chick down collected in spring 2022 most closely aligns with fish collected in winter 2022. Similarly adult moult feathers collected in summer 2023/24 were grown in summer 2022/23, i.e. aligns with fish collected in summer 2022, and chick down/contour feathers collected in spring 2023 most closely aligns with fish collected in winter 2023.

1c) Comparison of SI values of $\delta^{15}N$, $\delta^{13}C$ in fish samples with and without lipid removed from the fish sample.

2) Results of the surveys for beach-washed penguins, and the results of any necropsies

3) Maps of the foraging habitat and home range of the penguins. Note that this aspect of the study has not been funded by Westport, and as such no associated data other than jpgs of the maps will be supplied.

3 Materials and Methods

3.1 Diet

3.1.1 DNA analysis of faeces

Sample collection

A total of 128 little penguin faecal samples were collected from June 2020 to July 2023 from chicks and adults with 107 samples selected for analysis in the study (Table 1). Not all samples collected could be analysed due to the financial costs of the DNA metabarcoding approach. Faecal samples were collected either directly from penguins or substrates and stored in 1.5 ml Eppendorf tubes in 100% ethanol until analysis.

Table 1. Summary of all samples included in the study. GI = Garden Island, PI = Penguin Island. The Penguin Island samples were included as positive faecal samples (see text for more details). 16S Dataset A/Dataset B and 18S Dataset A/Dataset B = whether the sample passed the filtering for Dataset A/Dataset B criteria for that dataset as laid out in the methods. FS = faecal sample, PFS = positive faecal sample, FP = fish positive, SP = shark positive, SDP = squid positive, JP = jellyfish positive, GP = gastropod positive, CP = crab positive, PP = prawn positive and N = DNA negative. *fish mix contains sandy sprat, pilchard, sardine and blue sprat.

Sample ID	Sample type	Date collected	Island	16S Dataset A/Dataset B	18S Dataset A/Dataset B
GI 2020 01	FS	28/02/2020	GI	No/Yes	
GI 2020 02	FS	28/02/2020	GI	Yes/Yes	
GI 2020 03	FS	15/05/2020	GI	Yes/Yes	
GI 2020 04	FS	15/05/2020	GI	Yes/Yes	
GI 2020 05	FS	12/06/2020	GI	Yes/Yes	
GI 2020 06	FS	10/07/2020	GI	Yes/Yes	
GI 2020 07	FS	10/07/2020	GI	Yes/Yes	
GI 2020 08	FS	10/07/2020	GI	Yes/Yes	
GI 2020 09	FS	10/07/2020	GI	Yes/Yes	
GI 2020 10	FS	5/08/2020	GI	Yes/Yes	
GI 2020 11	FS	4/09/2020	GI	No/No	
GI 2020 12	FS	4/09/2020	GI	No/Yes	
GI 2020 13	FS	2/10/2020	GI	Yes/Yes	
GI 2020 14	FS	2/10/2020	GI	Yes/Yes	
GI 2020 15	FS	27/10/2020	GI	No/No	
GI 2020 16	FS	27/10/2020	GI	Yes/Yes	
GI 2020 17	FS	27/11/2020	GI	Yes/Yes	
GI 2020 18	FS	27/11/2020	GI	Yes/Yes	
GI 2021 19	FS	6/04/2021	GI	Yes/Yes	
GI 2021 20	FS	19/05/2021	GI	Yes/Yes	
GI 2021 21	FS	1/06/2021	GI	Yes/Yes	
GI 2021 22	FS	1/06/2021	GI	Yes/Yes	
GI 2021 23	FS	1/06/2021	GI	Yes/Yes	
GI 2021 24	FS	1/06/2021	GI	Yes/Yes	
GI 2021 25	FS	1/06/2021	GI	Yes/Yes	
GI 2021 26	FS	1/06/2021	GI	Yes/Yes	
GI 2021 27	FS	1/06/2021	GI	Yes/Yes	Yes/Yes
GI 2021 28	FS	1/06/2021	GI	Yes/Yes	Yes/Yes
GI 2021 29	FS	1/06/2021	GI	Yes/Yes	
GI 2021 30	FS	2/06/2021	GI	Yes/Yes	
GI 2021 31	FS	2/06/2021	GI	Yes/Yes	
GI 2021 32	FS	2/06/2021	GI	Yes/Yes	
GI 2021 33	FS	16/06/2021	GI	Yes/Yes	
GI 2021 34	FS	16/06/2021	GI	Yes/Yes	
GI 2021 35	FS	13/07/2021	GI	No/No	
GI 2021 36	FS	21/09/2021	GI	Yes/Yes	
GI 2021 37	FS	19/10/2021	GI	Yes/Yes	

GI 2021 38	FS	19/10/2021	GI	Yes/Yes	
GI 2021 39	FS	20/10/2021	GI	Yes/Yes	
GI 2021 40	FS	16/11/2021	GI	Yes/Yes	No/Yes
GI 2021 41	FS	16/11/2021	GI	No/Yes	
GI 2021 42	FS	30/11/2021	GI	Yes/Yes	
GI 2021 43	FS	14/12/2021	GI	Yes/Yes	No/Yes
GI 2021 44	FS	14/12/2021	GI	Yes/Yes	
GI 2021 45	FS	28/12/2021	GI	Yes/Yes	Yes/Yes
GI 2021 46	FS	29/12/2021	GI	Yes/Yes	Yes/Yes
GI 2022 01	FS	24/01/2022	GI	Yes/Yes	No/Yes
GI 2022 02	FS	2/02/2022	GI	Yes/Yes	No/No
GI 2022 03	FS	3/05/2022	GI	Yes/Yes	
GI 2022 04	FS	31/05/2022	GI	Yes/Yes	Yes/Yes
GI 2022 05	FS	31/05/2022	GI	Yes/Yes	
GI 2022 06	FS	14/06/2022	GI	Yes/Yes	
GI 2022 07	FS	14/06/2022	GI	Yes/Yes	
GI 2022 08	FS	12/07/2022	GI	Yes/Yes	
GI 2022 09	FS	12/07/2022	GI	Yes/Yes	
GI 2022 10	FS	9/08/2022	GI	Yes/Yes	
GI 2022 11	FS	23/08/2022	GI	Yes/Yes	
GI 2022 12	FS	7/09/2022	GI	Yes/Yes	
GI 2022 13	FS	9/08/2022	GI	Yes/Yes	
GI 2022 14	FS	20/09/2022	GI	Yes/Yes	
GI 2022 15	FS	20/09/2022	GI	Yes/Yes	
GI 2022 16	FS	4/10/2022	GI	Yes/Yes	Yes/Yes
GI 2022 17	FS	18/10/2022	GI	Yes/Yes	No/No
GI 2022 18	FS	19/10/2022	GI	No/No	No/Yes
GI 2022 19	FS	19/10/2022	GI	Yes/Yes	No/No
GI 2022 20	FS	21/10/2022	GI	No/No	No/Yes
GI 2022 21	FS	1/11/2022	GI	Yes/Yes	No/Yes
GI 2022 22	FS	27/12/2022	GI	No/No	
GI 2022 23	FS	19/04/2022	GI	Yes/Yes	
GI 2022 24	FS	19/04/2022	GI	Yes/Yes	
GI 2022 60	FS	24/01/2022	GI	Yes/Yes	
GI 2022 61	FS	3/05/2022	GI	Yes/Yes	
GI 2022 62	FS	3/05/2022	GI	Yes/Yes	
GI 2022 63	FS	23/08/2022	GI	Yes/Yes	
GI 2022 64	FS	7/09/2022	GI	Yes/Yes	
GI 2022 65	FS	7/09/2022	GI	Yes/Yes	
GI 2022 66	FS	26/10/2022	GI	No/Yes	
GI 2022 67	FS	1/11/2022	GI	Yes/Yes	
GI 2022 68	FS	17/05/2022	GI	Yes/Yes	
GI 2022 69	FS	4/10/2022	GI	No/Yes	
GI 2023 01	FS	21/02/2023	GI	Yes/Yes	
GI 2023 02	FS	21/02/2023	GI	Yes/Yes	Yes/Yes
GI 2023 03	FS	21/02/2023	GI	No/No	

GI 2023 04	FS	4/04/2023	GI	Yes/Yes	
GI 2023 05	FS	4/04/2023	GI	Yes/Yes	Yes/Yes
GI 2023 06	FS	2/05/2023	GI	Yes/Yes	
GI 2023 07	FS	2/05/2023	GI	Yes/Yes	Yes/Yes
GI 2023 08	FS	16/05/2023	GI	Yes/Yes	Yes/Yes
GI 2023 09	FS	16/05/2023	GI	Yes/Yes	Yes/Yes
GI 2023 10	FS	16/05/2023	GI	Yes/Yes	
GI 2023 11	FS	13/06/2023	GI	Yes/Yes	
GI 2023 12	FS	13/06/2023	GI	Yes/Yes	No/Yes
GI 2023 13	FS	13/06/2023	GI	Yes/Yes	Yes/Yes
GI 2023 14	FS	19/06/2023	GI	Yes/Yes	
GI 2023 15	FS	27/06/2023	GI	Yes/Yes	Yes/Yes
GI 2023 16	FS	27/06/2023	GI	No/No	
GI 2023 17	FS	11/07/2023	GI	Yes/Yes	Yes/Yes
GI 2023 18	FS	24/07/2023	GI	Yes/Yes	Yes/Yes
GI 2023 19	FS	24/07/2023	GI	No/Yes	
GI 2023 20	FS	24/07/2023	GI	Yes/Yes	Yes/Yes
GI 2023 21	FS	24/01/2023	GI	Yes/Yes	
GI 2023 22	FS	27/06/2023	GI	Yes/Yes	
GI 2023 23	FS	25/07/2023	GI	No/No	
GI 2023 30	FS	13/06/2023	GI	Yes/Yes	
GI 2023 31	FS	11/07/2023	GI	Yes/Yes	
GI 2023 32	FS	8/02/2023	GI	Yes/Yes	
GI 2023 33	FS	13/06/2023	GI	Yes/Yes	
P1 02	PFS	14/06/2012	GI	NA/NA	
P1 102	PFS	11/10/2017	PI	NA/NA	
P1 120	PFS	10/11/2017	PI	NA/NA	
P1 139	PFS	24/10/2018	PI	NA/NA	
P1 142	PFS	5/11/2018	PI	NA/NA	
P1 160	PFS	22/05/2015	GI	NA/NA	
P1 177	PFS	24/07/2015	PI	NA/NA	
P1 194	PFS	25/07/2016	GI	NA/NA	
P1 20	PFS	22/08/2014	GI	NA/NA	
P1 76	PFS	27/08/2014	PI	NA/NA	
P1 78	PFS	31/08/2014	PI	NA/NA	
P1 88	PFS	21/10/2016	PI	NA/NA	
P1 89	PFS	1/11/2016	PI	NA/NA	
P1 91	PFS	10/07/2017	PI	NA/NA	
Carcharhinus plumbeus	SP			NA	
Coxiella sp.	GP				NA/NA
Four fish mix*	FP			NA/NA	
H. vittatus	FP			NA/NA	
Sardinella sp.	FP			NA/NA	
S. sagax	FP			NA/NA	
Sepioteuthis sp.	SDP			NA	NA/NA
S. robustus	FP			NA/NA	NA/NA

Penaeus latisulcatus	PP				NA/NA
Portunus armatus	CP				NA/NA
Pseudorhiza haeckeli	JP				NA/NA
Aurelia cf. *aurita*	JP				NA/NA
DNA Neg 01	N			NA/NA	NA/NA
DNA Neg 02	N			NA/NA	NA/NA
DNA Neg 03	N			NA/NA	NA/NA
DNA Neg 04	N			NA/NA	NA/NA
DNA Neg 05	N			NA/NA	
DNA Neg 06	N			NA/NA	
DNA Neg 07	N			NA/NA	
DNA Neg 08	N			NA/NA	
DNA Neg 09	N			NA/NA	
DNA Neg 10	N			NA/NA	
DNA Neg 11	N			NA/NA	
DNA Neg 12	N			NA/NA	
DNA Neg 13	N			NA/NA	
DNA Neg 14	N			NA/NA	
DNA Neg 15	N			NA/NA	
DNA Neg 16	N			NA/NA	
DNA Neg 17	N			NA/NA	
DNA Neg 18	N			NA/NA	
DNA Neg 19	N			NA/NA	
DNA Neg 20	N			NA/NA	
DNA Neg 21	N			NA/NA	
DNA Neg 22	N			NA/NA	
DNA Neg 23	N			NA/NA	
DNA Neg 24	N			NA/NA	
DNA Neg 25	N			NA/NA	
DNA Neg 26	N			NA/NA	
DNA Neg 27	N			NA/NA	
DNA Neg 28	N			NA/NA	
DNA Neg 29	N			NA/NA	
DNA Neg 30	N			NA/NA	

Sample processing and DNA extractions

Prior to extraction, each faecal sample was homogenised into a consistent paste through vigorous grinding with metal probes and repeated vortexing. Since DNA may not be uniformly distributed throughout a faecal sample (Mumma et al. 2016), two subsamples were taken from each faecal sample and put into different 1.5 mL Eppendorf tubes. Each subsample consisted of approximately 50 ng of faecal material. To remove ethanol, each subsample was washed with ddH_20, centrifuged and had the supernatant removed. This process was repeated twice. These assays were then subjected to whole genomic DNA extraction using a Maxwell ® 16 Tissue DNA Purification kit (Promega) following the manufacturer's instructions.

DNA metabarcoding

We selected a total of 107 faecal samples for analysis (see Table 1). DNA was extracted from two subsamples per sample and each subsample was sequenced twice, giving four assays per sample (Table 2; Figure 1). Subsampling and assay replication is crucial for metabarcoding studies due to the variability in the DNA extraction, PCR and sequencing processes (Mata et al. 2019).

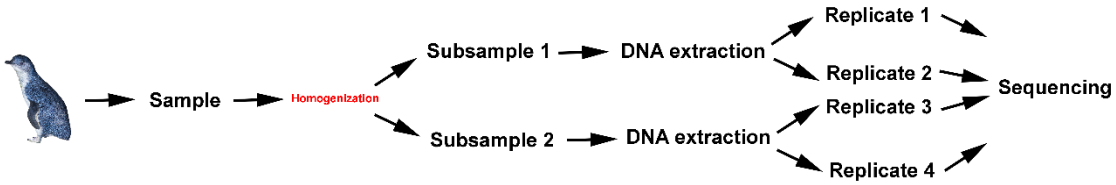


Figure 1. Example of workflow for the 107 sequenced faecal samples used in this study.

A 150 bp section of the mitochondrial 16S region was used to assess the fish diet of little penguins. This gene region was amplified using the 16S SHORT forward (5' TCA or ATGCGAGAAGACCCTRTGGAGCT 3') and reverse (5' TATCCTNGGTCGCCCCAAC 3') primers of Deagle et al. (2010). A blocking primer (5' GTGGAACTTGAAAATCAGCGACCACCA C3 3') also from Deagle et al. (2010) that inhibits the annealing of the 16S SHORT forward primer to little penguin DNA was used to prevent little penguin DNA from dominating the sequencing results. The 16S SHORT primers were chosen because fish have been shown to comprise a significant portion of the diet of little penguins (Cullen et al. 1991, Klomp and Wooller 1988, Murray et al. 2011) and these primers have been shown to be able to amplify the target region from a variety of different fish taxa (Deagle et al. 2010). In order to gain a preliminary understanding of the broader (e.g. invertebrate) diet of little penguins a 140 – 170 bp region of the nuclear 18S gene was also amplified for a subset of samples (26 samples; Table 1; Table 2) using the SSU3'F (CACCGCCCGTCGCTACTACCG) and SSU3'R (GGTTCACCTACGGAAACCTTGTTACG) primers from Jarman et al. (2013). These primers have been shown to amplify broadly across a range of eukaryotes with taxonomic resolution to above order or family level depending on the taxa (Jarman et al. 2013). A blocking primer (5' CCTTGTTACGACTTTTACTTCCTCTAGATAG# '3) that is designed to suppress the amplification of all tetrapods (including penguins) was also used.

DNA extractions were sent to the commercial laboratory Australian Genome Research Facility for library preparation and sequencing on an Illumina MiSeq platform.

Table 2. Breakdown of sampling design used to produce 16S and 18S data included in this study. Unique samples are independent samples. Also see Figure 1.

Sample type	Unique sample	Subsample	Replicates	Total assays
16S				
Faecal	107	2	2	428
Faecal positive	13	1	2-4	35
Fish positive	5	1	1-2	15
Shark positive	1	1	NA	3
Squid positive	1	1	NA	1
Negative	30	0	NA	30
Total				512
18S				
Faecal	26	2	2	104
Gastropod positive	1	1	2	2
Fish positive	1	1	2	2
Jellyfish positive	2	1	1	2
Squid positive	1	1	2	2
Decapoda positive	2	1	2	4
Negative	4	0	NA	4
Total				120

Controls

In addition to the DNA from the unknown faecal samples, the following samples were sequenced as positive or negative controls for the 16S and 18S datasets.

16S

(1) Little penguin positives. Thirteen faecal samples (one subsample with two to four replicates; Table 1) from little penguins that had been assayed in a separate study and found to contain one or more fish species. These samples were used to assess the consistency of results between sequencing runs and were found to contain the same prey items in all assays in which they were included.

(2) Fish positives. Seven DNA samples of extracts from four fish species that had previously been identified in the diet of little penguins from the Perth area i.e. blue sprat, pilchard, sardine and sandy sprat. Most extracts contained the DNA of only a single species, but we also included a mixed sample with DNA from all four fish species in approximately similar amounts (Table 1). The fish positives were used to determine if our 16S assays would detect these likely prey items, including in cases when the DNA of multiple species was present in a sample. The results confirmed that these fish species were detected by the assays and to test for cross contamination. We also included DNA from sandbar shark (*Carcharhinus plumbeus*) which was extracted and handled independently of the penguin faecal samples and other fish samples, to evaluate whether any cross-contamination of samples occurred during the PCR and/or sequencing (which was done in a commercial laboratory). Both the shark and fish positives comprised 99.9% of reads of the expected species suggesting that levels of cross contamination across samples was very low. We also included a squid positive (*Sepioteuthis* sp.) in one assay to assess whether our 16S methods could detect cephalopod DNA using our 16S assay methods

(Table 1). This sample contained negligible reads and did not amplify the expected taxa which suggests that the 16S assay used is unable to determine the presence of cephalopod prey items in little penguin diets.

(3) DNA negatives. A total of 30 'samples' that had been subjected to the DNA extraction process as the faecal samples but did not contain any faecal sample or added DNA. These were used to test for contamination. All but six negative assays contained negligible PCR product/sequence reads, as expected. Of the six exceptions, two contained noticeable amounts of product/sequences from anchovy. We believe this is due to a human labelling error and have not used these samples in the 16S data filtering (see below). The remaining four DNA negatives contained high numbers of sequences of sea mullet (*Mugil cephalus*), tailor (*Pomatomus saltatrix*), *Caranx* sp., sandy sprat, sardine and *Thunnus* sp. This raises the possibility that our dataset includes some false positives (identifies a species in a sample when it was not present) although we think this is unlikely given the filtering methods used (see below).

18S

(1) Seven positive DNA samples were included in the 18S controls with one subsample with two replicates taken from each sample. Given the broad taxonomic resolution of the 18S assay, potential prey representatives at the Class or Order level were selected e.g. Actinopterygii (blue sprat), Cephalopoda (*Sepioteuthis* sp.), Gastropoda (*Coxiella* sp.), Scyphozoa (*Aurelia* cf. *aurita*, *Pseudorhiza haeckeli*) and Decapoda (*Portunus armatus* and *Penaeus latisulcatus*). At least 97% of reads in these positives corresponded with the anticipated Class or Order except *Portunus armatus* which contained 75%.

(2) Four of the DNA negatives included in the 16S assays were also run in the 18S assay. In this assay, the DNA negatives all contained variable but generally higher numbers of reads than in the 16S assay. However, in three cases, the DNA negatives were comprised of non-target taxa e.g. fungi or mammal sequences not considered likely to be real components of the diet of the little penguins. The same DNA negative that contained high amounts of anchovy reads in the 16S assay (as described above) also showed high numbers (99%) of Actinopterygii reads in the 18S assay. As above, we believe that this is due to human labelling error and have not included this sample in the 18S data filtering (see below).

Data processing

Bioinformatics

Paired ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al. 2014). Primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al. 2010) USEARCH (Edgar 2010, Edgar et al. 2011) (version 8.0.1623) and UPARSE software.

Using USEARCH tools sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using "rdp_gold" database as a reference. To obtain number of reads in each operational taxonomic unit (OTU), reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using the NCBI Blast database. Where genus or species identification was possible (e.g. in the 16S data) FishBase [http://fishbase.org] was used to determine the most likely species but where multiple species may have been identified, identifications have been limited to genus.

Data filtering

Filtering of high-throughput sequencing data is crucial to mitigate the effects of contamination which is unavoidable in metabarcoding studies using highly sensitive PCR amplifications. Filtering is a trade-off between a loss of information (false negatives) and a lack of stringency (false positives) (Drake et al. 2022). We took a conservative approach as we considered the inclusion of false positives (e.g., reporting a prey species not actually present in the diet) to be of greater concern than false negatives (e.g., not detecting a prey species that is actually present). The following steps were used to filter the high throughput sequence data. These steps are based off the recommendations of Drake et al. (2022) but include additional filtering steps (1 and 4).

1) For both positive and negative control assays, the highest number of reads of non-target taxon (e.g. any taxa in the negative or not the intended species in the positive) were subtracted from reads from the penguin assays *sensu* Drake et al. (2022) 'Maximum Contamination'.
2) The percentage contribution of each prey item identified within each faecal assay was calculated. Prey species had to contribute to at least 10% (Dataset A) or 1% (Dataset B) to the total reads of that assay were considered. Although no threshold value is universally accepted in metabarcoding studies, a threshold as low as 1% has been shown to greatly reduce the effect of contaminating sequences (Ando et al. 2020).
3) Only those species that were present in two or more replicates and satisfied step (3) were recorded for a sample (Alberdi et al. 2018).
4) For the 18S dataset, only those samples that had at least one match to a taxon within the phylum Animalia that was considered a likely prey item were considered passed. This excluded host DNA (e.g. bird), terrestrial taxa such as mites, collembola and parasites known from fish (Multivalvulida) as well as a range of macroscopic and microscopic algae, fungi, and higher plants (Appendix 8.1 Table A1).

Dataset A (10% contribution threshold) provides a conservative assessment of the little penguin diet and given the high stringency of the filtering likely includes some false negatives (e.g., excludes rare but present species). Dataset B (1% contribution threshold) may be more sensitive to false positives than Dataset A but a 1% threshold has been used in similar dietary studies on little penguins (Cavallo et al. 2018) and suggested more broadly as a suitable cut-off presence/absence point in dietary faecal studies (Ando et al. 2020).

Quantitative data

We calculated the frequency of occurrence (FoO), i.e. the percentage of all samples in which a specific prey taxon was found, and the number of species detected per sample.

3.1.2 SI analysis

Fish samples

Little penguin potential prey samples were collected in November 2021 and 2022, and May 2022 and 2023 by WAMSI Westport Marine Science Program (WWMSP) Theme 4: Fisheries and aquatic resources projects. These prey were identified using prior knowledge of penguin diet composition from Cockburn Sound (Murray et al. 2011, Cannell unpubl. data), as well as using length criteria for various prey species. For example, skipjack trevally (*Pseudocaranx wrighti*) and yellowtail scad (*Trachurus novazeaelandiae*) <120 mm long and garfish <200 mm long were included. These criteria were based on the maximum length and width of a fish that can be swallowed by a penguin (Cannell 1994). Flesh samples from fish identified as likely penguin prey were obtained (Table 3) and dried at 60°C until constant weight. The dried samples were ground into a powder using a mill and ball grinder (SPX

Sample Prep Genogrinder 2010). Samples were analysed for 1) $\delta^{15}N$, $\delta^{13}C$ without lipid removal (WWMSP Project *Trophic pathways and food web structure of Cockburn Sound and Owen Anchorage* for penguin prey samples collected in November 2021 and May 2022, and this project for samples collected in November 2022 and May 2023), 2) for $\delta^{15}N$ and $\delta^{13}C$ after lipids had been removed (this project, all samples collected) and 3) $\delta^{34}S$ (lipid removal not required, WWMSP Project *Trophic pathways and food web structure of Cockburn Sound and Owen Anchorage* for penguin prey samples collected in May 2022, and this project for samples collected in November 2022 and May 2023; Table 3). For the $\delta^{34}S$ analysis collected by WWMSP Project *Trophic pathways and food web structure of Cockburn Sound and Owen Anchorage*, the majority of the fish prey samples were not the same as those obtained for the $\delta^{15}N$ and $\delta^{13}C$ analysis. However, all the potential penguin fish prey samples collected in November 2022 and May 2023 were analysed for all three stable isotopes.

Table 3. Potential penguin prey species collected for analysis of $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$, month and year of collection and location of analysis.

Genus and Species	Common name	Month and year collected	Location of analysis $\delta^{15}N$, $\delta^{13}C$, lipids not removed	Location of analysis $\delta^{15}N$, $\delta^{13}C$, lipids removed	Location of analysis $\delta^{34}S$
Atherinomorus vaigiensis	common hardyhead	November 2021	UC Davis	UWA	
Engraulis australis	anchovy	November 2021	UC Davis	UWA	
Hyperlophus vittatus	sandy sprat	November 2021	UC Davis	UWA	
Mugil cephalus	sea mullet	November 2021	UC Davis	UWA	
Parequula melbournensis	silverbelly	November 2021	UC Davis	UWA	
Sardinops sagax	pilchard	November 2021	UC Davis	NA	
Spratelloides robustus	blue sprat	November 2021	UC Davis	UWA	
Trachurus novaezelandiae	yellowtail scad	November 2021	UC Davis	UWA	
Atherinomorus vaigiensis	common hardyhead	May 2022	UC Davis	UWA	University of Hawaii
Engraulis australis	anchovy	May 2022	UC Davis	UWA	
Hyperlophus vittatus	sandy sprat	May 2022	UC Davis	UWA	University of Hawaii
Hyporhamphus melanochir	garfish	May 2022	UC Davis	UWA	
Parequula melbournensis	silverbelly	May 2022	UC Davis	UWA	University of Hawaii
Spratelloides robustus	blue sprat	May 2022	UC Davis	UWA	
Trachurus novaezelandiae	yellowtail scad	May 2022	UC Davis	UWA	
Aldrichetta forsteri	yelloweye mullet	November 2022	UWA	UWA	UWA
Atherinomorus vaigiensis	common hardyhead	November 2022	UWA	UWA	UWA
Hyporhamphus melanochir	garfish	November 2022	UWA	UWA	UWA
Hyperlophus vittatus	sandy sprat	November 2022	UWA	UWA	UWA
Leptatherina prebyteroides	silverfish	November 2022	UWA	UWA	UWA

Mugil cephalus	sea mullet	November 2022	UWA	UWA	UWA
Parequula melbournensis	silverbelly	November 2022	UWA	UWA	UWA
Spratelloides robustus	blue sprat	November 2022	UWA	UWA	UWA
Engraulis australis	anchovy	May 2023	UWA	UWA	UWA
Parequula melbournensis	silverbelly	May 2023	UWA	UWA	UWA
Pentapodus vitta	western butterfish	May 2023	UWA	UWA	UWA
Pseudocarax wrighti	skipjack trevally	May 2023	UWA	UWA	UWA
Trachurus novaezelandiae	yellowtail scad	May 2023	UWA	UWA	UWA
Upeneus australiae	Australian goatfish	May 2023	UWA	UWA	UWA

Feather samples

Moulted contour feathers (hereafter referred to as 'moult feathers') were collected directly from adult penguins (Figure 2a) or from within nest sites. Mesoptyle down (hereafter referred to as 'chick down') was collected from chicks as they were growing their contour feathers (Figure 2b). The feathers were collected from January 2020 - December 2023. In 2023, several penguins were found deceased on Garden Island. Some feathers were sampled from several of these birds, as they would have become the moult feathers later in the year. A few deceased chicks were also found in various stages of fledging, and both the down and their contour feathers were sampled. The down and the contour feathers from the chicks represent the diet of breeding adults the year they were collected. The feathers from adults represents the diet they consumed the year before moulting.

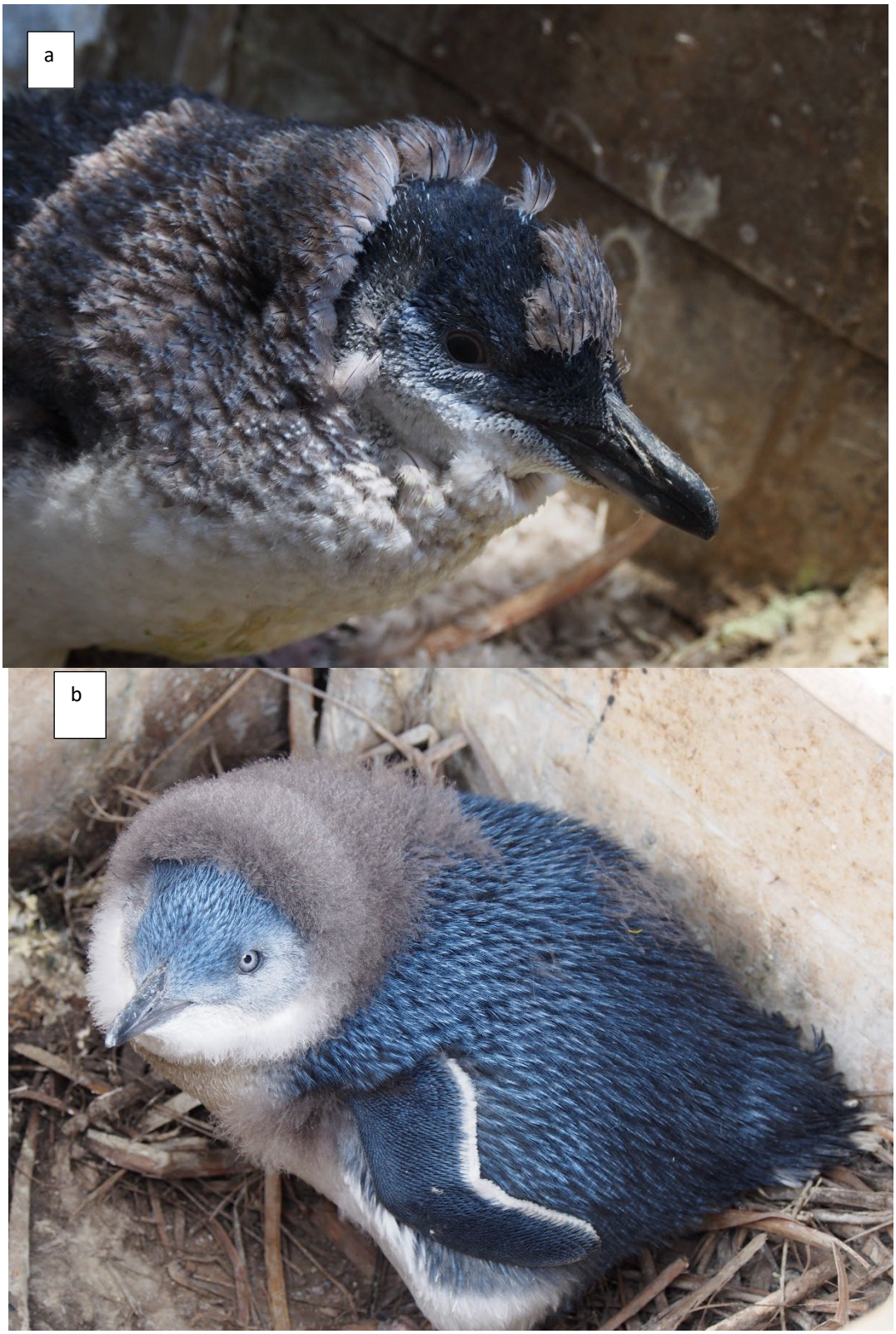


Figure 2 a). A moulting adult little penguin on Garden Island. The moulted contour feathers are pushed out by the new feathers growing underneath, and b) a fledgling little penguin on Garden Island, showing the chick mesoptyle down (brown/grey colour), which is pushed out by the growing contour feathers. *Photographs: Dr Belinda Cannell*

To prepare the feathers for SIA, they were first cleaned for three minutes in a 2:1 choloroform:methanol solution in a hydrasonic bath, then washed in two successive methanol rinses (*sensu* Jaeger et al. 2013). The feathers and down were then air dried, cut into fine pieces using stainless steel scissors and dried in an oven for 48 hours at 50°C.

SI Analysis of Fish Samples (both with lipid and lipid removed) and Feather Samples

To extract lipids from the fish samples, dried powdered samples were placed in polyethylene centrifuge tubes and immersed in a 2:1 chloroform:methanol solution with a solvent volume about three to five times greater than sample volume. Each sample was mixed for 30 sec and left to stand for at least 30 mins, then centrifuged at 2500 rpm for 10 mins. The supernatant containing lipids and solvent was then discarded. This process was repeated at least three times until the supernatant was clear following centrifugation. The samples were then rinsed with chloroform:methanol and dried at 50°C.

Feather and fish samples were analysed for $\delta^{15}N$ and $\delta^{13}C$ using 0.5-0.6 mg of homogenised subsamples loaded into tin cups and combusted at 1020°C in a Thermo Flush 1112 Elemental Analyzer. The yielded N_2 and CO_2 gases were introduced into a Delta V Plus Isotope Ratio Mass Spectrometer via Conflo IV (Thermo-Finnigan/Germany) as transient peaks. Feather and fish samples were analysed for $\delta^{34}S$ using an Automated Nitrogen Carbon Analyzer system consisting of a Sercon 20-22 mass spectrometer connected with an EA (SERCON, UK). The samples were combusted at 1080°C. Measurement was based on SO masses 48/49/50. Multi-point normalization of the raw isotopic data to an isotope international reference scale was performed using both international standards provided by the International Atomic Energy Agency ($\delta^{13}C$ - NBS22, USGS24, NBS19, LSVEC; $\delta^{15}N$ - N1, N2, USGS32, $\delta^{34}S$: IAEA-S1 (-0.30 ‰), IAEA-S2 (+22.62 ‰), IAEA-S3 (-32.49‰) and NBS127 (+21.12 ‰) (Brand et al. 2014, Krause and Coplen 1997) and laboratory standards (Skrzypek et al. 2010, Skrzypek 2013). Stable nitrogen, carbon and sulphur isotope compositions are reported using standard δ-notation (Hobson et al. 1994), and the uncertainty associated with stable isotope analyses (1 standard deviation) was not more than 0.10‰ ($\delta^{15}N$ and $\delta^{13}C$) and 0.40‰ ($\delta^{34}S$). The analyses were conducted at the West Australian Biogeochemistry Centre, University of Western Australia.

3.2 Beach surveys and causes of penguin mortality

A program was established to regularly survey beaches on the eastern shore of Cockburn Sound by community volunteers and employees of businesses that are located adjacent to the foreshore. The volunteers were recruited via social media or direct contact with the businesses. The eastern shore was divided into approximately 1 km stretches, and volunteers choose a section to walk once a week (not necessarily on the same day), but ensuring all sections are covered (where possible) (Figure 3). In addition, dead penguins may have also be found by Dr Cannell (and other Department of Defence personnel) on Garden Island (not part of the beach surveys). Any dead penguins found were taken to DPIRD Diagnostics and Laboratory Services (DDLS) for necropsy (note that Murdoch University was not conducting the necropsies due to lack of available staff). The necropsies identified, where possible, all causes of mortality such as trauma, starvation, and parasitic loads. They were also tested for Avian Influenza and Newcastle Disease. A body condition score was determined for each bird, based on its body weight, fullness of pectoral musculature and the presence of subcutaneous and abdominal fat stores (Campbell et al. 2022).

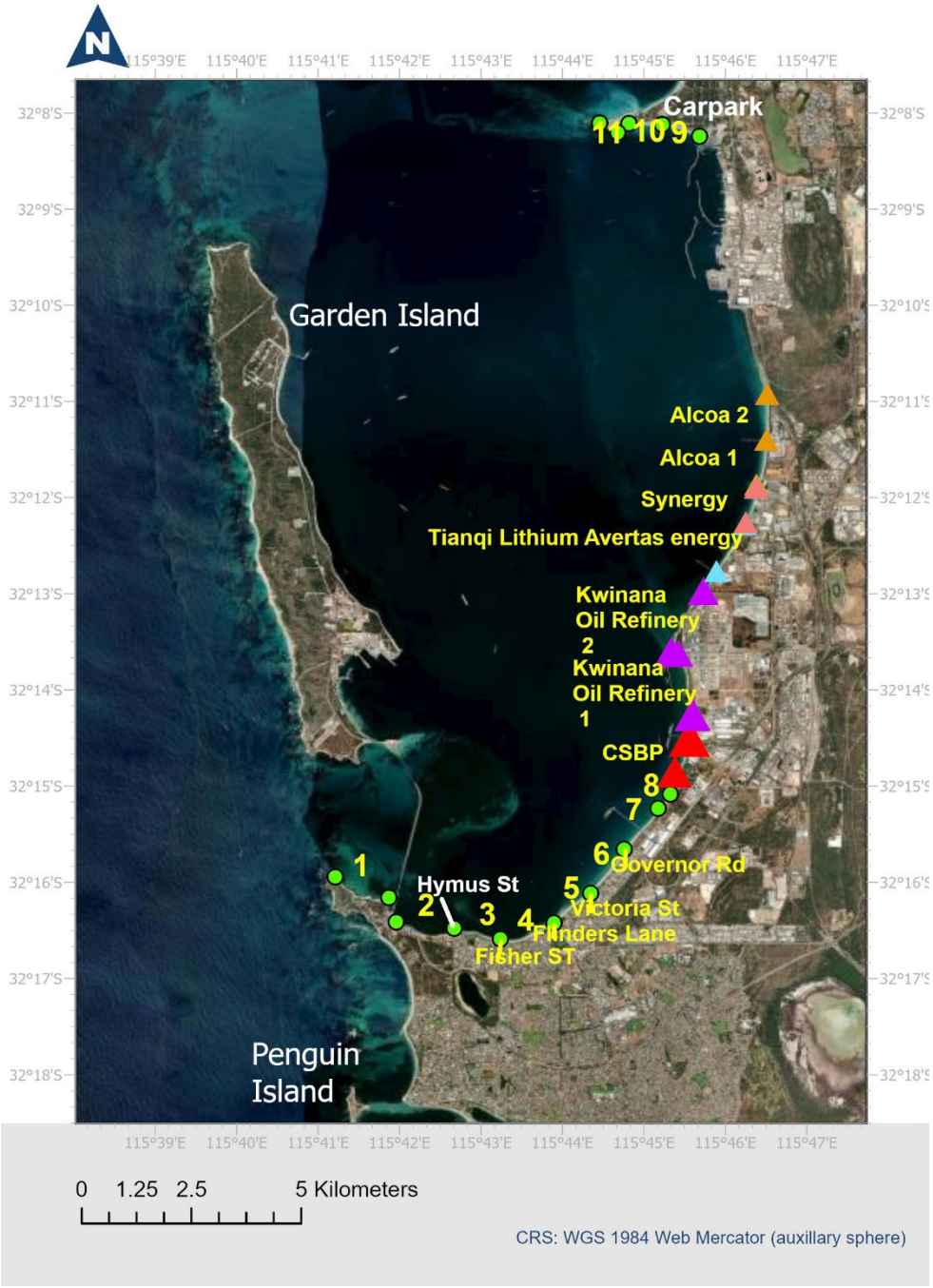


Figure 3. Map of the eastern shore of Cockburn Sound used to conduct regular beach surveys to find dead penguins. The numbers or names refer to the identity of each beach section for those beaches not adjacent to a business. The symbols denote the start and end of each section. Note there is no walkable beach in areas where there are no numbers or names e.g. north of Alcoa 2 and the start of number 9 beach

At the conclusion of the survey programme, all volunteers were invited to send any comments about their surveys.

3.3 Foraging habitat and home range of little penguins

To study the foraging movements of the birds, satellite tags (Kiwisat PTT 202 K2G 173A, 32g, 60 x 27 x 17 mm, Antenna angle 45°, duty cycle 1900-1400 UTC, repetition rate 35s, or Kiwisat PTT K4G 154, 17g,

74 x 21 x 11 mm, Antenna angle 45°, duty cycle 1900-1400 UTC, repetition rate 35s) were attached to 10 little penguins at Garden Island during the incubation phase of breeding in 2022 (N = 6) and 2023 (N = 4). Usable data were obtained from eight of the 10 tags, as one tag malfunctioned, and the data quality was poor from another tag. Tags which obtain GPS positions of the penguins at a very high frequency (Axy-Trek Marine,26g, 56 x 23 x 12 mm) were deployed on four penguins during the chick-guard stage (chicks up to two weeks old) in 2022 (N = 3) and 2023 (N = 1). The number of deployments of both the satellite and GPS tags was limited by the presence of breeding little penguins in accessible sites on Garden Island.

Data from satellite tags are collected by Argos and were downloaded from the Argos website, whereas the GPS tags log the location data on the tag itself. This means the data from GPS tags can only be obtained if the tag is retrieved and the data are then downloaded. Location data were analysed using different methodologies, dependent on two things:

a) the type of tag deployed on the penguins, and
b) if there were sensible outcomes from the analysis.

Satellite tags

The location data obtained from both single and multiple day trips were analysed using a Bayesian State-space model (SSM) to account for location uncertainty (Jonsen et al. 2005, Patterson et al. 2008). A correlated randomised walk model was fitted to the SSM, using the R (R Core team 2023) package 'animotum' (Jonsen et al. 2023). The 50% kernel density area represents the area where there is a 50% chance of finding that animal, and generally represents core foraging habitat. The 95% kernel density area represents home range (Hooge et al. 2001). For individual birds, these kernel density areas were calculated using the Brownian Bridge kernel method implemented in the function 'kernelbb' of the R package 'adehabitatHR' (Calenge, 2006).

Data from satellite tags deployed on penguins that completed foraging trips but could not be analysed using the SSM (due to non-sensible model outputs), were analysed separately. The study area was gridded into 500 m x 500 m square grids, and the total time spent in each grid cell was determined using the 'trip' package (Sumner & Luque, 2015) in R (R core team 2019). To represent spatial use, the grid cells was ranked in order of time spent in each cell, and the core foraging area was defined as the cells which covered the first 50% of the cumulative frequency distribution (Ferreira et al. 2021).

GPS tags

As the data from the GPS tags had greater position accuracy, and the locations were obtained at a much higher frequency rate, the raw data were analysed without preprocessing. However, due to this high frequency rate of location detection, the data are autocorrelated, and conventional kernel density estimation tends to underestimate the space used for home range (Fleming et al. 2015). Therefore, only the 50% kernel density area is determined analysed using the hplugin value implemented in the function 'kd' of the R package 'ks' (Doung, 2014).

The 50% and 95% kernel density areas (where appropriate) were overlaid on benthic habitat data, the Stage 3 preferred Option-Port Footprint and existing shipping channels, obtained from the Westport ArcGIS platform. For the GPS data, a line was also drawn between each point. Mapping was conducted in ArcGIS Pro 3.2. The kernel density areas for each bird were colour coded, and for those birds with multiple trips, a different line type was used for each trip. A single trip is taken from the time the bird leaves the colony until it returns. Birds can undergo either multiple single day trips, or a multi-day trip.

3.4 Statistical Analysis

3.4.1 DNA analysis of faeces

We investigated the annual diversity of fish species present within the faecal samples of little penguins collected from 2020-2023, with each year considered as a separate assemblage, and using Dataset B. We used diversity indices based on Hill numbers (*q*D), using the three most popular values of *q* (0, 1, and 2; Chao et al. 2010). These represent 1) the species richness index (*q* = 0), which is insensitive to the frequency of species and emphasises rare species, 2) the exponential form of the Shannon entropy index (*q* = 1), which does not favour either common or rare species (i.e. is the number of "typical species" in an assemblage; Chao et al. 2010) and 3) the inverse Simpson concentration (*q* = 2), which favours more dominant species (Jost 2006, Jost et al. 2011, Gotelli & Chao 2013).

We used a two-step approach for the diversity analysis: the first step was to develop diversity profiles (sensu Gotelli & Chao 2013) of the diet for each assemblage (i.e. each year). The profiles were obtained using iNEXT online (iNterpolation/EXTrapolation, https://chao.shinyapps.io/iNEXTOnline/; Chao et al. 2016). The second step was to compare diversity estimates between assemblages by constructing sample-size-based rarefaction and extrapolation curves with 95% confidence intervals (Chao et al. 2014, Hsieh et al. 2016). We used the R package 'iNEXT' (Hsieh et al. 2024). Both rarefaction and extrapolation standardise uneven samples to an equal size based on a reference sample size so they can be compared (further information on Hill numbers and the diversity analysis are available in Appendix 8.1).

We used a multivariate random forest (RF) regression model to determine the environmental variables that influenced the presence/absence of fish prey in the faecal samples, but due to limited availability of some of the data, we were only able to do this for the samples collected in 2021-2023. RF are robust to the inclusion of correlated predictor variables (Fox et al. 2017). Furthermore, robust variable selection is obtained using a recursive feature elimination algorithm (Gregorutti et al. 2017). We included 28 environmental variables as predictors in our models, broadly categorised into annual rainfall, volume of discharged water from the river which feeds into Cockburn Sound, monthly Fremantle Sea Level (a proxy for Leeuwin Current), and water quality variables (collected from the DWER buoys, and data only available from 2021-2023). The water quality variables included dissolved oxygen, salinity, temperature and turbidity, and were obtained from buoys CS11 in 2021, CS11 and CS13 in 2022 and Mangles Bay in 2023 (as no data were available from CS11 or CS13 in 2023, and this was the closest buoy to CS11 and CS13) (Appendix 1, Figure A1). We also included 1 - 3 month lags of all the monthly data, and 1 - 3 year lags for the rainfall data (Appendix 8.1, Table A2). The lags are included as the environmental variables may not have an immediate effect on the prey species. The response variables were the presence/absence of all the fish prey found across the three years, and then the seven most common fish prey found, in Dataset B. We used the function 'rfsrc' in the R package 'randomforestSRC' (Ishwaran & Kogalur 2022) and 'rfe' in the R package 'caret' (Kuhn 2008). We ran models using 500 trees, with three environmental variables considered at each split, and a minimum terminal node was set to N = 5. The models were checked for convergence by plotting the number of trees incrementally against model error to make sure that the plot asymptotes. To evaluate the performance of the RF models, we obtained an out-of-bag explained variance value (OOB R^2) and performance error.

3.4.2 SI Analysis

All stable isotope data were tested for normality using the Shapiro-Wilk test. We investigated potential differences in $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values in feathers between years using linear models (LMs) or Kruskal-Wallis (KW) test if model assumptions were not met. We compared differences in each SI value in 1) the feathers of the non-breeding adults between years, 2) the chick down/contour feathers collected during the breeding season in 2020, 2021 and 2022 with the adult feathers that were synthesized following that breeding season, i.e. between the breeding and non-breeding/pre-moult penguins, and 3) in the chick down/contour feathers collected in the breeding seasons of 2020, 2021, 2022 and 2023. We used the function 'lm' or if the data did not meet normality assumptions, a 'kruskal.test' or 'glm'

in the R package 'stats' (R Core Team 2021). A KW test was run if there was only one response variable, and a Generalized Linear Model was used when there were multiple predictors and interaction terms. For the stable isotopes in the fish prey, Student's t-test or Wilcoxon rank test for non-normal data were conducted to determine differences in the $\delta^{15}N$ and $\delta^{13}C$ values in the penguin fish prey samples with and without lipid removal. Differences in the $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values between the penguin prey species within a season, and within a penguin prey species between seasons were determined using ANOVA or KW test for non-normal data. For the SIA in both the feathers and fish, post hoc comparisons were conducted with Tukey's test when significant results from the ANOVA were found using the function 'glht' in the R package 'multcomp' for normally distributed data (Hothorn et al, 2008), or the Dunn Test when significant results were found from the KW test, using the R package 'rstatix' (Kassambra 2023). For those models with significant interactions, post-hoc tests using the package *emmeans* (Lenth 2022) were performed to determine significant pairwise comparisons. All data were analysed using R version 4.1.3 (R Core Team 2021).

We compared the isotopic niche width of 1) the non-breeding/pre-moult penguins between years, 2) the breeding penguins and non-breeding/pre-moult penguins in 2020, 2021, and 2022, and 3) the breeding penguins in 2020-2023. We also calculated the corrected Standard Ellipse Area (SEA_C) of the stable isotopes in the feathers and down, and estimated the percentage of overlap in the isotopic niches of 1) non-breeding penguins between years (2019/20, 2020/21, 2021/22, 2022/23, but unable to include 2018/19 due to sample size limitations), 2) breeding and non-breeding penguins in 2020, 2021, and 2022) breeding penguins in 2020, 2021, 2022, and 2023. There are two estimates of overlap for each comparative pair, e.g. amount of overlap of isotopic niche in 2019/20 with that of 2020/21, and the amount of overlap of isotopic niche in 2020/21 with that of 2019/20. For isotopic niche comparison between the groups, we calculated the Bayesian approximation of the standard ellipse area (SEA_B) and corresponding 95% confidence intervals (Jackson et al. 2011). To determine significant differences in the sizes of the isotopic niche between the groups, pair-wise comparisons on the proportion of SEA_B that differed between the groups were performed (Jackson et al. 2011, Reid et al. 2016). We used the package 'SIBER' (Stable Isotope Bayesian Ellipses in R, Jackson et al. 2011) to estimate both the SEA_C and SEA_B. Even though we were not able to statistically compare the data of the non-breeding penguins in 2018/19, we have presented the SEA_C in the figures.

To estimate the relative consumption of different prey in the penguins' diet in the breeding penguins in 2023, we fitted Bayesian isotope mixing models using the 'MixSIAR' R package (Stock and Semmens 2016, Stock et al. 2018). Only the null model was run, which considers all individuals to have the same diet composition. This is because there were no covariates such as age (all feather samples came from chicks, whose parents are of indeterminate age), or sex (chicks are fed by both parents). The mixing models were fit using Markov chain Monte Carlo (MCMC) which produce a likelihood framework of plausible values of dietary composition based on the available data, and incorporate variability in consumer and prey isotope values, as well as any covariate information (Moore and Semmens 2008, Parnell et al. 2010, de Vries et al. 2016). They also account for uncertainty in trophic discrimination factors, concentration dependence (i.e. different contributions of a prey source to a mixture for both C and N (Phillips and Koch 2002)) and the variability in predator stable isotope values that results from predators finitely "sampling" from prey isotope distributions many times (Moore and Semmens 2008, deVries et al. 2016). We compared the results from models run with uninformative Dirilecht priors (where the penguins consumed all prey types in equal proportions) and informative priors (Stock et al. 2018). The informative priors were based on the results from the molecular analysis of the penguin faeces collected in 2023, and prior dietary studies conducted for little penguins (Murray et al. 2011) but using data collected only for penguins that would be feeding within Cockburn Sound (Cannell unpubl. data).

We ran models using two different isotopic mean discrimination factors (TDF, the difference in isotope values between consumer and source tissues) for $\delta^{15}N$ and $\delta^{13}C$. The first TDF was based on fractionation values obtained from little penguins experimentally fed a diet consisting solely of sprats

(*Sprattus sprattus*; MacKenzie 2011), with a TDF value of 3.9‰ for $\delta^{15}N$ and 0.2‰ for $\delta^{13}C$. However, a SD value was not available for these data. The second TDF value was obtained using the R package 'SIDER' (Healy et al. 2018; *sensu* Morganthaler et al 2021), with mean and SD $\delta^{15}N$ of 3.99‰ and 1.11‰, and mean and SD $\delta^{13}C$ of 2‰ and 1.24‰.

The models were run using a "normal" combination for MCMC (three chains with a length of 100,000, a burn-in of 50,000 and a thinning interval of 50), "long" (three chains with a length of 300,000, a burn-in of 200,000 and a thinning interval of 100) and "very long" (three chains with a length of 1,000,000, a burn-in of 500,000 and a thinning interval of 500). The "long" and "very long" iterations were only used if the previous model failed to converge. Model convergence was checked using the Gelman-Rubin and Geweke diagnostics (Gelman et al. 2003, Stock & Semmens 2016). Gelman-Rubin diagnostics analyse multiple simulated MCMC chains by comparing the variance between each chain and the variance between multiple chains. Confidence intervals close to one indicate model convergence, and values < 1.1 are acceptable for most cases (Stock & Semmens 2016 and refs within). The Geweke test is a 2-sided z-test that compares the means of the first part and last part of each Markov chain. The means should be the same at convergence, with ≤ 5% of variables in each chain outside of ± 1.96 (Stock & Semmens 2016). Additionally, a multiplicative error term for each isotope, ξ (epsilon), should equal 1 if there are no additional sources of tracer variability beyond consumer integration of the source uncertainty. If the values are < 1, this indicates that the consumers are sampling multiple times from each source pool. If the values are much greater than 1, this indicates that one or more of the basic assumptions of the mixing model have not been met, e.g. the model is missing a non-negligible source (Stock et al. 2018).

The prey used for the Bayesian mixing model included the majority of those initially identified (see 3.1.2 - SI Analysis, fish samples), as well as additional prey sources 1) identified by the molecular analysis of the faecal samples but which were larger than those considered to be eaten by the penguins (i.e. they were used for the dolphin dietary analyses), and 2) identified in the diet of little penguins known to feed in Cockburn Sound (Murray et al. 2011, Cannell unpubl. data). The isotope signature of each prey type was for that prey caught in the season and year of the feather growth, unless those prey had not been caught in that season and year but was observed in the diet studies (Table 4). In such cases, the SI signatures of that species caught in the closest temporal season were used. Finally, as mixing models work better when there are six or less prey data (Stock et al. 2018), after initially running the models with all prey species, data were aggregated between species where there was little difference between their SI signatures. For the breeding penguins in 2023 prey were initially aggregated from 13 species to form seven groups (Table 4). However, the models were unable to converge, even when the very long iteration option was chosen. We then removed group E (which included goatfish and western butterfish), given that they had not been observed in any of the faecal samples, nor in any previous dietary samples for penguins foraging in Cockburn Sound. The $\delta^{34}S$ values were not obtained for all the "dolphin" prey species, and hence $\delta^{34}S$ values were not included in the mixing models.

Table 4. Little penguin prey species used in the Bayesian mixing models for diet composition of breeding penguins from Garden Island, Western Australia in 2023, based on prior knowledge and outcomes of the molecular analysis of penguin faeces (this project). The season and year the prey were collected and whether they were used for the dolphin dietary analysis are detailed.

Prey type	Season x Year	Used for dolphin diet	Group
anchovy	Winter 2023	N	B
Australian goatfish	Winter 2023	N	E
pilchard	Summer 2022/23	Y	D
blue sprat	Summer 2022/23	N	F
jellyfish	Winter 2023	N	A
sandy sprat	Summer 2022/23	N	B
sardine	Winter 2023	Y	B
sea mullet	Summer 2022/23	N	G
silverbelly	Winter 2023	N	C
skipjack trevally	Winter 2023	N	B
garfish	Winter 2023	Y	B
western butterfish	Winter 2023	N	E
yellowtail scad	Winter 2023	N	C

4 Results

4.1 Diet

4.1.1 DNA analysis of faeces

16S

A total of 512 sequencing assays were completed. These assays comprised subsamples and replicates from 107 faecal samples (n= 18 in 2020, 28 in 2021, 34 in 2022 and 27 in 2023), as well as positive and negative controls (Tables 1 and 2).

A total of 32,391,966 reads were retained after quality control for all the 512 assays. The vast majority of reads from faecal samples were from bony fishes (91.5%) while 6% of sequences were from non-target sources e.g., human, bird or shark DNA (Table 5). A total of 2.5% reads could not be matched to a reference sequence in GenBank (Table 5).

Table 5. Summary of reads from HTS sequencing from little penguin faecal samples collected from Garden Island, Western Australia, from 2020 - 2023 after quality control. % is the relative contribution of sequences from each group to the total number of reads. Data do not include reads from positive and negative controls. NA = unassigned sequences were those that did not have a 97% similarity in the reference database.

Group	N° reads	%
bony fishes	29,653,822	91.5
birds	1,888,665	5.8
NA	800,193	2.5
humans	49,282	0.2
sharks & rays	3	< 0.1
Total	**32,391,966**	

Diet summary

Of the 107 faecal samples that were assayed, 92 unique faecal samples passed the filtering steps for Dataset A while 95 unique faecal samples passed for Dataset B (see methods).

Dataset A - 10% threshold

A total of 14 prey taxa from 10 different families were detected in 92 unique samples (Table 6).

Across the four years, pilchard (47.8%) and sandy sprat (50%) along with anchovy (40.2%) were the three most commonly detected prey items, with at least one of these three fish species detected in 90.2% of samples. Garfish and sardine were detected in 12% of samples, whilst the remaining nine species (sea mullet, tailor, common buffalo bream (*Kyphous sydneyanus*), blue sprat, skipjack trevally (*Pseudocaranx wright*), hardyheads (*Atherinomorus* sp.), *Carangoides* sp., rough leatherjacket (*Scobinichthys granulatus*) and jack mackerel (*Trachurus* sp.) were found in < 3.3% of samples (Table 6).

There were interannual differences in the most commonly detected species in the faecal samples. For example, in 2020 anchovy was the most common, when it was found in 85.7% of samples, but thereafter the percentage declined (Table 6). Pilchard was found in > 57.1% of samples in 2020 and 2021, but 46.6% of samples in 2022 and 33.3% of samples in 2023 (Table 6). Sandy sprat was only found in 20.8% of samples in 2023 but had been found in > 53.6% of samples in the preceding three years (Table 6). Sardine (likely scaly mackerel, *S. lemuru*) were found in samples every year except 2022.

Table 6. Summary of all prey taxa detected in faecal samples from little penguins collected from Garden Island, Western Australia, from 2020 - 2023 for Dataset A in the 16S assay. Count is the number of faecal samples that the species was detected in. % is the percentage of faecal samples that the species was found in indicated by N samples. Generic identifications are based off BLAST results. Species identifications are based off BLAST results and distributional data (see methods).

Family	**Genus**	**Species**	**Common name**	**2020**	**N = 14**	**2021**	**N = 26**	**2022**	**N = 28**	**2023**	**N = 24**	**Overall**	**N = 92**
				Count	%	Count	%	Count	%	Count	%	Count	%
Clupeidae	*Hyperlophus*	*vittatus*	sandy sprat	8	57.1	18	69.2	15	53.6	5	20.8	46	50
Clupeidae	*Sardinops*	*sagax*	pilchard	8	57.1	15	57.7	13	46.4	8	33.3	44	47.8
Engraulidae	*Engraulis*	*australis*	anchovy	12	85.7	10	38.5	8	28.6	7	29.2	37	40.2
Clupeidae	*Sardinella*	sp.	sardine	1	7.1	3	11.5			7	29.2	11	12
Hemiramphidae	*Hyporhamphus*	*melanochir*	garfish	2	14.3	1	3.8	4	14.3	4	16.7	11	12
Mugilidae	*Mugil*	*cephalus*	sea mullet							3	12.5	3	3.3
Pomatomidae	*Pomatomus*	*saltatrix*	tailor	1	7.1					2	8.3	3	3.3
Carangidae	*Pseudocaranx*	*wrighti*	skipjack trevally							2	8.3	2	2.2
Kyphosidae	*Kyphosus*	*sydneyanus*	common buffalo bream					1	3.6	1	4.2	2	2.2
Spratelloididae	*Spratelloides*	*robustus*	blue sprat					1	3.6	1	4.2	2	2.2
Atherinidae	*Atherinomorus*	sp.	hardyheads					2	7.1			2	2.2
Carangidae										1	4.2	1	1.1
Monacanthidae	*Scobinichthys*	*granulatus*	rough leatherjacket			1	3.8					1	1.1
Carangidae	*Trachurus*	sp.	jack mackerels							1	4.2	1	1.1
10	**14**			**32**		**48**		**44**		**42**		**166**	

The majority of samples contained one (40.2%) or two taxa (41.3%), with the remaining containing three (16.3%) or four (2.2%) prey taxa (Table 7).

Table 7. The percentage (and total number) of little penguin faecal samples collected from Garden Island, Western Australia from 2020-2023 in which 1 – 4 prey taxa were detected for Dataset A (N = 92). The total number of taxa found in all samples was 14.

N prey items	2020	2021	2022	2023	Overall
1	7.1 (1)	42.3 (11)	53.6 (15)	41.7 (10)	40.2 (37)
2	64.3 (9)	30.8 (8)	39.3 (11)	41.7 (10)	41.3 (38)
3	21.4 (3)	26.9 (7)	3.6 (1)	16.7 (4)	16.3 (15)
4	7.1 (1)		3.6 (1)		2.2 (2)
Total	**15**	**26**	**28**	**24**	**92**

Dataset B - 1% threshold

A total of 19 prey species from 13 different families were detected in the 95 faecal samples for Dataset B (Table 8).

Overall, anchovy (56.8%), pilchard (54.7%) and sandy sprat (60%) were the most commonly detected prey species while sardine and garfish were found in approximately 20% of samples (Table 8). Sea mullet, skipjack trevally, tailor and jack mackerel were found in 9.5 to 12.6% of samples and approximately 2.1 to 4.2% of samples contained mosaic leatherjackets (*Eubalichthys mosaicus*), rough leatherjackets, blue sprat, hardyheads and buffalo bream. Carangidae sp., morwongs (*Cheilodactylus* sp.), chub mackerel (*Scomber japonicus*) and round herring (*Etrumeus acuminatus*) were identified once (Table 8). There were other interannual differences including that anchovy was the most common species in 2020 and 2022, and sandy sprat was the most common in 2021 (Table 8). Sardine were not present in samples in 2022, were rare in 2020, but were the joint most common species, along with pilchard and anchovy in 2023 (Table 8).

Table 8. Summary of all prey taxa detected in faecal samples from little penguins for Dataset B in the 16S assay. Count is the number of faecal samples that the species was detected in. % is the percentage of faecal samples that the species was found in indicated by N samples (the total number of samples which passed the filtering criteria laid out in the methods). Generic identifications are based off BLAST results. Species identifications are based off BLAST results and distributional data (see methods).

Family	**Genus**	**Species**	**Common name**	**2020**	**N = 15**	**2021**	**N = 27**	**2022**	**N = 28**	**2023**	**N = 25**	**Overall**	**N = 95**
				Count	%	Count	%	Count	%	Count	%	Count	%
Clupeidae	*Hyperlophus*	*vittatus*	sandy sprat	10	66.7	22	81.5	16	57.1	9	36	57	60
Engraulidae	*Engraulis*	*australis*	anchovy	14	93.3	12	44.4	17	60.7	11	44	54	56.8
Clupeidae	*Sardinops*	*sagax*	pilchard	10	66.7	16	59.3	15	53.6	11	44	52	54.7
Clupeidae	*Sardinella*	sp.	sardine	1	6.7	7	25.9			11	44	19	20
Hemiramphidae	*Hyporhamphus*	*melanochir*	garfish	2	13.3	1	3.7	7	25	7	28	17	17.9
Mugilidae	*Mugil*	*cephalus*	sea mullet	2	13.3	1	3.7	2	7.1	7	28	12	12.6
Carangidae	*Pseudocaranx*	*wrighti*	skipjack trevally	3	20	2	7.4	3	10.7	3	12	11	11.6
Pomatomidae	*Pomatomus*	*saltatrix*	tailor	3	20	2	7.4			5	20	10	10.5
Carangidae	*Trachurus*	sp.	Jack mackerel	2	13.3	2	7.4	1	3.6	4	16	9	9.5
Monacanthidae	*Eubalichthys*	*mosaicus*	mosaic leatherjacket	1	6.7	1	3.7			2	8	4	4.2
Monacanthidae	*Scobinichthys*	*granulatus*	rough leatherjacket			2	7.4			2	8	4	4.2
Spratelloididae	*Spratelloides*	*robustus*	blue sprat			1	3.7	1	3.6	1	4	3	3.2
Atherinidae	*Atherinomorus*	sp.	hardyheads					3	10.7			3	3.2
Kyphosidae	*Kyphosus*	*sydneyanus*	common buffalo bream					1	3.6	1	4	2	2.1
Scombridae	*Scomber*	*japonicus*	chub mackerel	1	6.7							1	1.1
Latridae	*Cheilodactylus*	sp.	morwong							1	4	1	1.1
Dussumieriidae	*Etrumeus*	*acuminatus*	round herring			1	3.7					1	1.1
Carangidae										1	4	1	1.1
13	**17**			**49**		**70**		**66**		**76**		**261**	

Across all four years, most faecal samples had more than one prey item with two thirds of penguin samples having two or three prey items and seven prey items were detected in two samples. (Table 9).

Table 9. The percentage (and total number) of little penguin faecal samples collected from Garden Island, Western Australia from 2020-2023 in which 1 – 4 prey taxa were detected for Dataset B (N = 95). The total number of taxa found in all samples was 19.

N prey items	2020	2021	2022	2023	Overall
1		22.2 (6)	10.7 (3)	12 (3)	12.6 (12)
2	26.7 (4)	25.9 (7)	60.7 (17)	28 (7)	36.8 (35)
3	46.7 (7)	33.3 (9)	25 (7)	40 (10)	34.7 (33)
4	13.3 (2)	7.4 (2)		4 (1)	5.3 (5)
5	6.7 (1)	11.1 (3)	3.6 (1)	4 (1)	6.3 (6)
6				8 (2)	2.1 (2)
7	6.7 (1)			4 (1)	2.1 (2)
Total	**15**	**27**	**28**	**25**	**95**

18S

A total of 120 sequencing assays were completed. These assays comprised subsamples and replicates from 26 faecal samples (N = 6 in 2021, 9 in 2022 and 11 in 2023), as well as positive and negative controls (Tables 1 and 2).

A total of 4,263,788 reads were retained after quality control for 120 assays. The vast majority of reads from faecal samples were from animals (87.5%) with chordates (80.5%) the most frequently identified phyla (Table 10). Within the chordates, fish (33.2% of all reads) and birds (47.1% of all reads) constituted the bulk of the reads (data not shown). A total of 12.5% of all reads were from non-target sources e.g., fungi, higher order plants, Protozoa and Chromista (Table 10). A total of 2.5% reads could not be matched to a reference sequence in GenBank (Table 10).

Table 10. Summary of reads from HTS sequencing from little penguin faecal samples after quality control for the 18S assay. % is the relative contribution of sequences from each group to the total number of reads. Data do not include reads from positive and negative controls. Unassigned sequences were those that did not have a 97% similarity in the reference database.

Taxa	Reads	%
Animalia		85.7
Chordata	2,936,623	80.5
Arthropoda	128,289	3.5
Cnidaria	50,699	1.4
Platyhelminthes	8,354	0.2
Mollusca	3,382	0.1
Rotifera	517	0.01
Annelida	451	0.01
Brachiopoda	278	0.01
Echinodermata	9	< 0.1
Chaetogtha	3	< 0.1
Dicyemida	2	< 0.1
Porifera	2	< 0.1
Plantae	276,774	7.59
Streptophyta	9	< 0.1
NA	95,186	2.61
Fungi	72,265	1.98
Chromista	70,055	1.92
Protozoa	2,796	0.08
Protista	2,441	0.07
Archaeplastida	361	0.01
Opisthokonta	111	< 0.1
Amoebozoa	42	< 0.1

Diet summary

Of the 26 faecal samples that were assayed, 16 unique faecal samples passed the filtering steps for Dataset A while 23 unique faecal samples passed for Dataset B as set out in the methods (Table 1).

Dataset A – 10% threshold

Prey from three phyla were detected in this dataset. Fish was the most commonly detected prey item found in 82.4% of samples (Table 11). Squid and copepods were also found in one and two samples respectively. The detection of copepods in two samples could be an example of secondary predation. Alternatively, given the low taxonomic resolution of the 18S marker and gaps in the 18S database it is possible that the sequence in question has come from a different type of crustacean that is included in the diet of the little penguin.

Table 11. Phylum, class and order of animals detected in all faecal samples of little penguins collected from Garden Island, Western Australia 2021-2023 using metabarcoding of 18S using a 10% threshold of occurrence. Count is the number of faecal samples that the species was detected in. % is the percentage of the faecal samples that passed the filtering steps for Dataset A that each species was found in.

Phylum	**Class**	**Order**	**2021**		**2022**		**2023**		**Total**	
			Count	%	Count	%	Count	%	Count	%
Arthropoda	Copepoda	Calanoida	2	50					2	12.5
Chordata	Actinopterygii		2	50	1	100	11	100	14	87.5
Mollusca	Cephalopoda	Myopsida					1	9.1	1	6.3
Total			**4**		**1**		**12**			
N samples			**4**		**1**		**11**		**16**	

Dataset B – 1% threshold

Prey from five different phyla were detected (Table 12). As with Dataset A, the vast majority of samples (87%) contained fish (Table 12), however, copepods, squid and jellyfish were also detected in one to five samples (Table 12).

Table 12. Phylum, class and order of animals detected in faecal samples of little penguins using metabarcoding of 18S using a 1% threshold of occurrence (Dataset B). Count is the number of faecal samples that the prey taxon was detected in. % is the percentage of the faecal samples that passed the filtering steps for Dataset B that each taxon was found in.

Phylum	**Class**	**Order**	**2021**		**2022**		**2023**		**Total**	
			Count	%	Count	%	Count	%	Count	%
Arthropoda	Copepoda	Calanoida	2	33.3	1	20	2	18.1	5	21.7
Arthropoda	Copepoda	Harpacticoida			3	60			3	13
Chordata	Actinopterygii		5	83.3	3	60	11	100	20	87
Chordata	Appendicularia	Copelata					1	9	1	4.3
Cnidaria	Scyphozoa	Semaeostomeae					1	9	1	4.3
Mollusca	Cephalopoda	Myopsida					1	9	1	4.3
Platyhelminthes	Trematoda	Azygiida			1	20	2	18.1	3	13
Total			**7**		**8**		**19**		**34**	
N samples			**6**		**5**		**11**		**23**	

Diversity analysis

Overall, the dietary diversity profile of the diet for the little penguins in 2020 – 2022 were similar, but their diet in 2023 consisted of significantly more typical (q = 1) and dominant (q=2) species (Figures 4 and 5). However, Figure 5 reveals that the sampling curves for species richness (q = 0), extrapolated up to double the sample size, did not reach an asymptote in any year, although it came close in 2020, suggesting that the current data represents a minimum species richness in all years. For all years except 2023, only approximately one typical species was not detected, and two species not detected in 2023 (Table 13). Finally, only approximately one dominant species was not detected from the samples collected in 2020 and 2023 (Table 13).

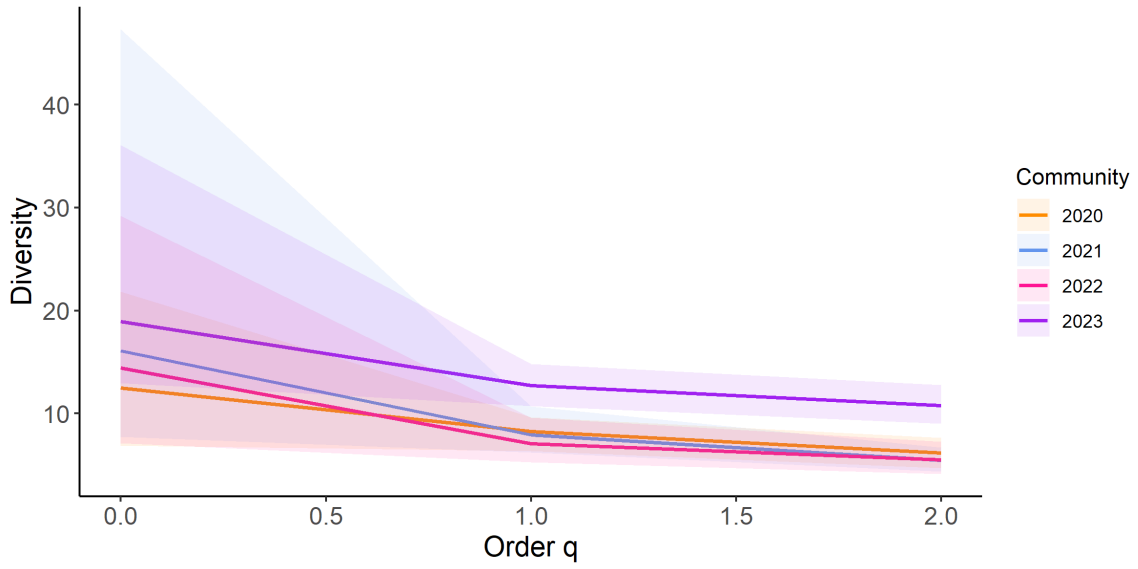


Figure 4. Diversity profile of the diet composition of little penguins, obtained from DNA analysis of faecal samples, collected from 2020 – 2023 at Garden Island as a function of order q(0,1,2) in the Hill numbers, where q0 represents species richness index and emphasises rare species, q1 is the number of 'typical species' in an assemblage, and q2 favours more dominant species. The shaded areas are the 95% confidence intervals, based on a bootstrap method of 50 replications.

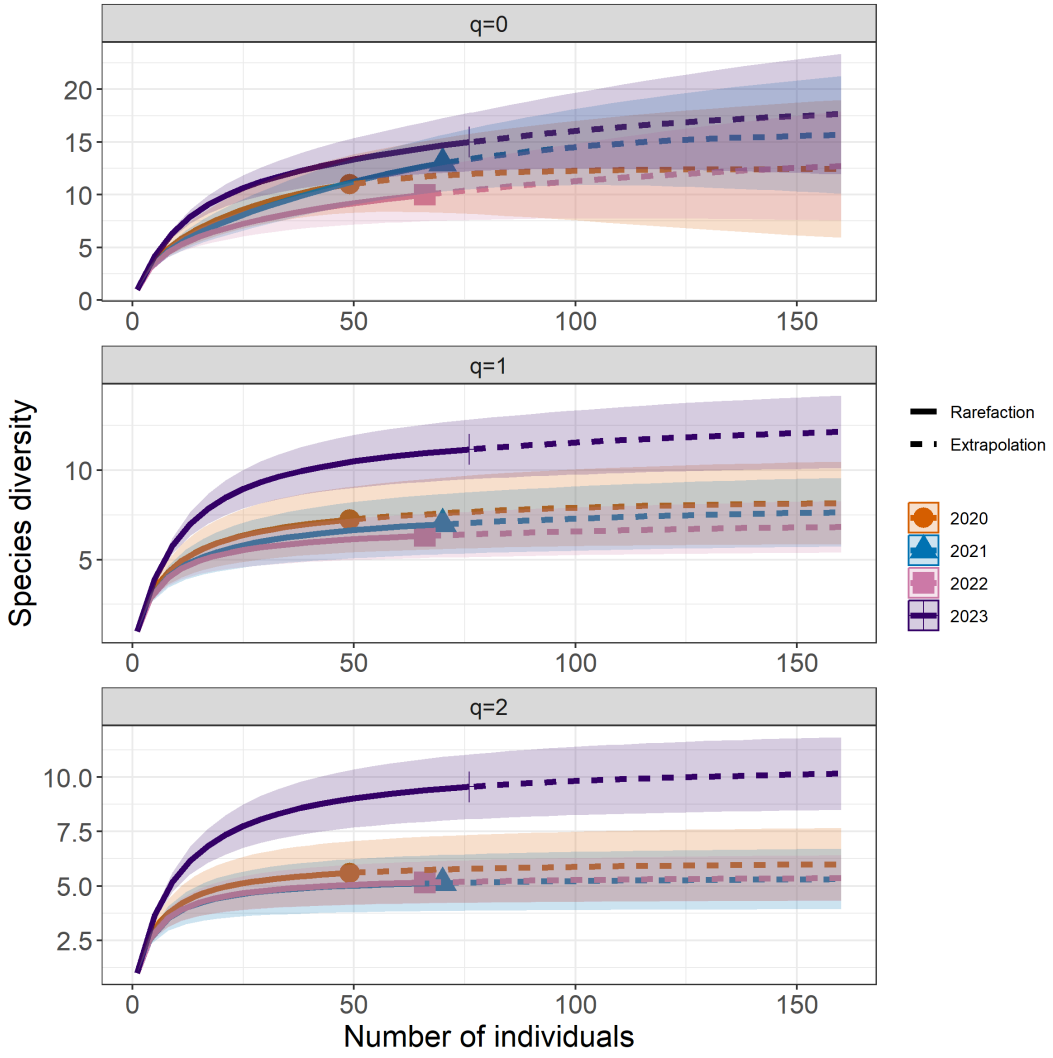


Figure 5. Sample-size-based rarefaction and extrapolation curves of the diet composition of little penguins from molecular analysis of faecal samples collected on Garden Island in 2020 – 2023 for *q* = 0, 1 and 2 (defined as in Figure 4). The solid symbols represent the reference samples

Table 13. The numerical values for *q* = 0, 1 and 2 for the abundance of fish species in little penguin faecal samples collected from Garden Island from 2020 – 2023.

Diversity	*q* = 0	*q* = 1	*q* = 2
2020			
Asymptotic	12.5	8.3	6.2
Estimated	11	7.2	5.6
Undetected	1.2	1.1	0.6
2021			
Asymptotic	16.1	7.9	5.5
Estimated	13	7	5.1
Undetected	3.1	0.9	0.4
2022			
Asymptotic	14.4	7.1	5.5
Estimated	10	6.3	5.2
Undetected	4.4	0.8	0.3
2023			
Asymptotic	18.9	12.7	10.8
Estimated	15	11.2	9.6
Undetected	3.9	1.5	1.2

RF modelling

Neither the model using the presence/absence of all species, or that with the seven species from Dataset B with the greatest frequency of occurrence (anchovy, pilchard, sandy sprat, sardine, garfish, sea mullet and jack mackerels) performed well. The OOB R^2 and performance error for the first model was 0.006 and 3617.74, and 0.02 and 3564.62 for the second model. As such, we were unable to predict the influence of any of the environmental variables on the presence/absence of fish species in the diet of the penguins from 2021 – 2023.

4.1.2 SI analysis

Moult feathers were collected from 66 adults from the summers of 2018/19 - 2023/24 (Table 14). Chick down was collected from 36 chicks during the breeding season in the years 2020-2023 (Table 14). Additionally, contour feathers were collected from four deceased chicks in 2023.

Comparison of non-breeding/pre-moult penguins between years

Isotopic composition of the adult penguin feathers varied significantly between years for all three isotopes ($\delta^{15}N$ values LM: $F_{4,61} = 5.845$, $P < 0.001$, $\delta^{13}C$ values LM: $F_{4,61} = 10.65$, $P < 0.001$, $\delta^{34}S$ values KW: $H_{(4)} = 16.499$, $P < 0.005$, Table 7). However, the years that differed varied, dependent on the isotopes. The $\delta^{15}N$ values of the non-breeding/pre-moult penguins in 2022/23 were significantly lower than those from in 2018/19, 2019/20 and 2020/21 (Tukey's post-hoc for $\delta^{15}N$, $P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively; Table 14). The $\delta^{13}C$ values in 2020/21 and 2021/22 were significantly less enriched compared to both 2019/20 (Tukey's post-hoc for $\delta^{13}C$, $P < 0.001$ and $P < 0.001$, respectively). and 2022/23 (Tukey's post-hoc for $\delta^{13}C$, $P < 0.01$ and $P < 0.001$ respectively). Additionally, the $\delta^{13}C$ values in 2021/22 were significantly less enriched compared to 2018/2019 (Tukey's post-hoc for $\delta^{13}C$, $P < 0.05$; Table 14). The $\delta^{34}S$ values were significantly higher in 2020/21 and 2021/22 compared to 2019/20 (Dunn's test for $\delta^{34}S$, $P < 0.05$, $P < 0.001$ respectively; Table 14). There were no significant differences between other years (all $P > 0.05$).

Table 14. Stable isotope values for carbon (δ13C), nitrogen (δ15N) and sulphur (δ34S) in the adult contour feathers, chick mesoptyle down and chick contour feathers of little penguins on Garden Island, Western Australia, collected in 2020-2023. Values are means (SD). Stable isotope values in the adult feather samples represent diet composition of non-breeding/pre-moult adult penguins during the summer period a year prior to collection, whilst those in the chick down samples represent diet composition of breeding adult penguins a few weeks prior to collection. Chick contour feathers were collected from deceased fledglings and, thus, still represent breeding from that time period.

Foraging season and year	N samples	Adult/chick	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Summer 2018/19	4	Adult	-19.7 (0.3)	14.5 (0.4)	19.4 (0.6)
Summer 2019/20	7	Adult	-19.2 (0.6)	14.0 (0.6)	18.2 (0.2)
Summer 2020/21	25	Adult	-20.2 (0.4)	13.7 (0.4)	19.1 (0.6)
Summer 2021/22	10	Adult	-20.5 (0.3)	13.7 (1.1)	19.6 (0.8)
Summer 2022/23	20	Adult	-19.7 (0.7)	13.1 (0.7)	19.0 (0.9)
Winter-Spring 2020	11	Chick	-20.4 (0.3)	13.1 (0.2)	18.4 (0.1)
Spring-Summer 2021	5	Chick	-19.6 (0.3)	13.1 (0.4)	17.9 (0.3)
Spring 2022	10	Chick	-20.4 (0.8)	13.1 (0.3)	18.8 (0.3)
Winter-Spring 2023	10	Chick	-20.0 (0.6)	13.1 (0.6)	18.1 (1.0)
Winter-Spring 2023	4	Chick contour	-20.3 (1.4)	13.2 (0.4)	17.0 (1.7)

The narrowest isotopic niche for $\delta^{13}C$ and $\delta^{15}N$ of the non-breeding/pre-moult penguins was in 2020/21 (Figure 6a). The probability that the $\delta^{13}C$ and $\delta^{15}N$ isotopic niche was wider in 2019/20, 2021/22 and 2022/23 compared to 2020/21 was 0.94, 0.99 and 1, respectively. The 2020/21 ellipse overlapped most with that of 2021/22 (77%) and 2022/23 (88%; Table 15). There was no difference in the isotopic niche between 2019/20 and 2021/22 (probability= 0.67), but the $\delta^{13}C$ values tended to be less enriched whilst the range of $\delta^{15}N$ values was wider in 2021/22, and as such there was little overlap between the ellipses (Figure 6d). Only 29% of the ellipse for 2019/20 overlapped with 2021/22 and 20% of that for 2021/22 overlapped with 2019/20 (Table 15). The $\delta^{13}C$ and $\delta^{15}N$ isotopic niche in 2022/23 was wider than 2019/20 but not 2021/22 (probability = 0.87 and 0.77 respectively; Figure 6a). The ellipse for 2022/23 overlapped least with each of these years (Table 15, Figure 6d).

The narrowest isotopic niche of $\delta^{13}C$ and $\delta^{34}S$ was in 2019/20 (Figure 6b), driven by a very narrow range of $\delta^{34}S$ values (Figure 6e). The probability that the isotopic niche of $\delta^{13}C$ and $\delta^{34}S$ was wider in 2020/21, 2021/22 and 2022/23 compared to 2019/20 was 0.97, 0.99 and 0.99, respectively. The isotopic niche in 2022/23 was wider than that in 2020/21 and 2021/22 (probability = 0.99 and 0.96 respectively), and the ellipses for all years prior to 2022/23 were almost completely, if not wholly, within the 2022/23 ellipse (Figure 6e, Table 15). There was no difference in the isotopic niche between 2021/22 and 2020/21 (probability = 0.76, Figure 6b), but there was less overlap of 2021/22 ellipse with that of 2020/21 (59%) than vice versa (82%).

The narrowest isotopic niche of $\delta^{34}S$ and $\delta^{15}N$ was in 2019/20 (Figure 6c). The probability that the isotopic niche of $\delta^{34}S$ and $\delta^{15}N$ in 2020/21, 2021/22 and 2022/23 was wider than that in 2019/20 was 0.98, 0.99 and 1 respectively. The isotopic niche in 2020/21 was narrower than that in 2021/22 and 2022/23 (probability = 0.99 and 1 respectively, Figure 6c). There was no difference between 2021/22 and 2022/23 (probability = 0.44, Figure 6c), yet there was only approximately 50% overlap between the ellipses for each year. This was largely driven by a narrow range of $\delta^{34}S$ in 2021/22 and of $\delta^{15}N$ in 2022/23. The ellipses for 2019/20 and 2020/21 were mostly encompassed within the ellipse for 2022/23, and the least overlap (7%) occurred between 2021/22 and 2019/20 (Figure 6f, Table 15).

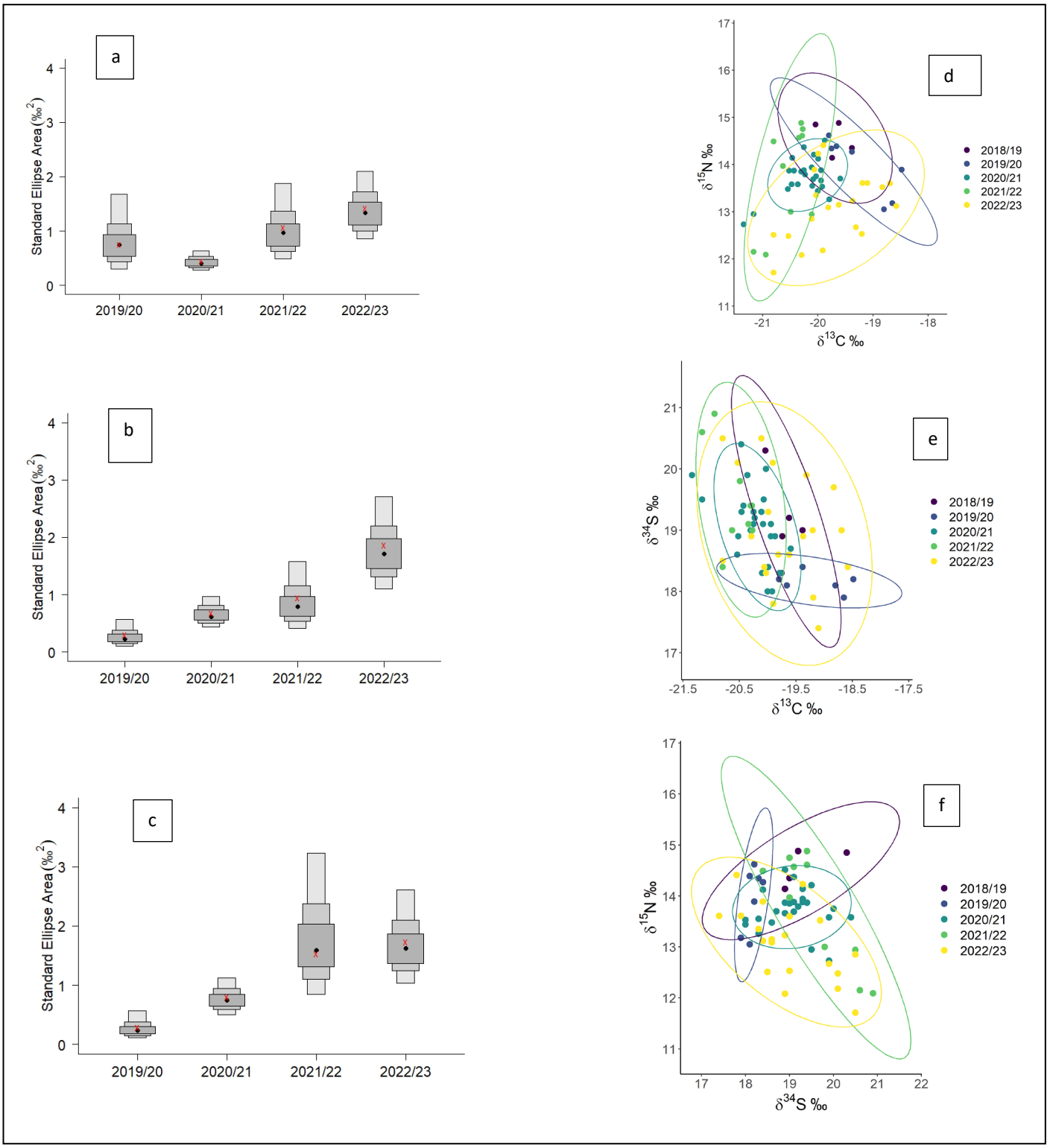


Figure 6. (a-c) Bayesian standard ellipse area (SEA_B), and **(d-f)** sample size-corrected standard ellipse areas (SEA_C) calculated for $\delta^{13}C$ and $\delta^{15}N$ (a and d), $\delta^{13}C$ and $\delta^{34}S$ (b and e), and $\delta^{34}S$ and $\delta^{15}N$ values (c and f) for non–breeding/pre-moult little penguins from Garden Island, Western Australia in 2019/20, 2020/21, 2021/22 and 2022/23. For the SEA_B, black dots represent their mode, red crosses are the true population mean and the shaded boxes represent the 50%, 75% and 95% credible intervals from dark to light grey (after Jackson et al. 2011). Note that SI values for 2018/19 have been included in the sample size-corrected standard ellipse areas (SEA_C) but have not been in the Bayesian standard ellipse area (SEA_B) due to the small sample size for this year.

Table 15. Pairwise dietary niche overlap between non-breeding/pre-moult little penguins from Garden Island, Western Australia in each sampling year. Expressed as the percentage of overlap in relation to the corrected standard ellipse area (SEA_C).

Year comparison	SI Pair		
	$\delta^{13}C$ and $\delta^{15}N$	$\delta^{13}C$ and $\delta^{34}S$	$\delta^{34}S$ and $\delta^{15}N$
2019/20 with 2020/21	21	48	56
2020/21 with 2019/20	36	21	20
2019/20 with 2021/22	29	29	37
2021/22 with 2019/20	20	10	7
2019/20 with 2022/23	64	93	80
2022/23 with 2019/20	34	15	13
2020/21 with 2021/22	77	82	67
2021/22 with 2020/21	32	59	35
2020/21 with 2022/23	88	100	84
2022/23 with 2020/21	27	37	39
2021/22 with 2022/23	50	87	52
2022/23 with 2021/22	37	44	45

Comparison between breeding and non-breeding/pre-moult penguins in 2020, 2021 and 2022

To compare the stable isotopes of breeding and non-breeding/pre-moult penguins in each year (hereafter breeding stage), we used the chick down (and chick contour feathers where appropriate, i.e. from dead fledglings), collected in the year, and the moult feathers collected the following summer (e.g. chick down collected in 2020 and moult feathers collected in the summer of 2020/21).

For the $\delta^{15}N$ values, there was a significant effect of breeding stage, year, and an interaction between breeding stage and year (breeding stage LM: $F_{1,75} = 8.273$, $P < 0.01$, year LM: $F_{2,75} = 5.012$, $P < 0.01$; breeding stage X year LM $F_{2,75} = 2.586$, $P < 0.1$). The $\delta^{15}N$ values of the breeding adults were significantly lower than that of the non-breeding/pre-moult adults in 2020 and 2021, but there was no difference in 2022 (2020: $t = 2.996$, $P < 0.01$, 2021: $t = 2.024$, $P < 0.05$, 2022: $t = -0.066$, $P = 0.948$; Table 14).

For the $\delta^{13}C$ values, there was a significant effect of year and an interaction between breeding stage and year (breeding stage LM: $F_{1,75} = 2.076$, $P = 0.1538$, year LM: $F_{2,75} = 3.823$, $P < 0.05$; breeding stage X year LM $F_{2,75} = 11.441$, $P < 0.001$). The $\delta^{13}C$ values of the non-breeding/pre-moult adults was significantly less enriched than the breeding adults in 2021, but significantly more enriched than the breeding adults in 2022 (2020: $t = 0.997$, $P = 0.337$, 2021: $t = -3.240$, $P < 0.05$, 2022: $t = -3.703$, $P < 0.001$; Table 14).

For the $\delta^{34}S$ values, there was a significant effect of breeding stage and an interaction between breeding stage and year (breeding stage GLM: $F_{1,75} = 18.349$, $P < 0.001$, year GLM: $F_{2,75} = 0.328$, $P = 0.722$; breeding stage X year GLM $F_{2,75} = 6.660$, $P < 0.005$). The $\delta^{34}S$ values of the breeding adults was significantly lower than the non-breeding/pre-moult adults in 2020 and 2021, but there was no difference in 2022 (2020: $t = 2.697$, $P < 0.01$, 2021: $t = -4.914$, $P < 0.001$, 2022: $t = -0.635$, $P = 0.527$; Table 14).

The isotopic niche of the breeding penguins in 2020, 2021 and 2022 was narrower than that of the non-breeding penguins for all isotope pairs ($\delta^{13}C$ and $\delta^{15}N$, probability = 1, 0.99 and 0.93 for 2020, 2021 and 2022 respectively; $\delta^{13}C$ and $\delta^{34}S$, probability = 1, 0.98 and 0.99 for 2020, 2021 and 2022 respectively; $\delta^{34}S$ and $\delta^{15}N$, probability = 1, 0.99 and 1 for 2020 , 2021 and 2022 respectively; Figures 7 a-c). The overlaps in the ellipses of each isotope pair ranged from 0 to 100% (Table 16). For the

breeding and non-breeding/pre-moult birds in 2021, there was very little overlap in the ellipses of $\delta^{13}C$ and $\delta^{15}N$, and no overlap of $\delta^{34}S$ and $\delta^{15}N$ (Figures 7d and 7f, Table 16). In all other pairs, there was always greater overlap between breeding and non-breeding/pre-moult than vice versa (Figure 7 d-f, Table 16).

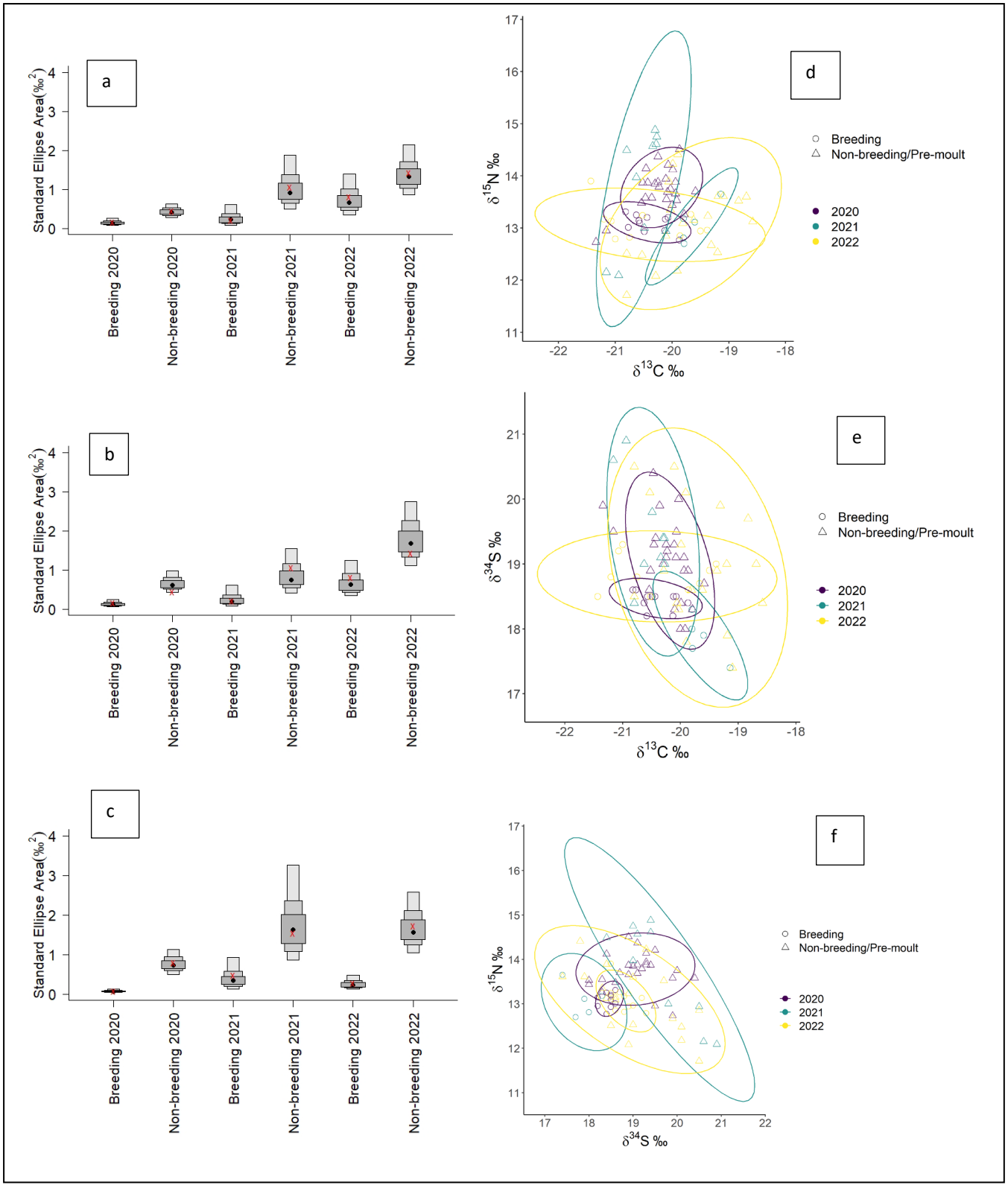


Figure 7. **(a-c)** Bayesian standard ellipse area (SEA_B), and **(d-f)** sample size-corrected standard ellipse areas (SEA_C) calculated for $\delta^{13}C$ and $\delta^{15}N$ (a and d), $\delta^{13}C$ and $\delta^{34}S$ (b and d), and $\delta^{34}S$ and $\delta^{15}N$ (c and f) values for breeding and non–breeding/pre-moult little penguins from Garden Island, Western Australia in 2020, 2021 and 2022. For the SEA_B, black dots represent their mode, red crosses are the true population mean and the shaded boxes represent the 50%, 75% and 95% credible intervals from dark to light grey (after Jackson et al. 2011)

Table 16. Pairwise dietary niche overlap between breeding and non-breeding/pre-moult little penguins from Garden Island, Western Australia in 2020, 2021 and 2022. The overlap is expressed as the percentage of overlap in relation to the corrected standard ellipse area (SEA_C).

Year comparison	SI Pair		
	$\delta^{13}C$ and $\delta^{15}N$	$\delta^{13}C$ and $\delta^{34}S$	$\delta^{34}S$ and $\delta^{15}N$
Breeding 2020 with non-breeding/pre-moult 2020/21	75	80	81
Non-breeding/pre-moult 2020/21 with breeding 2020	26	17	8
Breeding 2021 with non-breeding/pre-moult 2021/22	15	37	0
Non-breeding/pre-moult 2021/22 with breeding 2021	3	10	0
Breeding 2022 with non-breeding/pre-moult 2022/23	71	75	100
Non-breeding/pre-moult 2022/23 with breeding 2022	41	30	16

Comparison between breeding penguins 2020, 2021, 2022 and 2023

There was no difference in the $\delta^{15}N$ values in chick down (representing breeding adults) in the four breeding seasons (KW $H_{(3)}$ = 0.249, P = 0.9692, Table 14). However, there was a difference in the values of $\delta^{13}C$ between years ($F_{3,31}$= 4.796, P < 0.01), with the values in 2021 more enriched than those in 2020, 2022 and 2023 (t = 3.190, P < 0.05, t =3.572, P < 0.01, and t =3.034, P < 0.05. respectively, Table 14). There were no differences between any other year combinations (all P > 0.05). The values of $\delta^{34}S$ varied between the breeding seasons (KW $H_{(3)}$ = 14.66, P < 0.005), with the values in 2022 more enriched than those in 2021 and 2023 (z = -3.48, P < 0.01 for 2022 v 2021, and z = -2.94, P < 0.05 for 2022 v 2023). There were no other significant differences between year pairs.

The isotopic niche for $\delta^{13}C$ and $\delta^{15}N$ of the breeding penguins in 2023 was greater than all other years (probability that it was wider than 2020 = 0.85, than 2021 = 1 and 2022 = 0.92, Figure 8a). The isotopic niche in both 2021 and 2022 was wider compared to 2020 (probability = 1 and 1 respectively), and that in 2022 was wider than in 2021 (probability = 0.97; Figure 8a). The isotopic niche of $\delta^{13}C$ and $\delta^{34}S$ in 2021, 2022 and 2023 was wider compared to 2020 (probability = 0.89, 1 and 1 respectively), the isotopic niche in 2022 and 2023 was wider than in 2021 (probability = 0.95 and 1 respectively), and 2023 was wider than 2022 (probability = 1; Figure 8b). The isotopic niche of $\delta^{34}S$ and $\delta^{15}N$ in 2021, 2022 and 2023 were wider than that in 2020 (probability = 0.99, 1 and 1 respectively), but unlike the other isotopic niches, that of $\delta^{34}S$ and $\delta^{15}N$ in 2021 was wider than in 2022 (probability = 0.79; Figure 8c). The isotopic niche of $\delta^{34}S$ and $\delta^{15}N$ in 2023 was wider than in 2021 and 2022 (probability = 1 and 1 respectively; Figure 8 c). There was overlap in the ellipses for all pairs (Figure 8 d-f), but the amount of overlap varied between years and isotope pair, from 3% to complete overlap (Table 17). The ellipses for all pairs of stable isotopes for 2020, 2021 and 2022 were almost entirely, if not entirely, encompassed within those for 2023. The very little overlap of all the ellipses in 2023 with those in 2020 and 2021 (ranging from 3-14%, Table 17), indicates that the penguins in 2023 occupied a very different isotopic space compared to these other two years.

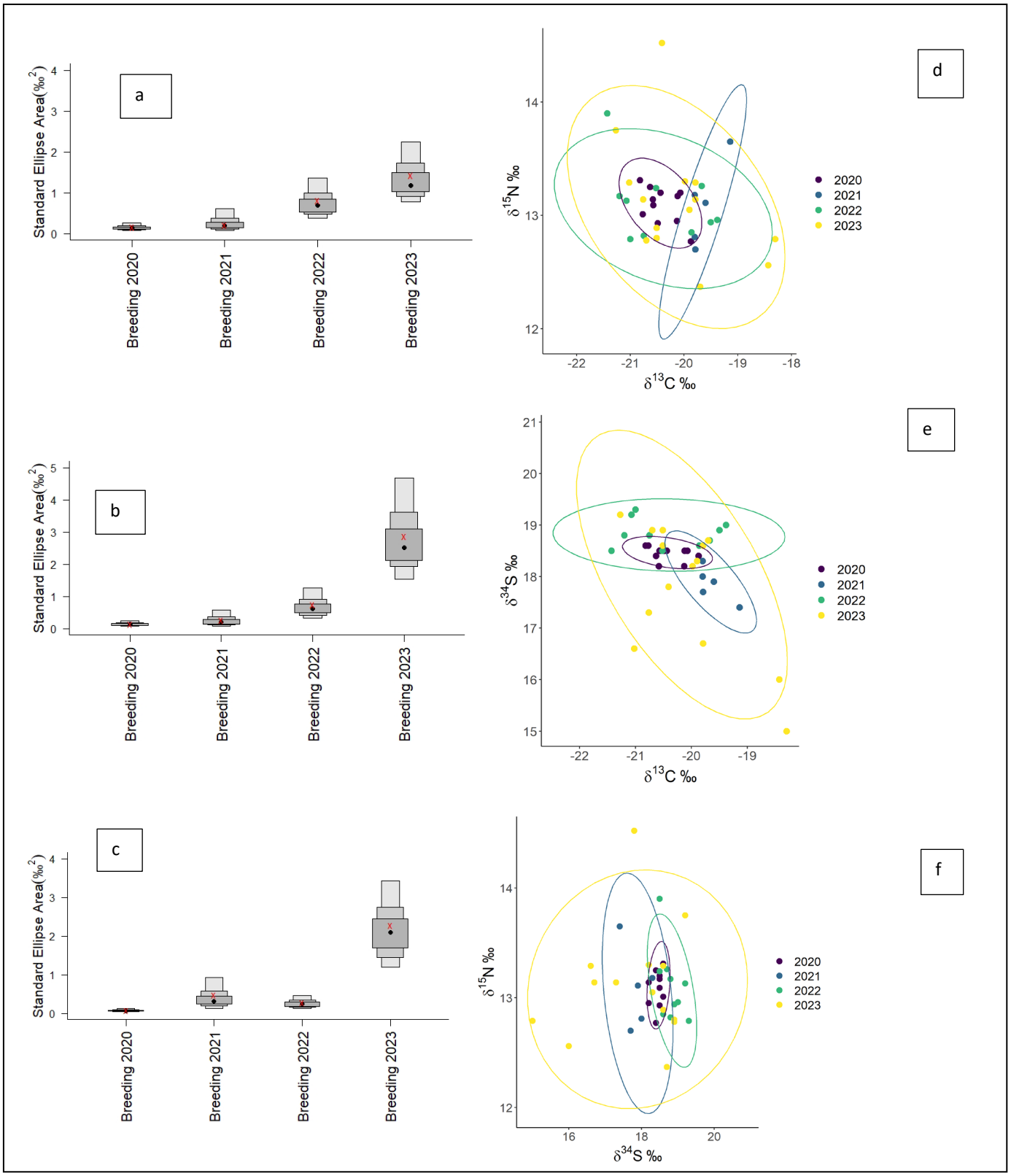


Figure 8. **(a-c)** Bayesian standard ellipse area (SEA_B), and **(d-f) s**ample size-corrected standard ellipse areas (SEA_C) calculated for $\delta^{13}C$ and $\delta^{15}N$ (a and d), $\delta^{13}C$ and $\delta^{34}S$ (b and d), and $\delta^{34}S$ and $\delta^{15}N$ (c and f) values for breeding little penguins from Garden Island, Western Australia in 2020, 2021, 2022 and 2023. For the SEA_B, black dots represent their mode, red crosses are the true population mean and the shaded boxes represent the 50%, 75% and 95% credible intervals from dark to light grey (after Jackson et al. 2011).

Table 17. Multiple pairwise dietary niche overlap between breeding little penguins from Garden Island, Western Australia between 2020, 2021, 2022 and 2023. Expressed as the percentage of overlap in relation to the corrected standard ellipse area (SEA_C).

Year comparison	SI Pair		
	$\delta^{13}C$ and $\delta^{15}N$	$\delta^{13}C$ and $\delta^{34}S$	$\delta^{34}S$ and $\delta^{15}N$
2020 with 2021	25	48	88
2021 with 2020	17	27	14
2020 with 2022	100	98	77
2022 with 2020	19	19	21
2020 with 2023	100	100	100
2023 with 2020	11	5	3
2021 with 2022	70	31	17
2022 with 2021	19	11	31
2021 with 2023	95	100	100
2023 with 2021	14	7	8
2022 with 2023	97	89	100
2023 with 2022	56	23	11

4.1.3 Effect of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$

The effect of lipid extraction on the $\delta^{13}C$ and $\delta^{15}N$ in penguin prey samples was dependent on the stable isotope, when they were collected and the fish species. For the penguin prey species collected in November 2021 and May 2022, there was a significant **decrease** in $\delta^{13}C$ in all the fish species following lipid extraction (Tables 18 - 20). There was also a significant decrease in the values of $\delta^{15}N$ in three of the six fish species collected in November 2021, and a significant increase in one of the species collected in May 2022 (Tables 18 - 20). The significant decrease in values of both $\delta^{13}C$ and $\delta^{15}N$ following lipid extraction is contrary to what is typically observed (e.g. Post et al. 2006, Logan et al. 2008), and is possibly due to the analyses being conducted in different laboratories. For the penguin prey species collected in November 2022 and May 2023, there was a significant **increase** in $\delta^{13}C$ after lipid extraction in 10 of the 14 species collected, and a significant **increase** in $\delta^{15}N$ in eight of the 14 species collected (Tables 18 - 20).

4.1.4 Prey SI ratios

Thirteen potential penguin prey species were captured across the four sampling periods, eight of which were caught in more than one sampling period. As all the samples were analysed for normalised (i.e. following lipid removal) values of $\delta^{13}C$ and $\delta^{15}N$ in the same laboratory, these values have been used for statistical analyses.

The summer 2021 stable isotope values of prey collected ranged from -21.3 to -15.3‰ for $\delta^{13}C$ and 8.8 to 12.3 ‰ for $\delta^{15}N$ (Table 19). There was a significant difference in the normalised values $\delta^{13}C$ and $\delta^{15}N$ values between fish species ($F_{6,36}$=85.96, $P < 0.001$ for $\delta^{13}C$ and $F_{6,36}$=11.91, $P < 0.001$ for $\delta^{15}N$). Sea mullet had the most enriched mean values for $\delta^{13}C$ (Table 19), and significantly differed from all other penguin prey species caught in summer 2021 (Tukey's post hoc test for $\delta^{13}C$, all $P < 0.005$), but there were no other significant differences between any of the other species (Tukey's post hoc test for $\delta^{13}C$, all $P > 0.05$). Hardyheads had the most enriched values of $\delta^{15}N$ (Table 19) but was only significantly different to the sea mullet (Tukey's post hoc test for $\delta^{13}N$ between hardyheads and sea mullet, $P < 0.005$). Furthermore, sea mullet had significantly lower values of $\delta^{15}N$ compared to all the other penguin fish prey species (Tukey's post hoc test for $\delta^{13}N$, all $P < 0.005$; Table 19).

The mean winter 2022 stable isotope values of prey ranged from -21.0 to -19.0‰ for $\delta^{13}C$, 9.1 to 11‰ for $\delta^{15}N$ (Table 19) and 17.0 to 17.6‰ for $\delta^{34}S$ (Table 21). There was a significant difference in the normalised values $\delta^{13}C$ and $\delta^{15}N$ values between fish species ($H_{(6)}$=20.612, $P < 0.01$ for $\delta^{13}C$, $H_{(6)}$=20.287, $P < 0.01$ for $\delta^{15}N$) but not in the values of $\delta^{34}S$ between species ($H_{(3)} = 0.769$, P=0.857). Hardyheads had the most enriched mean values for $\delta^{13}C$ (Table 19) but were only significantly more enriched than sandy sprat and yellowtail scad (Tukey's post hoc test, both $P < 0.05$). Yellowtail scad, with the least enriched value of $\delta^{13}C$, were only significantly less enriched than whitebait and blue sprat (Tukey's post hoc test, both $P < 0.01$). Yellowtail scad had the most enriched values of $\delta^{15}N$ (Table 19) but were not significantly different to the values of $\delta^{15}N$ of any of the other penguin prey species (Tukey's post hoc test, all $P > 0.01$).

The mean summer 2022 stable isotope values of prey ranged from -21.6 to -12.2‰ for $\delta^{13}C$, 7.5 to 13.1‰ for $\delta^{15}N$ (Table 19), and 10.4 to 19.5‰ for $\delta^{34}S$ (Table 21). There was a significant difference in the normalised $\delta^{13}C$and $\delta^{15}N$ values between fish species ($H_{(7)}$=30.198, $P < 0.001$ for $\delta^{13}C$, $H_{(7)}$=25.386, $P < 0.001$ for $\delta^{15}N$) and in the $\delta^{34}S$ between fish species ($F_{7,27}$= 94.2, $P<0.001$). Sea mullet had the most enriched mean values for $\delta^{13}C$ (Table 19) and were significantly different from blue sprat, hardyheads, silverbelly and yelloweye mullet (Dunn's test, all $P < 0.05$). Blue sprat had the most enriched values of $\delta^{15}N$ (Table 19), but was only significantly different from sea mullet, which had the lowest value of $\delta^{15}N$. Hardyheads had significantly different values of $\delta^{15}N$ compared to sea mullet (Dunn's test, both $P < 0.05$). The $\delta^{34}S$ in sea mullet, with the least enriched value, was significantly different to all other seven species tested that season (Table 21, Tukey's post hoc test, all $P < 0.05$ or less). Blue sprat, with the most enriched $\delta^{34}S$ value, was significantly different to hardyheads and sandy sprat (Table 21, Tukey's post hoc test, $P < 0.01$ and $P < 0.05$, respectively). In addition to the significant difference in the $\delta^{34}S$ value of hardyheads and sea mullet, hardyheads were significantly different to garfish, silverbelly and yelloweye mullet (Table 21, Tukey's post hoc test, all $P< 0.05$).

The mean winter 2023 stable isotope values of prey ranged from -20.2 to -17.5‰ for $\delta^{13}C$, 9.5 to 10.8‰ for $\delta^{15}N$ (Table 19), and 14.5 to 19.2‰ for $\delta^{34}S$ (Table 21). There was a significant difference in the normalised values $\delta^{13}C$ and $\delta^{15}N$ values between fish species ($H_{(5)}$=20.43, $P < 0.01$ for $\delta^{13}C$, $H_{(5)}$=17.782, $P < 0.01$ for $\delta^{15}N$) and in the values of $\delta^{34}S$ between fish species ($H_{(5)} = 22.285$, $P< 0.001$). Western butterfish had the most enriched mean values for $\delta^{13}C$ (Table 19), and was significantly different from anchovy, skipjack trevally and yellowtail scad (Dunn's test, all $P < 0.05$). Silverbelly had the most enriched values of $\delta^{15}N$ but were only significantly different from goatfish (Dunn's test, $P < 0.05$). Goatfish also had a significantly lower $\delta^{15}N$ value than yellowtail scad (Dunn's test, $P < 0.05$). Western butterfish also had the least enriched values of $\delta^{34}S$ and were significantly different to skipjack trevally and yellowtail scad (Dunn's test, both $P < 0.05$).

Table 18. Summary of mean change in $\delta^{13}C$ and $\delta^{15}N$ (±SD) following lipid extraction in the muscles of various little penguin prey species caught in November 2021, May 2022, November 2022 and May 2023 in Cockburn Sound. Significant P-values are marked in bold, ^{W} denotes Wilcoxon rank test

Genus and Species	Common Name	Month and Year	n	$\delta^{13}C$	t statistic	P	$\delta^{15}N$	t statistic	P
Atherinomorus vaigiensis	common hardyhead	November 2021	4	-1.68(0.07)	-46.308	**<0.001**	-0.20 (0.05)	-8.802	**<0.005**
Engraulis australis	anchovy	November 2021	7	-1.49 (0.17)	-23.592	**<0.001**	0.06 (0.08)	2.015	0.075
Hyperlophus vittatus	sandy sprat	November 2021	8	-1.47 (0.14)	-29.785	**<0.001**	0.21 (0.10)	5.806	**<0.001**
Mugil cephalus	sea mullet	November 2021	5	-1.47 (0.13)	-24.978	**<0.001**	-0.12 (0.09)	-3.000	**<0.05**
Parequula melbournensis	silverbelly	November 2021	5	-1.58 (0.08)	-46.979	**<0.001**	-0.08 (0.13)	-1.423	0.228
Spratelloides robustus	blue sprat	November 2021	8	-1.32 (0.19)	-19.247	**<0.001**	0.04 (0.68)	0.171	0.869
Trachurus novaezelandiae	yellowtail scad	November 2021	6	-1.36 (0.15)	-22.004	**<0.001**	-0.07 (0.20)	-0.900	0.409
Atherinomorus vaigiensis	common hardyhead	May 2022	9	-1.19 (0.13)	-28.22	**<0.001**	0.08 (0.20)	1.581	0.282
Engraulis australis	anchovy	May 2022	2	-1.21 (0.07)	-24.200	**<0.05**	0.17 (0.03)	8.500	0.074
Hyperlophus vittatus	sandy sprat	May 2022	9	-1.35 (0.21)	-19.272	**<0.001**	0.05 (0.20)	28^{W}	0.573
Hyporhamphus melanochir	garfish	May 2022	5	-1.14 (0.16)	-16.234	**<0.001**	0.02 (0.08)	0.648	0.552
Parequula melbournensis	silverbelly	May 2022	5	-1.47 (0.21)	-15.833	**<0.001**	0.00 (0.19)	0.048	0.964
Spratelloides robustus	blue sprat	May 2022	11	-0.97 (0.19)	-17.124	**<0.001**	0.02 (0.18)	0.392	0.703
Trachurus novaezelandiae	yellowtail scad	May 2022	6	-1.36 (0.30)	-11.274	**<0.001**	0.25 (0.11)	5.222	**<0.005**
Aldrichetta forsteri	yelloweye mullet	November 2022	5	0.42 (0.12)	15^{W}	0.063	0.50 (0.08)	15^{W}	0.063
Atherinomorus vaigiensis	common hardyhead	November 2022	5	0.65 (0.09)	15^{W}	0.063	0.50 (0.09)	14.37	**<0.001**
Hyporhamphus melanochir	garfish	November 2022	4	0.72 (0.17)	8.457	**<0.01**	0.59 (0.11)	10^{W}	0.125
Hyperlophus vittatus	sandy sprat	November 2022	5	0.43 (0.01))	66.36	**<0.001**	0.30 (0.12)	15^{W}	0.063

Leptatherina prebyteroides	silverfish	November 2022	4	0.73 (0.09)	15.696	**<0.001**	0.53 (0.07)	15.849	**<0.001**
Mugil cephalus	sea mullet	November 2022	4	0.35 (0.15)	4.724	**<0.05**	0.46 (0.11)	8.049	**<0.01**
Parequula melbournensis	silverbelly	November 2022	3	0.47 (0.29)	2.849	0.104	0.44 (0.03)	21.757	**<0.01**
Spratelloides robustus	blue sprat	November 2022	5	0.57 (0.09)	14.927	**<0.001**	0.46 (0.11)	8.904	**<0.001**
Engraulis australis	anchovy	May 2023	2	0.37 (0.03)	15.294	**<0.05**	0.29 (0.02)	22.123	**<0.05**
Parequula melbournensis	silverbelly	May 2023	5	0.44 (0.14)	7.2608	**<0.01**	0.29 (0.15)	15^{W}	0.063
Pentapodus vitta	western butterfish	May 2023	6	0.37 (0.26)	3.505	**<0.05**	0.39 (0.12)	8.254	**<0.001**
Pseudocarax wrighti	skipjack trevally	May 2023	5	0.76 (0.18)	9.302	**<0.001**	0.38 (0.21)	15^{W}	0.063
Trachurus novaezelandiae	yellowtail scad	May 2023	5	0.44 (0.38)	2.574	0.062	0.55 (0.10)	11.732	**<0.001**
Upeneus australiae	Australian goatfish	May 2023	4	0.40 (0.25)	3.188	**<0.05**	0.17 (0.19)	1.773	0.174

Table 19. The mean (±SD) value of normalized (i.e. lipid removed) $\delta^{13}C$ and $\delta^{15}N$ in each of the potential little penguin prey species collected in November 2021, May 2022, November 2022 and May 2023 from Cockburn Sound. All samples were analysed at the University of Western Australia.

Genus and species	Common Name	$\delta^{13}C$				$\delta^{15}N$			
		Nov 2021	May 2022	Nov 2022	May 2023	Nov 2021	May 2022	Nov 2022	May 2023
Aldrichetta forsteri	yelloweye mullet			-21.0 (0.37)				10.5 (0.83)	
Atherinomorus vaigiensis	common hardyhead	-20.4 (0.21)	-19.0 (0.22)	-19.9 (0.65)		12.2 (1.07)	10.9 (0.70)	11.7 (0.33)	
Engraulis australis	anchovy	-21.3 (0.22)	-19.8 (0.25)		-19.8 (0.30)	10.8 (0.45)	10.1 (0.16)		10.5 (1.12)
Hyperlophus vittatus	sandy sprat	-21.2 (0.15)	-19.8 (0.42)	-20.6 (0.57)		10.9 (0.33)	10.4 (0.94)	10.9 (0.38)	
Hyporhamphus melanochir	garfish		-19.3 (0.12)	-19.2 (0.35)			9.7 (0.28)	10.3 (1.28)	
Leptatherina prebyteroides	silverfish			-19.1 (0.26)				11.4 (0.21)	
Mugil cephalus	sea mullet	-15.3 (0.66)		-12.2 (0.26)		8.8 (0.47)		7.5 (0.37)	
Parequula melbournensis	silverbelly	-20.6 (0.90)	-19.7 (1.75)	-21.6 (0.74)	-19.0 (0.24)	11.6 (0.58)	10.2 (0.30)	11.0 (0.89)	10.9 (0.23)
Spratelloides robustus	blue sprat	-20.6 (0.28)	-19.2 (0.66)	-21.4 (0.17)		11.4 (0.83)	9.9 (0.83)	13.1 (0.50)	
Pentapodus vitta	western butterfish				-17.5 (0.42)				9.6 (0.78)
Pseudocarax wrighti	skipjack trevally				-19.2 (0.18)				10.3 (0.18)
Trachurus novaezelandiae	yellowtail scad	-21.3 (0.91)	-21.0 (0.11))		-20.2 (1.16)	11.9 (1.30)	11.0 (0.13)		10.8 (0.23)
Upeneus australiae	Australian goatfish				-18.4 (0.52)				9.5 (0.22)

Table 20. The mean (±SD) value of $\delta^{13}C$ and $\delta^{15}N$ without lipid removal in each of the potential little penguin prey species collected in November 2021, May 2022, November 2022 and May 2023 from Cockburn Sound. Samples from November 2021 and May 2022 were analysed at UC Davis, and those in November 2022 and May 2023 were analysed at UWA.

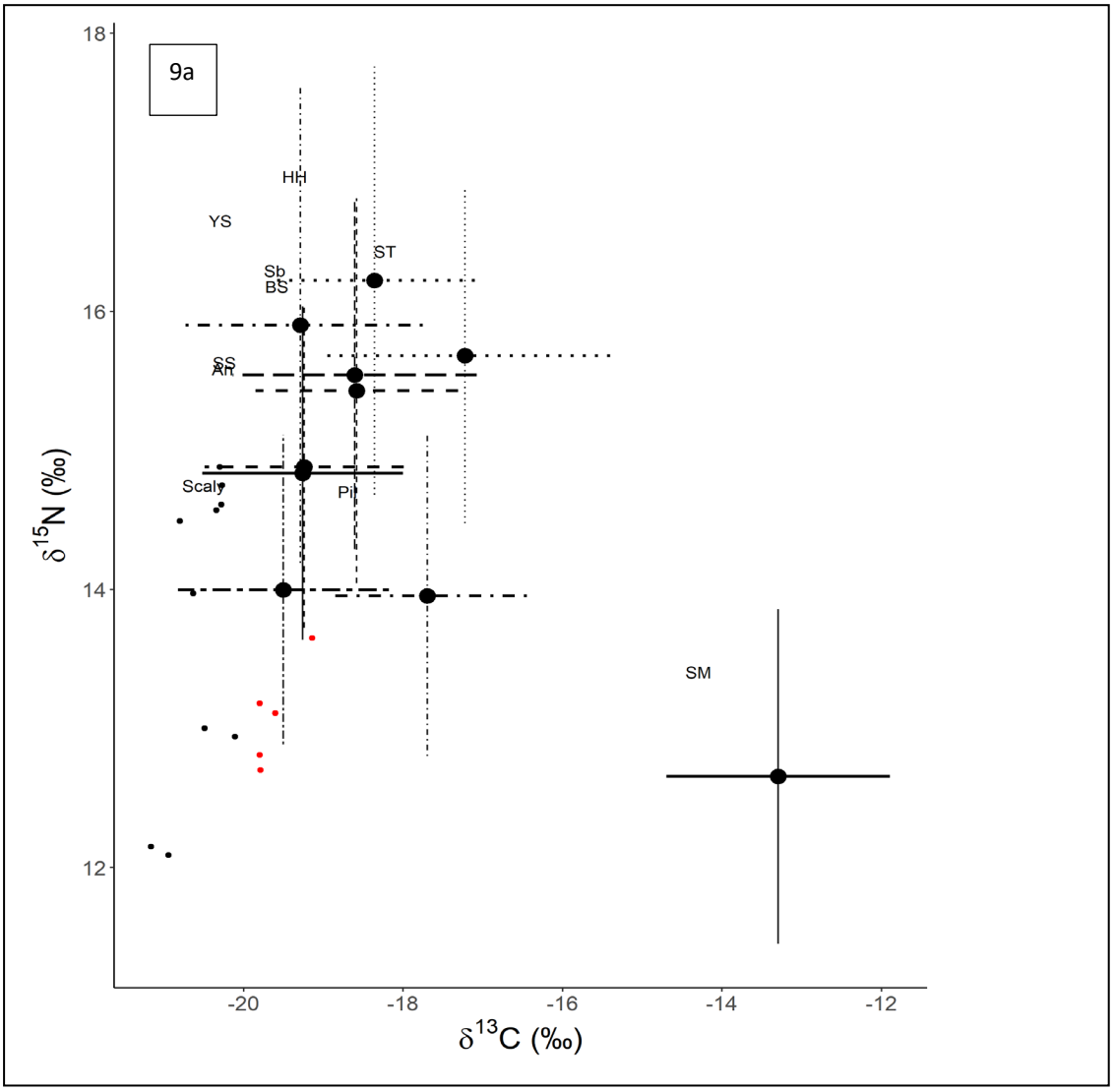
Genus and species	Common Name	$\delta^{13}C$				$\delta^{15}N$			
		Nov 2021	May 2022	Nov 2022	May 2023	Nov 2021	May 2022	Nov 2022	May 2023
Aldrichetta forsteri	yelloweye mullet			-21.4 (0.37)				10.0 (0.84)	
Atherinomorus vaigiensis	common hardyhead	-18.7 (0.20)	-17.9 (0.20)	-19.9 (0.65)		12.4 (1.09)	10.9 (0.55)	11.1 (0.31)	
Engraulis australis	anchovy	-19.8 (0.221)	-18.5 (0.32)		-20.2 (0.34),	10.8 (0.51)	9.9 (0.18)		10.2 (1.14)
Hyperlophus vittatus	sandy sprat	-19.8 (0.15)	-18.4 (0.44)	-21.0 (0.57)		10.7 (0.35)	10.3 (0.96)	10.6 (0.40)	
Hyporhamphus melanochir	garfish		-18.2 (0.18)	-19.9 (0.33)			9.7 (0.32)	9.7 (1.24)	
Leptatherina prebyteroides	silverfish			-19.9 (0.35)				10.9 (0.21)	
Mugil cephalus	sea mullet	-13.8 (0.74)		-12.6 (0.17)		8.8 (0.43)		7.0 (0.40)	
Parequula melbournensis	silverbelly	-19.0 (0.87)	-18.3 (1.57)	-22.0 (0.56)	-19.4 (0.32)	11.6 (0.47)	10.2 (0.42)	10.5 (0.92)	10.6 (0.28)
Spratelloides robustus	blue sprat	-19.3 (0.27)	-18.2 (0.71)	-22.0 (0.19)		11.4 (0.95)	9.9 (0.85)	12.6 (0.40)	
Pentapodus vitta	western butterfish				-17.8 (0.35)				9.2 (0.82)
Pseudocarax wrighti	skipjack trevally				-20.0 (0.28)				9.9 (0.18)
Trachurus novaezelandiae	yellowtail scad	-19.9 (0.93)	-19.6 (0.33)		-20.6 (0.82)	11.9 (1.16)	10.8 (0.15)		10.3 (0.19)
Upeneus australiae	Australian goatfish				-18.8 (0.43)				9.28 (0.14)

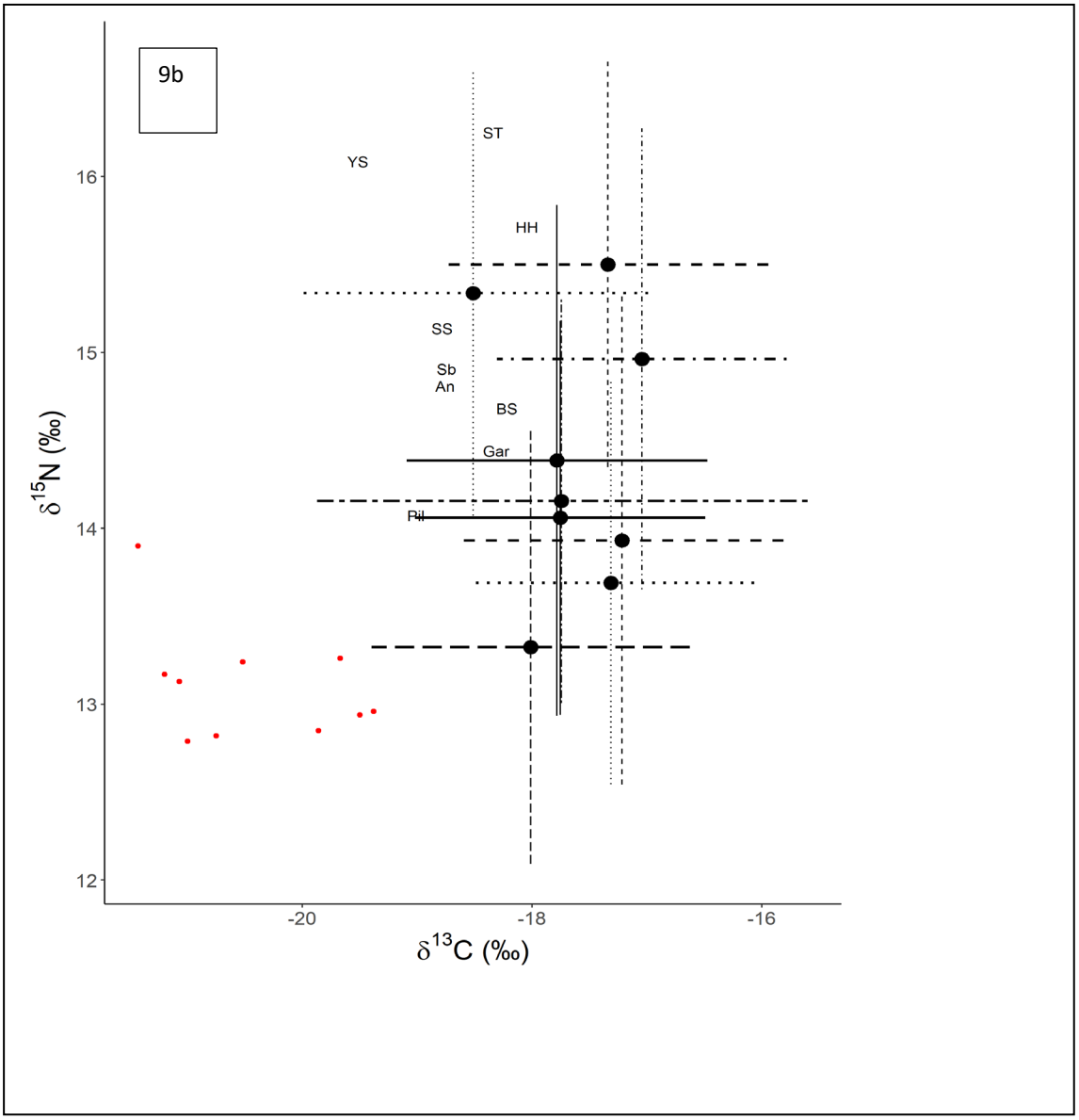
Table 21. The mean (±SD) value of $\delta^{34}S$ in each of the potential little penguin prey species collected May 2022, November 2022 and May 2023 from Cockburn Sound. Samples collected in May 2022 were analysed at the University of Hawaii, whilst those collected in November 2022 and May 2023 were analysed at University of Western Australia.

Genus and species	Common Name	$\delta^{13}S$					
		May 2022	n	Nov 2022	n	May 2023	n
Aldrichetta forsteri	yelloweye mullet			19.2 (0.5)	5		
Atherinomorus vaigiensis	common hardyhead	17.6 (0.5)	8	17.7 (0.7)	5		
Engraulis australis	anchovy	17.5	1			18.9 (0.2)	2
Hyperlophus vittatus	sandy sprat	17.4 (0.4)	9	17.9 (1.1)	5		
Hyporhamphus melanochir	garfish			19.1 (0.5)	4		
Leptatherina prebyteroides	silverfish			19.0 (0.2)	4		
Mugil cephalus	sea mullet			10.4 (0.7)	4		
Parequula melbournensis	silverbelly	17.0 (2.5)	8	19.3 (0.4)	3	17.4 (0.6)	5
Spratelloides robustus	blue sprat			19.5 (0.3)	5		
Pentapodus vitta	western butterfish					14.5 (1.4)	6
Pseudocarax wrighti	skipjack trevally					18.8 (0.2)	5
Trachurus novaezelandiae	yellowtail scad					19.2 (1.3)	5
Upeneus australiae	Australian goatfish					16.1 (0.4)	4

4.1.1 Mixing model outputs

For mixing models to provide solutions for diet composition from specific food sources, the isotopic values of the consumers must fall within the range of the corrected food source isotopic values (i.e. isotope value with diet-tissue discrimination factor added) (Phillips et al. 2014). This only occurred for the breeding penguins in 2023, and hence we were unable to run the mixing models for the breeding and non-breeding penguins from 2021, breeding penguins in 2022, and non-breeding penguins in 2022 (Figures 9 a - d).

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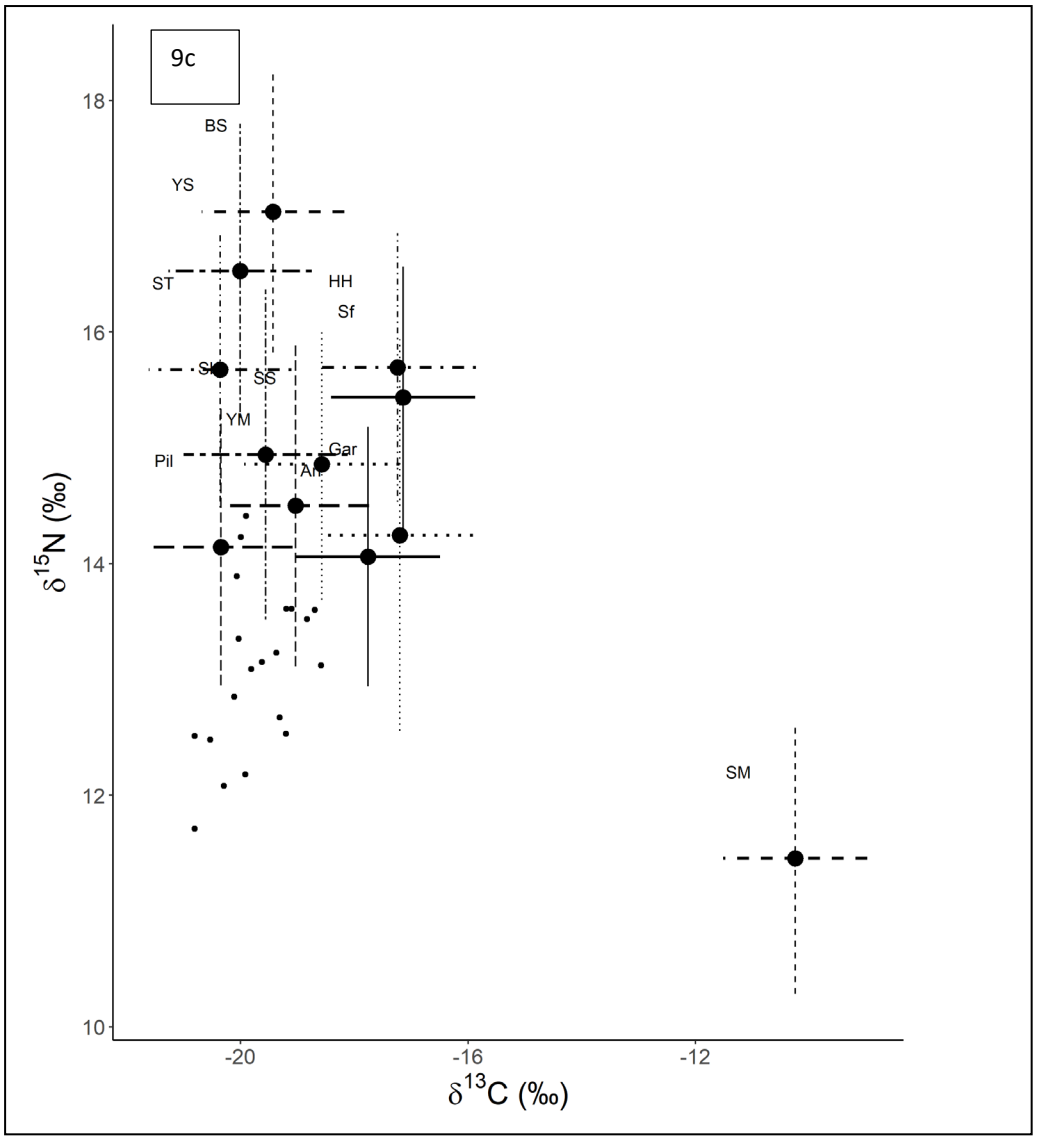
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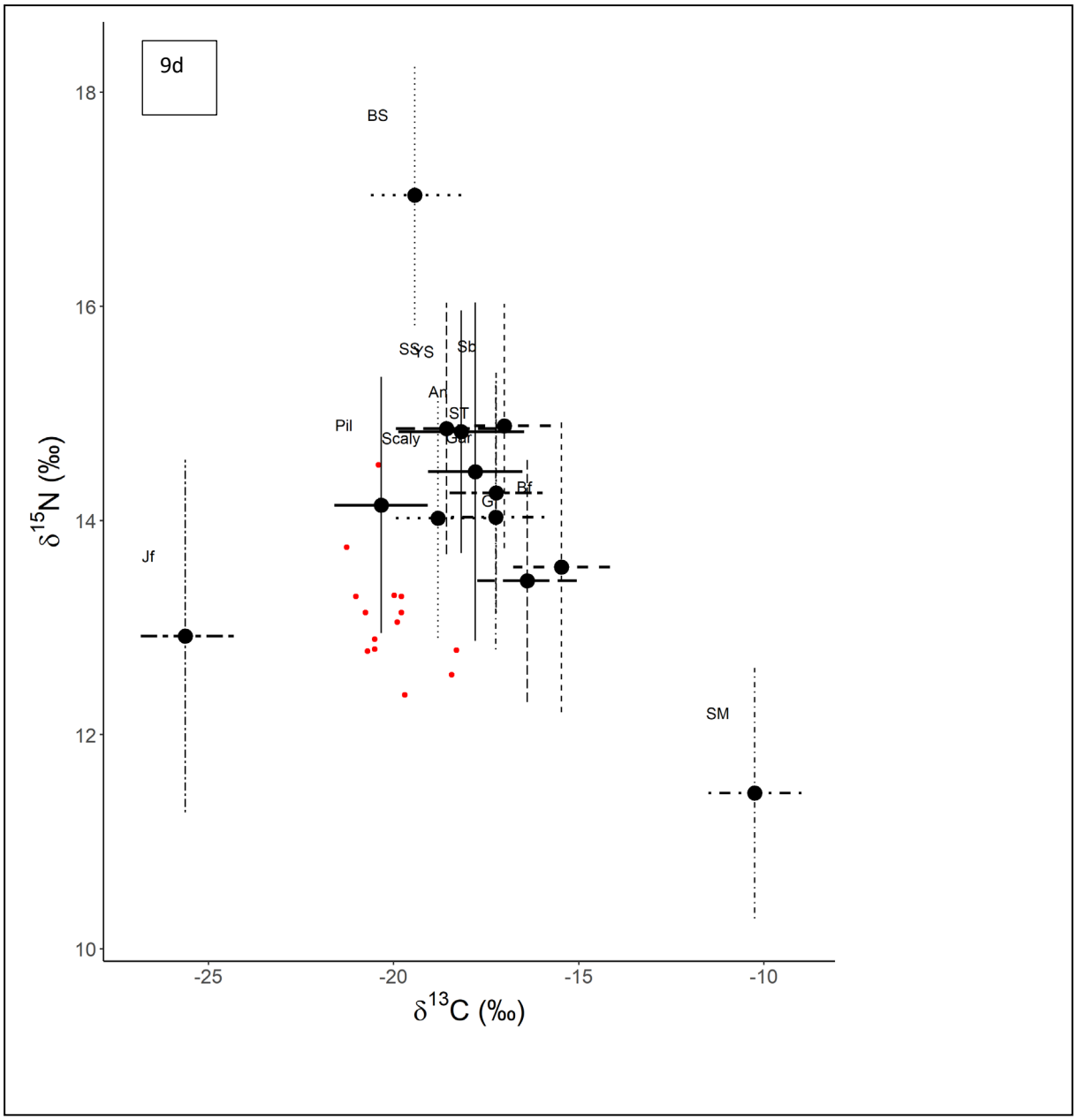

Figure 9. Biplot of stable isotope signatures of little penguins from Garden Island, Western Australia, and potential fish prey samples collected in Cockburn Sound, represented with the mean value of each group and the 95% confidence intervals which incorporate the error in the source isotopic signatures and in the diet-to-tissue discrimination factors. a) breeding (black) and non-breeding (red) penguins from summer 2021 b) breeding penguins in winter 2022. c) non-breeding penguins in summer 2022, and d) breeding penguins in winter 2023. An – anchovy; Bf- Western butterfish, BS – blue sprat; Gar –garfish, Gf – goatfish; Jf- jellyfish, HH – hardyheads; Pil – Pilchard, Sb - silverbelly, Scaly – sardine(scaly mackerel), SM - sea mullet; SS – sandy sprat, ST – skipjack trevally, S – yellowtail scad.

The only mixing model that converged included six prey groups using a very long iteration and the TDF obtained from SIDER (Table 22). From this mixing model, fish from Group B (anchovy, sandy sprat, sardine, garfish and skipjack trevally) composed the greatest proportion of the diet (mean 38%; 32 - 55% CI), with jellyfish the second most important (mean 26%; 21 - 36% CI; Figure 10). Blue sprat (group G) contributed the least to the diet (8%; 5 - 15% CI) of the breeding penguins in 2023 (Figure 10).

Table 22. Comparison of the mixing models fitted with MixSIAR on little penguin data from Garden Island, Western Australia, during breeding in 2023 (n= 10). Only the null model was run, where all individuals are assumed to have the same prey composition. TDF 1: 3.9‰ for $\delta^{15}N$ and 0.2‰ for $\delta^{13}C$ (MacKenzie 2011), 2: mean and SD $\delta^{15}N$ of 3.99‰ and 1.11‰, and mean and SD $\delta^{13}C$ of 2‰ and 1.24‰ (obtained using the R package SIDER). Geweke test: gives the number of variables outside +/-1.96, with ≤ 5% indicating the model has converged. Gelmen-Rubin: The percentage of variables > 1.1, all variables should be < 1.1, and if not, the model has not converged.

Model	MCMC iteration	TDF	Geweke test	Gelmen-Rubin test (%>1.1)	Epsilon 1	Epsilon 2
			Chain 1/chain 2/chain 3			
All fish species	normal	1	11%/9%/7%	11	11.265	4.515
All fish species	normal	2	20%/20%/14%	11	2.767	0.885
Fish species, 7 groups	normal	2	14%/11%/5%	4	9.409	4.192
Fish species, 7 groups	long	2	5%/19%/11%	5	1.861	0.665
Fish species, 7 groups	Very long	2	5%/5%/0	5	1.875	0.661
Fish species, 6 groups	Normal	2	3%/0/29%	0	1.840	0.651
Fish species, 6 groups	Long	2	0/0/0	0	1.873	0.655

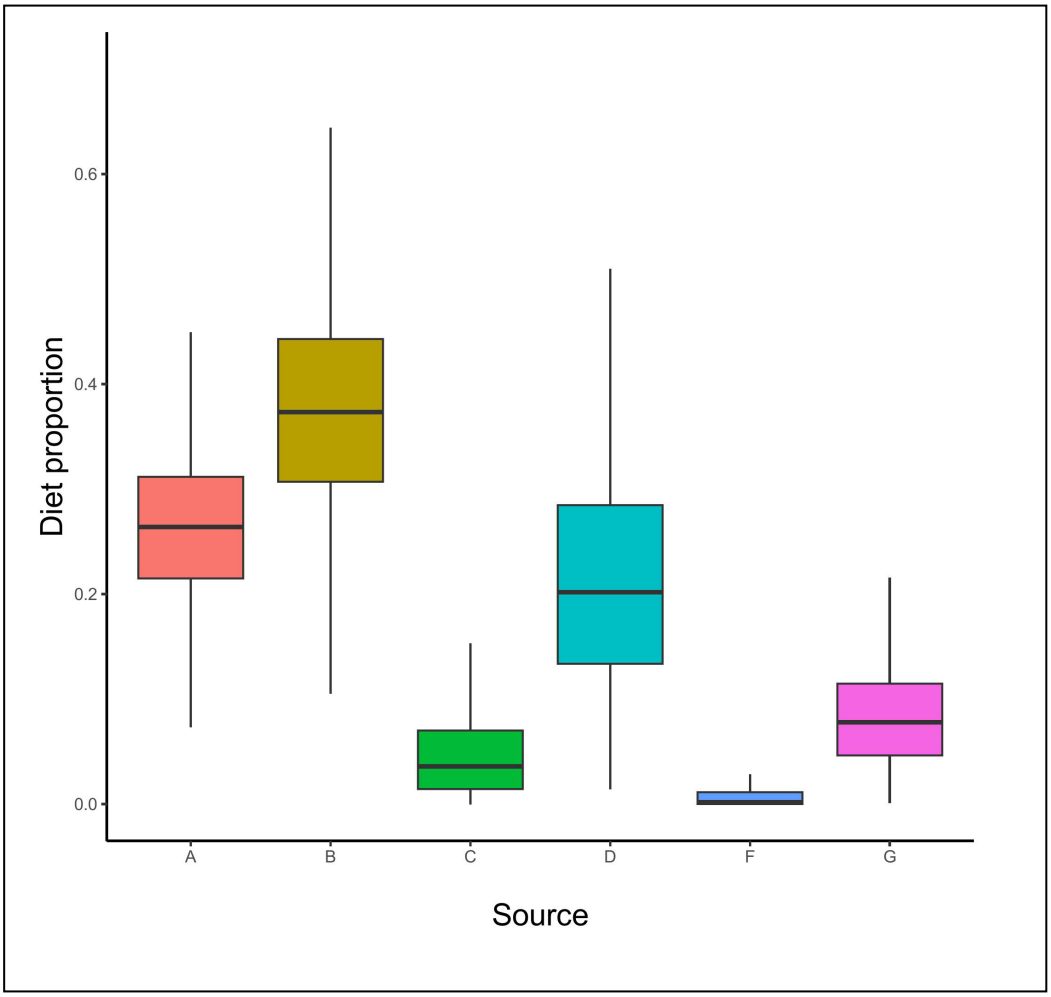

Figure 10. Median (lines in the centre of the box= median, box boundaries = 50% CI, error bars=95%CI), proportional contributions of prey groups to the diet of breeding little penguins from Garden Island, Western Australia, 2023. Fish were grouped based on similarity in stable isotope signatures, with one group containing multiple prey species, and the rest only one or two species. Group A – Jellyfish, B - anchovy, sandy sprat, sardine, garfish, skipjack trevally, C – silverbelly, yellowtail scad, D - pilchard, F – blue sprat, G - sea mullet.

4.2 Beach surveys and causes of mortality

A request for community volunteers for beach surveys was sent out in a media release in December 2021. Additionally, all the businesses adjacent to the eastern margin of Cockburn Sound were contacted. Volunteers were initially recruited for all the areas accessible by community members, and BP (Kwinana Oil in Figure 3) provided staff to walk along the sections adjacent to their industrial site. Industrial sites not covered include Tianqi Lithium/ Avertas Energy, Synergy and Alcoa. Not all volunteers continued with the surveys, and a couple conducted <3 surveys. A second social media campaign was organised in January 2023. Whilst six potential volunteers contacted Dr Cannell, only three were able to assist, and one has not sent in all the data despite being contacted on several occasions.

A protocol for the surveys and a seabird guide of the seabirds that are most likely to use Cockburn Sound), was developed. All volunteers were sent a survey kit, including the seabird guide.

A total of 865 surveys were conducted from February 2022 to the end of January 2024 (Table 23). No dead penguins were found during the beach surveys in Cockburn Sound, but several other dead fauna

were observed (Appendix 8.3 Tables A4 and A5). Notably large numbers of jellyfish were found in February and March 2023, as well as several uncommonly found dead fish in August 2023. Fifteen dead silver gulls were found on a jetty in the BP survey area in April 2023. They had signs of injury, with redness and blood under their wings. There had been a recent storm with winds up to ~60 km/h. Note that only a few volunteers recorded dead fauna other than seabirds or shore birds during their surveys.

Table 23. The number of beach surveys conducted in area along the eastern margin of Cockburn Sound, WA, from 1 February 2022 to 31 January 2024.

Beach survey area	2022	2023	2024
1	44	35	5
2	44	16	
3	3	46	4
4	53	44	4
5	19	44	4
6	29	26	8
7	60	8	
8		32	2
8a		32	2
9	13		
10	50	52	5
11	12		
CSBP	4	32	2
BP1 South	25	36	1
BP2 North	39	28	1
Jetty 2-3		1	
Total	**395**	**432**	**38**

Live penguins were occasionally observed by the volunteers in the water:

- In Mangles Bay on 28/11/22 (4 individuals)
- At the Point Peron boat ramp on 29/11/22 (1 individual),
- At the Mangles Bay fishing club boat ramp on 3/12/22 (1 individual),
- In Mangles Bay on 29/12/22 and 30/12/22 (see Figure 11). Schools of little silver fish were jumping out of the water where the penguin was (observed three times)

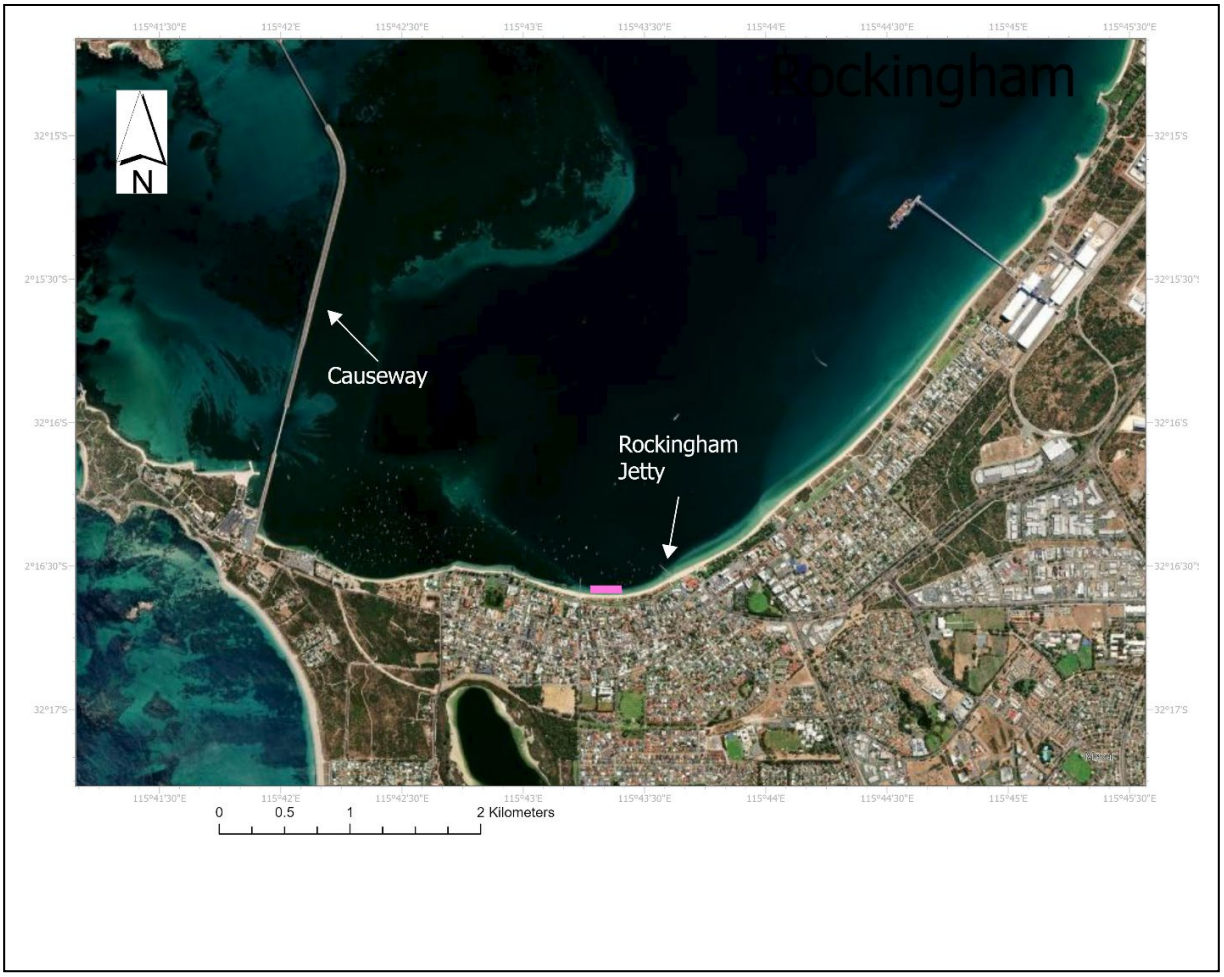

Figure 11. A little penguin was observed swimming in the pink highlighted area on 29/12/22

One penguin was found deceased on Garden Island in August 2022 (this was not part of the beach surveys). A basic necropsy was performed on it at the DDLS in October 2022 (the penguin had been kept frozen). The penguin was in a fair body condition (body condition score 2.5/5) with a fat pad present (mass = 4.9 g), indicating that it did not die from starvation. It had no external or internal injuries. PCR testing for Avian Influenza and Newcastle disease were negative. Its internal organs were not enlarged (Table 24), indicating that it did not have a protozoal parasite infection. Unfortunately, histopathology is not routinely conducted by DDLS. No definitive cause of death was identified.

Table 24. Mass (grams) of internal organs of necropsied dead penguin from Garden Island (GI), and comparison with the mean mass ± SD (number of penguins in brackets) of those penguins from the Perth region identified to have infections with *Toxoplasmosa* and/or *Haemoproteous* protozoal parasites (Cannell et al. 2013b, Campbell et al. 2022).

Organ	Mass of GI penguin 2022	Mean mass (and sample size) of penguins identified to have protozoal infections
Kidney	9.8	Not reported
Liver	23.9	66 ±7.4 (8)
Spleen	0.1	5.8 ± 2.1 (8)

An injured penguin was found on Garden Island on 23 January 2023. More than half of its left flipper had been amputated and it had a laceration to its left foot (Figures 12 - 14). These injuries were consistent with a boat strike. The penguin was euthanised.

Figure 12. Injured adult little penguin found on Garden Island 23/1/23. More than half the left flipper had been removed. It also had a foot laceration.

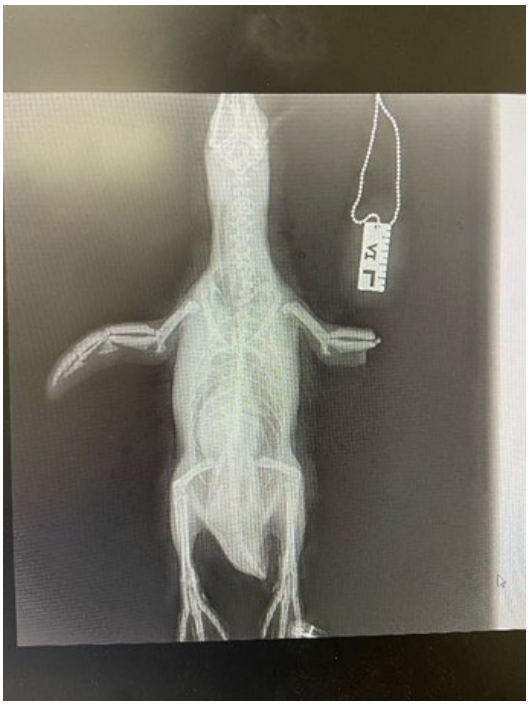

Figure 13. X-ray of injured adult little penguin found on Garden Island 23/1/23.

Figure 14. Injured adult little penguin found on Garden Island 23/1/23. Note, both the flipper and foot have been bandaged.

Many of the volunteers commented on the amount of rubbish that they found during their surveys, including plastic rope, plastic bottles, crates, face masks, toys, shoes etc. Many volunteers collected the rubbish. One volunteer logged the rubbish he collected through the AMDI app for Tangaroa Blue, and collected 20 kg of rubbish during his surveys. (See Appendix 3 for volunteer responses to the beach surveys).

4.3 Foraging habitat and home range of Garden Island little penguins

In 2022 and 2023, usable data were obtained from satellite tags deployed on eight penguins during the incubation stage of breeding. In 2022, the tags were deployed in July, August and October. Three birds conducted multiple foraging trips of varying duration, from one to nine days (Figures 15 and 16), and one bird conducted one foraging trip that was for three days (Figure 17). The SSM models did not converge for the latter, and hence core foraging habitat only was identified. In 2023, tags were deployed in May, June, and July. Each of the four penguins conducted multiple single day foraging trips (Figures 18 - 19). Regardless of how long each bird was at sea, they remained within Cockburn Sound, using the eastern margin, central basin, western margin and Kwinana Shelf (Figures 15 - 19). In 2022, the home range of two of the three penguins for which this information is available overlapped with the Stage 3 Preferred Option for the Port (Figure 15). In 2023, the home range of one of the four birds overlapped with the Stage 3 Preferred Option for the Port and one overlapped with the existing shipping channels (Figure 18). The home range of the penguins in 2022 ranged from 28 - 64 km^2, and from 20 - 107 km^2 in 2023. However, neither the analysis to predict the most probable locations of the penguins nor the kernel density areas can account for land masses. Thus, predicted points that are on land fall within Cockburn Sound, likely at a similar latitude. This would shift the home range to within Cockburn Sound and reduce the size of the ranges. Additionally, the home range of the first trip for bird 21625606 indicated that it went west of the Garden Island causeway. This is very unlikely based on previous tracking data (Cannell unpubl. data), and hence the size of the home range for this bird would also be reduced.

The core areas of the penguins incubating eggs in 2022 were located in the southern half of Cockburn Sound, including Mangles Bay, the central basin and the Kwinana Shelf (either within the footprint of the Stage 3 Preferred Option for the Port, or just adjacent to it; Figures 16 and 17). The size of the core areas ranged from 7 to 15 km^2. In 2023, the core areas were located mainly on the western margin of Cockburn Sound and Mangles Bay, although one penguin also foraged on the Kwinana Shelf (Figure 19). The size of the core areas ranged from 7 to 29 km^2.

Interestingly, neither the core foraging area, nor the size of the home range markedly differed, regardless of how long a penguin remained at sea.

GPS tags were deployed on four penguins whilst they were guarding chicks, three in 2022 and one in 2023. Each went to sea for a single day trip. The trips were typically divided into three phases, with the penguins initially leaving the colony before dawn and spending long periods of time on the surface of the water whilst heading in the direction towards their foraging grounds. The penguins then spent several hours foraging, identified by areas of high residence and sinuosity often interspersed by slower travel between areas. The third phase typically was a direct movement back to the colony but with little time on the surface. This often leads to few locations during the trip back to the colony and can be seen as a straight line on the map, though, in reality, it is unlikely to be this straight (Figure 20). The core foraging areas of the little penguins in 2022 occurred in the southern half of Cockburn Sound, including Mangles Bay and the central basin, with one foraging area approximately 600 m SW of the lower edge of the Stage 3 Preferred Option for the Port. The penguin spent approximately 10 hours in this core area. The core foraging area for the penguin in 2023 was in the NW of the Sound in Sulphur Bay (Figure 20).

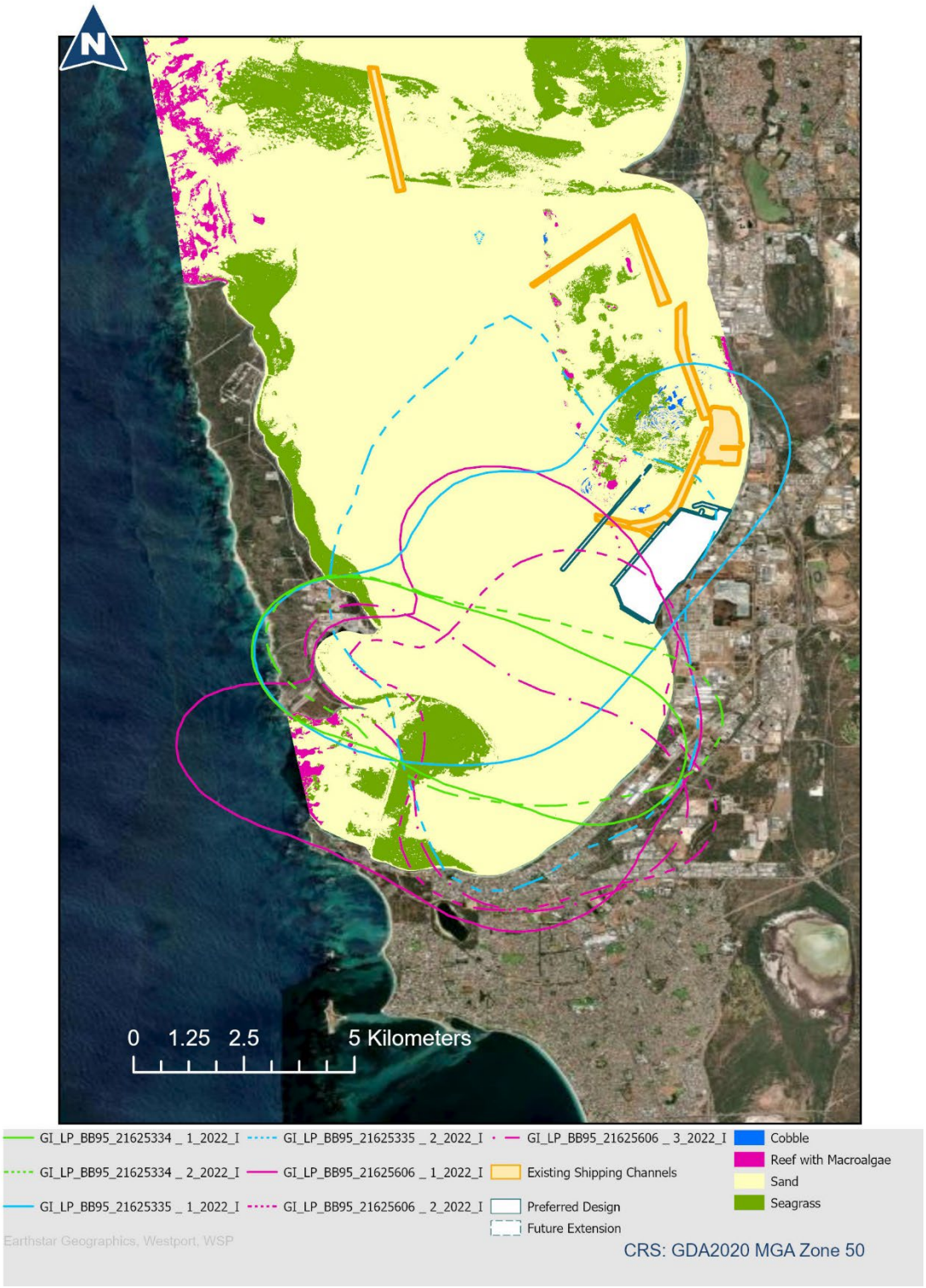

Figure 15. Home range (95% Kernel density area) of the little penguins from Garden Island, during the incubation stage of breeding in 2022. Birds are represented by distinct colours, and different trips are represented by different line patterns. Tags were deployed in July, August and October.

Figure 16. Core area (50% kernel density area) of the little penguins from Garden Island, during the incubation stage of breeding in 2022. Birds are represented by distinct colours, and different trips are represented by different line patterns. Tags were deployed in July, August and October.

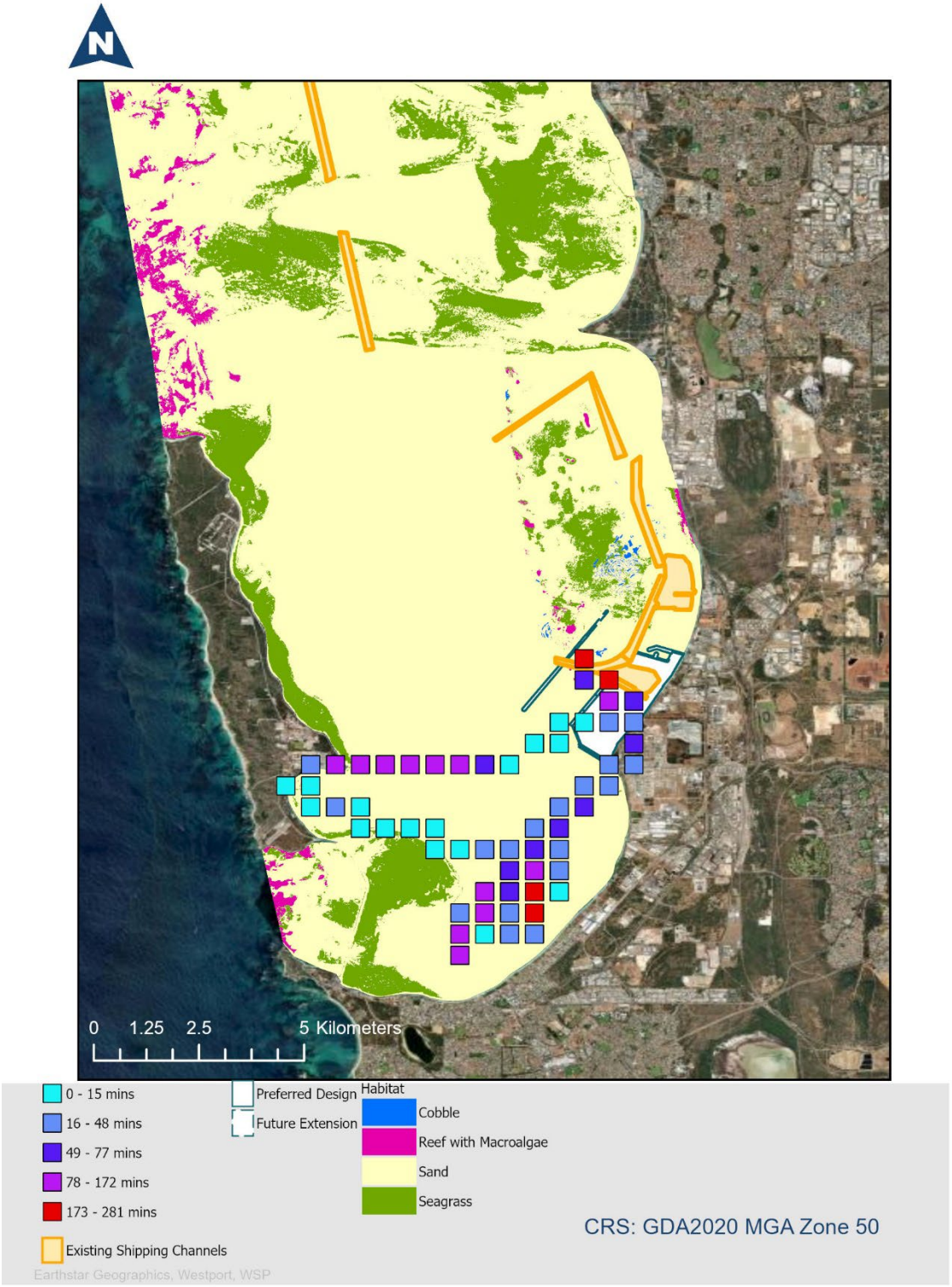

Figure 17. The total time spent in gridded cells (mins) by one little penguin from Garden Island, over a three-day foraging trip during the incubation stage of breeding in 2022. The tag was deployed in July.

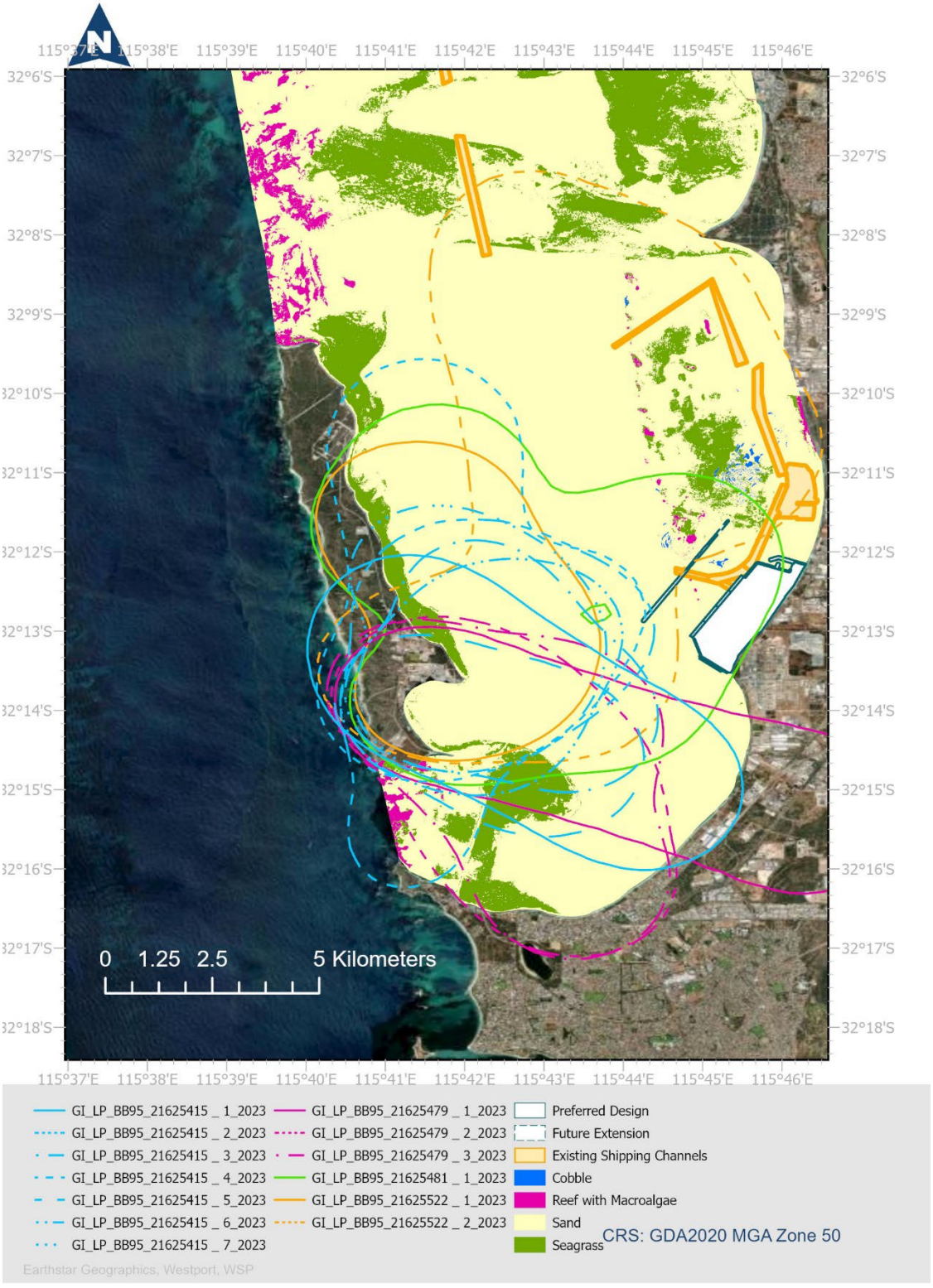

Figure 18. Home range (95% kernel density area) of the little penguins from Garden Island, during the incubation stage of breeding in 2023. Birds are represented by distinct colours, and different trips are represented by different line patterns. The tags were deployed in May, June, and July.

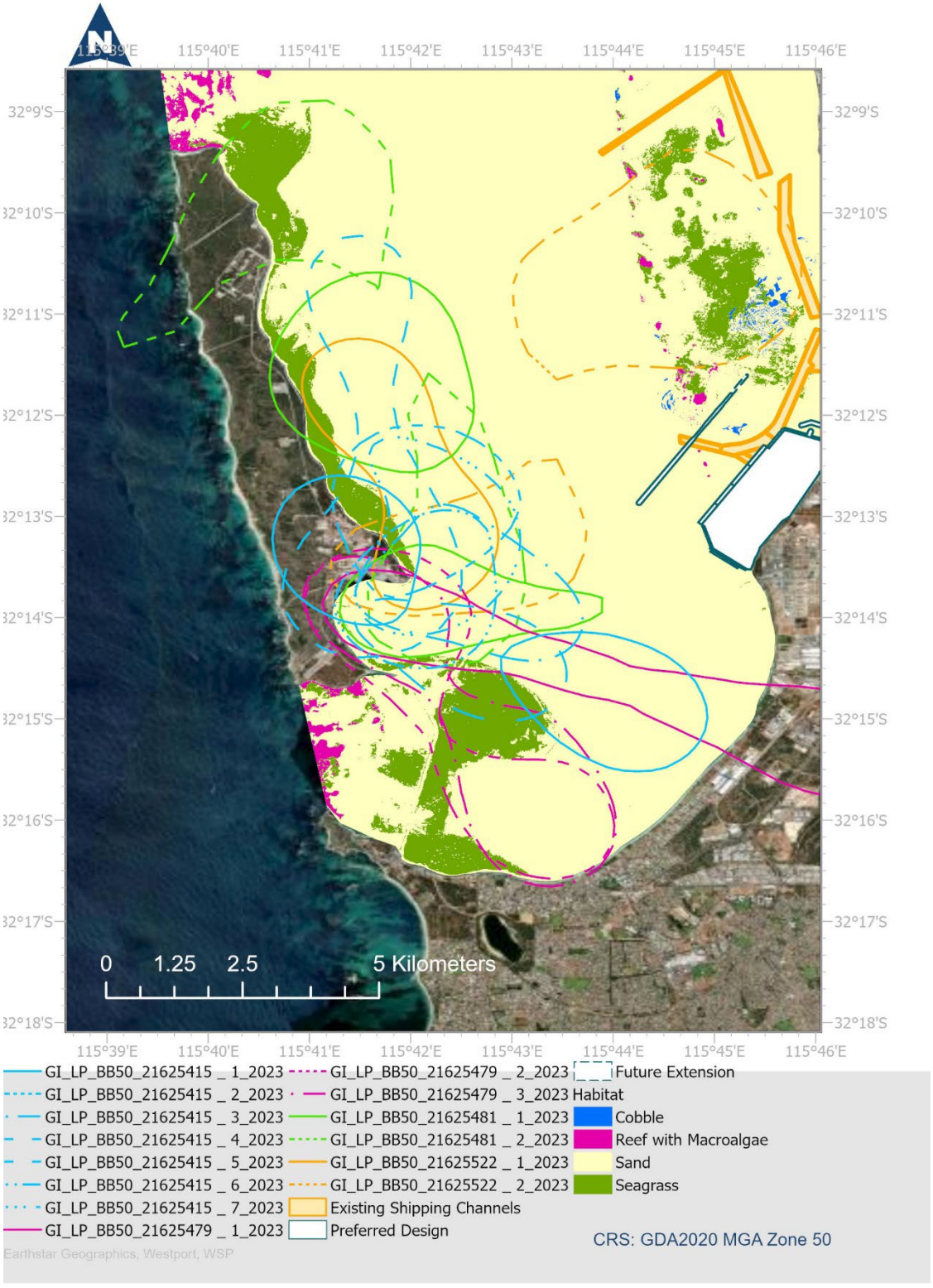

Figure 19. Core area (50% kernel density area) of the little penguins from Garden Island, during the incubation stage of breeding in 2023. Birds are represented by distinct colours, and different trips are represented by different line patterns. Tags were deployed in May, June, and July.

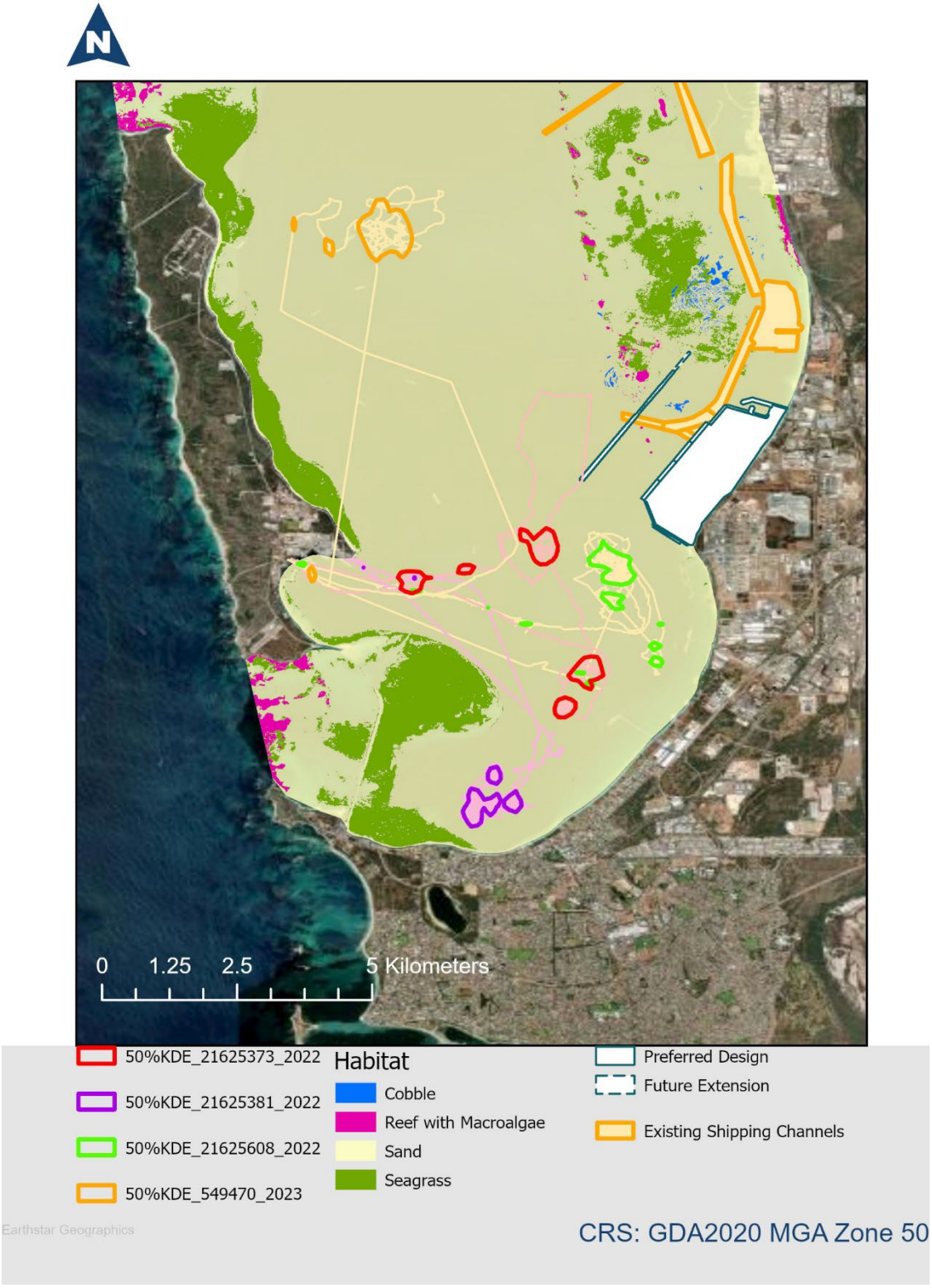

Figure 20. The GPS locations and core foraging habitat (50% kernel density area) of little penguins from Garden Island during the chick guard stage of breeding in 2022 (red, purple and green), and in 2023 (orange).

5 Discussion

5.1 Diet

5.1.1 DNA analysis of faeces

A total of 107 faecal samples from little penguins collected from Garden Island, Western Australia, between June 2020 and July 2023, were subjected to metabarcoding. All 107 samples were amplified for 16S to target the fish component of the diet of little penguins while the 18S region was also amplified from 26 samples to provide a more general assessment of the diet of little penguins. For the 16S assay, dietary data were generated for 92 or 95 samples depending on the filtering methodology used while 18S data were obtained for16 or 23 samples.

The 18S data support previous suggestions that fish are the most commonly detected components of the diet of little penguins from the Perth region, based on analysis of stomach contents, from which fish, crustaceans and cephalopods can be identified (Klomp and Wooller 1988, Wienecke 1989, Wooller et al. 1991, Connard 1995, Bradley et al. 1997). The 18S assay also detected the presence of squid (Cephalopoda) in one faecal sample and of jellyfish (Scyphozoa) in another. Small quantities of squid have also been recorded in the diet of Penguin Island penguins via an analysis of stomach contents (Klomp and Wooller 1988, Wienecke 1989, Wooller et al. 1991, Connard 1995, Bradley et al. 1997). However, jellyfish have not previously been documented as a diet item for penguins in the Perth region, likely due to the difficulty in identifying gelatinous material from stomach contents (Cavallo et al. 2018). Whilst the detection of soft-bodied prey was extremely low in our samples, Cavallo et al. (2018) found Scyphozoa in 7% of faecal samples, and salps in 25% of faecal samples, using a similar methodology to that used in this project. The 18S dataset has some limitations, including the detection of a high proportion of non-target taxa (because of the broad taxonomic range of the primers). This combined with the generally low taxonomic resolution could lead to errors. In addition, large amounts of 18S sequences from little penguins were found in many samples (i.e. the blocking primers did not work very effectively). In these samples, the amplification of little penguin DNA may have impeded the amplification of 18S sequences from prey items, i.e. increased the rate of false negatives (failure to detect prey when it is present) in our datasets. For the above reasons, after a trial run, we elected not to try for additional 18S data and hence the total sample size for this marker is relatively small.

Across the four years studied the 16S data suggested the most-commonly identified fish in the faecal samples were anchovy, pilchard and sandy sprat with one or two of these three species being the most common taxa in each year from 2020 to 2023. These findings are broadly consistent with the results of Murray et al. (2011) who identified sandy sprat, pilchard, anchovy and blue sprat as the most common prey items in little penguins from Penguin Island, based on a metabarcoding analysis of 27 faecal samples collected between October and December 2010. However, Murray et al.'s data samples were mainly from penguins that were likely to forage south of Penguin Island, and only included three samples from penguins that were likely to forage north (based on previous tracking data; Cannell 2016, Cannell 2019). Of the latter three samples, all contained pilchard, one contained anchovy, and none contained sandy sprat or blue sprat (Cannell unpubl. data). In our study, sandy sprat was most common in the diet in 2021, the year of unprecedented rainfall in July in Perth, which resulted in increased outflow from the Swan River and a surge of tannins and phytoplankton in Cockburn Sound (Pattiaratchi & Thomson 2021). There was also an increase in abundance of sandy sprat in Cockburn Sound in spring (Yeoh et al. 2025)

Whilst anchovy, pilchard and sandy sprat were the most common species overall, their frequency of occurrence in 2023 was lower than in any other year (apart from anchovy in 2021). Conversely, the highest frequency of occurrence of sardine, garfish, sea mullet and jack mackerel was in 2023, the year which had the most typical and dominant species. In fact, garfish and sea mullet were found in almost one third of samples compared to an average of 14% and 8% respectively from 2020 to 2022, and jack mackerel was found in 16% of samples, compared to an average of 8% across the other years. It was

not possible to determine the environmental variables related to the higher diversity of fish species in the diet of the penguins in 2023 due to the poor confidence from our RF models. However, an increase in penguin diet diversity has been associated with reduced availability of different prey types (Chairadia et al. 2010). Thus, it is possible that a suite of oceanographic variables in 2022 and 2023 resulted in decreased abundance of food for penguins.

We detected a total of 19 fish species in Dataset B compared to only 14 in Dataset A, which was subject to more stringent filtering. Filtering of metabarcoding data is essential to prevent false positives, however there is no consensus on how stringent that filtering should be (Drake et al. 2022). All the taxa identified here have been reported in other dietary studies of little penguins from Penguin Island (Klomp & Wooller 1988, Murray et al. 2011), thus, we are confident that the extended list of taxa in Dataset B is made up of real prey items rather than false positives. Dataset B suggests that individual penguins are typically feeding on multiple prey items at roughly the same time. Regardless of the differences, both datasets indicate that anchovy, pilchard and sandy sprat are the most common prey items in the assayed samples.

5.1.2 SI analysis

The non-breeding/pre-moult penguins foraging in 2019/20 and 2022/23 were feeding more inshore and/or on prey that have greater access to benthic carbon sources, i.e. seagrasses, comparative to 2018/19, 2020/21 and 2021/22 (higher average $\delta^{13}C$). From the trophic niche analyses, the non-breeding/pre-moult penguins in 2020/21 foraged on the smallest range of prey, whilst those in 2022/23 foraged on the widest range of prey. However, in 2022/23, they generally foraged on prey that were of a lower trophic level (lower $\delta^{15}N$) than in other years. In 2021/22, the prey were less enriched (had lower values of $\delta^{13}C$) and covered a greater range of trophic levels (i.e. greater range of $\delta^{15}N$) compared to 2019/20. Little penguins do not undergo migration post-breeding, although they visit the colony less frequently until pre-breeding, and they are most commonly found at their colony when they moult. It is not known where the little penguins from Garden Island forage in the period just prior to moult, when they must build up adequate fat reserves (often at least doubling their mass) to complete the 2 - 3 week moult. However, these data indicate that they do not always forage in the same area, or that other oceanographic variables could be driving changes in the isotopic signatures of some prey. For example, phytoplankton blooms result in temporary increases in $\delta^{13}C$ values in fish (Cobain et al. 2022).

In 2020, there was no difference in the values of $\delta^{13}C$ between the breeding and non-breeding/pre-moult penguins, but the values of both $\delta^{34}S$ and $\delta^{15}N$ were more enriched in the non-breeding/pre-moult penguins. As lower values of $\delta^{13}C$ and higher values of $\delta^{34}S$ typically indicate food sources derived from a more benthic environment or with greater freshwater input, it is not obvious why $\delta^{13}C$ did not change between the two time periods but $\delta^{34}S$ did. Despite this, it is likely that the breeding and non-breeding/pre-moult penguins were feeding on prey that originated from similar areas, but the latter were feeding on prey of higher trophic level and/or larger size. In 2021, non-breeding/pre-moult penguins had less enriched values of $\delta^{13}C$ and more enriched $\delta^{34}S$ and $\delta^{15}N$ values compared to breeding penguins in that year. This means that the penguins were feeding on prey that were more pelagic (or spawned in more offshore regions) and of higher tropic levels and/or larger size during the non-breeding/pre-moult period. In contrast, there was no difference in any of the isotopic values between non-breeding/pre-moult and breeding penguins in 2022, indicating that they were feeding in similar areas and on prey of similar trophic levels and/or size. However, the trophic niche of the non-breeding/pre-moult penguins in 2022, and indeed in the other years, was much greater than the breeding penguins in the same year, indicating that the penguins were feeding on a wider range of prey. In 2020 and 2021, there was marginal, if any, overlap in any of the isotope pair plots, indicating that the isotopic niche of the breeding penguins was completely distinct to that of the non-breeding/pre-moult penguins in these years. Thus, it is likely that non-breeding/pre-moult penguins in

2020 and 2021 were foraging further afield than the southern half of Cockburn Sound, where breeding little penguins from Garden Island predominantly foraged.

Comparing the breeding seasons of 2020, 2021, 2022 and 2023, the penguins were eating prey that were of equivalent trophic levels. However, the more enriched values of $\delta^{13}C$ in 2021 are intriguing. This would typically indicate that the penguins were foraging further inshore, but breeding penguins predominantly forage within the southern half of Cockburn Sound, regardless of year (Cannell unpubl. data). Thus, in this scenario, it is likely that the penguins were feeding on fish that spawned in the river and moved into Cockburn Sound or preyed on more benthic fish species. This is supported by the less enriched values of $\delta^{34}S$ in 2021, compared to 2022, which is due to either fish eating benthic fauna from areas of lower oxygen production, or from freshwater derived materials (Morkune et al. 2016). The above average winter rainfall in 2021 and the increased discharge in the Swan River (Appendix 1) have probably influenced the fish fauna present in the late spring and summer of 2021, and the delay of penguin breeding in that year (Cannell unpubl. data). The wider trophic niche for $\delta^{34}S$ and $\delta^{13}C$ prey in 2023 indicates that the prey came from a mixture of benthic and pelagic sources.

For the fish collected in November 2021 and May 2022, there was no significant increase in $\delta^{13}C$ or $\delta^{15}N$ following lipid extraction, and in fact there was an insignificant decrease in the values of both in most of the fish samples. In contrast, there was a significant increase in $\delta^{13}C$ and $\delta^{15}N$ following lipid extraction in many of the fish collected in November 2022 and May 2023. This contrasting result would seem to be due to the analyses of $\delta^{13}C$ and $\delta^{15}N$ with and without lipid extraction being conducted in different laboratories in November 2021 and May 2022, but in the same laboratory thereafter. Fish white muscle contains minimal lipids and, therefore, does not necessarily benefit from lipid extraction (Pinnegar and Polunin 1999, Post et al. 2007, Logan et al. 2008). Additionally, muscle C:N values < 3.4 are considered to not require lipid removal (Logan et al. 2008). However, this study has identified that there was not a consistent result for a significant increase in $\delta^{13}C$ and $\delta^{15}N$ following lipid removal between all fish species, despite the C:N values all < 3.4 (Appendix 8.1, Table A3). As lipid extraction for stable isotopes is costly and time-consuming, mathematical correction techniques for predicting values for lipid-extracted $\delta^{13}C$ and $\delta^{15}N$ have been proposed (e.g. Sweeting et al 2006). The results from this study indicate that a single mathematical correction may not be valid for all species. It was beyond the scope of this study to elucidate this any further.

The different temporal SI values within species reveals the importance of collecting prey samples for the same time period that the consumer isotopes were synthesized (Phillips et al. 2014). Furthermore, if the small sample size for each fish source is < 20 (in our case 4 - 5 fish of each species, due to cost constraints), then this can result in considerable uncertainty in the mean and variance values (Phillips et al. 2014). This is something to consider when modelling the diet composition of the penguins in different years. However, our results of the diet composition from the breeding penguins in 2023 mirrored those from the molecular analysis of the faeces, thus, we are confident in our mixing model results, despite the smaller sample sizes for each source, and using values for some fish species that were caught in a different time period to when the penguin feathers were collected.

The greatest proportion of the penguins' diet included a mix of anchovy, sandy sprat, sardine, garfish, and skipjack trevally. All these species were found in 12 - 44% of the faecal samples. Both the diet composition model and the molecular analysis of faeces found that blue sprat contributed the least to the diet. Interestingly, jellyfish contributed to approximately 25% as per the mixing models, and many jellyfish were observed during the beach surveys in 2023. Penguins elsewhere are known to feed on jellyfish (McInnes et al. 2016, Sutton et al. 2015, Cavallo et al. 2018, Petrovski 2023), despite their low nutritional value (Petrovski et al. 2023). Even though our 18S analysis did not indicate jellyfish were consumed by the penguins in 2021 or 2022, it is likely that jellyfish composed a proportion of their diet in these years. This is indicated by the biplots for 2021 and 2022, where a prey source of lower $\delta^{13}C$ and $\delta^{15}N$ values would be required for the penguin isotopic values to fall within the range of the prey sources. However, little penguins are generalist feeders, and the proportion of different prey types at any given time will be dependent on the abundance of each prey type (e.g. Bradley et al. 1997,

Chiaradia et al. 2010). Interestingly, silverbelly and yellowtail scad composed a small proportion of the diet, yet silverbelly were not identified in the penguin faecal samples in this study. From our diversity analysis, approximately four species were not accounted for in the species composition in 2023. This highlights the strength of using a combination of techniques to elucidate the diet composition of the little penguins.

5.2 Beach surveys and causes of mortality

There was a positive response to the request for community members to undertake beach surveys, and the majority of volunteers that conducted beach surveys after the initial request have continued the surveys through the more challenging winter months. One volunteer has had a hiatus in participation due to health issues but recommenced in September. All who have participated, regardless of the period, have commented that they have really enjoyed undertaking the beach surveys. BP Kwinana continued to be engaged, despite a transition in company on-site, and fewer personnel.

No dead penguins were found during the beach surveys on the foreshores of Cockburn Sound, however dead penguins have been previously found on these beaches, e.g. many dead penguins were found in 2017 and 2021. During the survey years, dead or injured penguins were found on Garden Island, with the latter being injured from a boat strike. The surveys have highlighted the issue with rubbish pollution on the beaches. This issue will be raised with local city councils, and hopefully, a regular beach cleaning program can be organised.

The mass deaths of jellyfish were reported to FishWatch, but not immediately, and, thus, it was not possible for any samples to be collected for investigation of potential causes.

5.3 Foraging habitat

The areas used by the little penguins from Garden Island that incubated eggs and raised chicks in 2022 tended to be concentrated in the southern half of Cockburn Sound, including the Kwinana Shelf and location of Stage 3 preferred option for the port. These results are consistent with historical foraging behaviour (Cannell unpubl. data). In 2023, penguins incubating eggs or raising chicks additionally used the western margin of Cockburn Sound, bounded by, and extending along the length of Garden Island. Consequently, the size range of both the core areas and home range was greater in 2023. Coinciding with this additional foraging area was the greater diversity in prey in the penguins' diet. Therefore, it is likely that a reduced abundance of different prey resulted in the extension of the penguins' home range and foraging areas.

The age class of all non-baitfish species found in the penguin samples in any of the years, such as garfish, sea mullet, tailor, leatherjackets, buffalo bream and morwongs, as well as the larger baitfish such as pilchards, anchovy and sardine, would be juveniles. This assumption is made from stomach samples which have identified otolith length to total length of fish (Klomp & Wooller 1988, Wienecke 1989, Connard 1995, Bradley et al. 1997), as well as the width of a penguin's mouth opening, which dictates the maximum ventral depth of a fish that a penguin can eat (Cannell 1994). It is well documented that seagrass beds are often associated with juveniles of many fish species, and whilst the penguins' core foraging areas were generally not directly over seagrass beds, they were often adjacent to them. This highlights the likely crucial role of seagrass to little penguins successful foraging within Cockburn Sound.

6 Conclusions

This project has highlighted the knowledge gain from using complementary techniques to elucidate the diet of the penguins across multiple years. Pilchard, anchovy sandy sprat and sardine were the most common prey across all years, but the composition of each species in the diet ranged from 7 - 70%. During the non-breeding/pre-moult period, when penguins are not central-placed foragers (i.e. not bound to regularly return to the island), their trophic niche increased. Whilst these penguins generally appeared to be feeding on prey of a higher trophic level, it is also possible that the elevated values of $\delta^{15}N$ are partially due to endogenous protein reserves during the fasting period the penguins undertake during feather synthesis. However, as we found that the isotopic niches $\delta^{15}N$ varied between years, we can assume that the penguins did indeed feed on fish of different trophic levels before their moult fast, and that these were typically higher than that of the breeding penguins.

The contribution by jellyfish to the diet of the penguins in 2023 was greater than anticipated. However, it is likely that they were also present in the diet in 2021 and 2022, given the wider trophic niches which included $\delta^{15}N$ in these years. It was unfortunate that we were not able to collect jellyfish from Cockburn Sound in those years, and, hence, obtain their SI values. If this had been achieved, it would have been possible to run the mixing models for the likely diet composition in these years. Future studies should assess the non-fish component of the diets of the penguins, including squid and jellyfish, using both SIA in feathers and analysis of 18S in faecal samples.

Whilst the RF models were unable to confidently identify the relationship between the presence/absence of prey type with environmental variables, it is important to continue the high temporal and spatial collection of water quality variables. Such data will be necessary for future modelling of penguin ecological values, including diet composition, prey abundance, and habitat use.

From a DoD funded project, the core habitat and home range of little penguins from Garden Island was determined in 2022 and 2023. There were some differences between years, but in both years, some of the penguins used the Kwinana Shelf and location of Stage 3 preferred option for the port.

It would be beneficial to continue the beach surveys, as dead penguins were found by community members on the Cockburn Sound foreshore prior to this study, and no doubt such events will happen again. Only then will it be possible to gain an understanding of the causes of mortality in this region, from necropsies on the dead penguins. Conducting the surveys will also continue to raise awareness of the penguins' utilization of Cockburn Sound, as well as the issue of plastic pollution.

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8 Appendices

8.1 Appendix 1 DNA analysis of faeces

Table A1: Total list of taxa detected using the 18S assay in all samples little penguin faecal samples analysed in this study. Count is the number of faecal samples that the species was detected in. % is the percentage of the 26 faecal samples that each species was found in.

Animalia	**%**	**Total**
Arthropoda		
Arachnida		
Sarcoptiformes	3.8	1
Trombidiformes	3.8	1
Collembola		
Poduromorpha	7.7	2
Copepoda		
Calanoida	19.2	5
Harpacticoida	11.5	3
Chordata		
Actinopteri	76.9	20
Appendicularia		
Copelata	3.8	1
Aves		
Apodiformes	96.2	25
Cnidaria		
Myxozoa		
Multivalvulida	11.5	3
Scyphozoa		
Semaeostomeae	3.8	1
Mollusca		
Cephalopoda		
Myopsida	3.8	1
Platyhelminthes		
Trematoda		
Azygiida	11.5	3
Chromista		
Apicomplexa		
Conoidasida		
Eucoccidiorida	7.7	2
Cercozoa	3.8	1
Ciliophora		
Colpodea		
Grossglockneriida		
Oligohymenophorea		
Philasterida	7.7	2
Sessilida		
Spirotrichea		
Sporadotrichida	7.7	2
Stichotrichida	3.8	1

Urostylida	3.8	1
Haptophyta		
Prymnesiophyceae		
Isochrysidales		
Fungi		
Ascomycota		
Dothideomycetes		
Dothideales	3.8	1
Microthyriales		
Mycosphaerellales		
Pleosporales	11.5	3
Eurotiomycetes		
Chaetothyriales	3.8	1
Eurotiales	3.8	1
Onygenales	3.8	1
Lecanoromycetes		
Lecanorales	3.8	1
Pezizomycetes		
Pezizales	3.8	1
Saccharomycetes		
Saccharomycetales	3.8	1
Bigyra		
Tubulinea		
Amphifilida		
Labyrinthulea		
Labyrinthulida	3.8	1
Chlorophyta		
Ulvophyceae		
Chlorocystidales	3.8	1
Streptophyta		
Magnoliopsida		
Brassicales	3.8	1
Caryophyllales	3.8	1
Malpighiales	3.8	1
Myrtales	30.8	8
Poales	3.8	1
Rosales	3.8	1
Saxifragales	3.8	1
Gyrista		
Chrysophyceae	3.8	1
Chromuliles	3.8	1
Myzozoa		
Dinophyceae		
Coccidiniales	7.7	2
Alveolata		
Dinoflagellata		
Gymnodiniales	3.8	1

Table A2. Environmental variables used in the analysis of diet composition of little penguin faecal samples collected from Garden Island, 2021 – 2023.

Variable	Description
Rainfall	Annual rainfall data (mm) (and 1-3 year lagged data) as measured at the Perth Airport. Data derived from http://www.bom.gov.au/climate/data/ There is an association between rainfall and riverine input, which can affect nutrient input in coastal marine systems (Molony et al. 2011). Additionally, rainfall had a positive influence on sandy sprat abundance in Warnbro Sound, the embayment immediately south of Penguin Island (Gaughan et al. 1996). Anecdotal evidence from fishers suggests that schools of sandy sprat move upstream into the Swan Estuary after the onset of winter rains, when freshwater outflow reaches the lower estuary (Gaughan et al. 1996). Finally, breeding participation in little penguins was associated with higher rainfall the previous year (Cannell et a. 2024ba
Stream flow from the Swan-Canning river	Monthly total levels of discharge (Megalitres) (and 1-3 monthly lags), from the Walyunga station. Derived from https://kumina.water.wa.gov.au/waterinformation/wir/reports/publish/616011/g02.htm
Fremantle Sea Level	Daily values of Fremantle (32° 3' S, 115° 44' E) sea level (FSL) were obtained from http://uhslc.soest.hawaii.edu/data/. From these data monthly means (and 1 – 3 month lagged data) were obtained.
Dissolved oxygen	Data were obtained every 15 minutes from buoys located in Cockburn Sound. From these data, monthly mean (and SD) Dissolved Oxygen (mg/L (and 1 - 3 month lags) were obtained. Data from Cockburn Sound Buoy Data, supplied by the Department of Water and Environmental Regulation, and accessed via https://uniwa.sharepoint.com/teams/EXT-WAMSIWestportReportsandData/Shared%20Documents/Forms/AllItems.aspx?id=%2Fteams%2FEXT%2DWAMSIWestportReportsandData%2FShared%20Documents%2FHistorical%5FData%5FCockburn%5FSound%2FWAMSI%5FWestport%5Fproject%5Fuse%5FONLY%2FDWER%2FCockburn%20Sound%20Mooring%20data&viewid=c4258036%2D8243%2D46aa%2Db02e%2D6bde95ff5f4d,.
Salinity	Data were obtained every 15 minutes from buoys located in Cockburn Sound. From these data, monthly mean (and SD) salinity (psu) at the bottom (and 1 - 3 month lags) were obtained. Data from Cockburn Sound Buoy Data, supplied by the Department of Water and Environmental Regulation, and accessed via https://uniwa.sharepoint.com/teams/EXT-WAMSIWestportReportsandData/Shared%20Documents/Forms/AllItems.aspx?id=%2Fteams%2FEXT%2DWAMSIWestportReportsandData%2FShared%20Documents%2FHistorical%5FData%5FCockburn%5FSound%2FWAMSI%5FWestport%5Fproject%5Fuse%5FONLY%2FDWER%2FCockburn%20Sound%20Mooring%20data&viewid=c4258036%2D8243%2D46aa

	%2Db02e%2D6bde95ff5f4d,.
Temperature	Data were obtained every 15 minutes from buoys located in Cockburn Sound. From these data, monthly mean (and SD) temperature (°C) at the bottom (and 1 - 3 month lags) were obtained. Data from Cockburn Sound Buoy Data, supplied by the Department of Water and Environmental Regulation, and accessed via https://uniwa.sharepoint.com/teams/EXT-WAMSIWestportReportsandData/Shared%20Documents/Forms/AllItems.aspx?id=%2Fteams%2FEXT%2DWAMSIWestportReportsandData%2FShared%20Documents%2FHistorical%5FData%5FCockburn%5FSound%2FWAMSI%5FWestport%5Fproject%5Fuse%5FONLY%2FDWER%2FCockburn%20Sound%20Mooring%20data&viewid=c4258036%2D8243%2D46aa%2Db02e%2D6bde95ff5f4d,.
Turbidity	Data were obtained every 15 minutes from buoys located in Cockburn Sound. From these data, monthly mean (and SD) turbidity (NTU) at the bottom (and 1 - 3 month lags) were obtained. Data from Cockburn Sound Buoy Data, supplied by the Department of Water and Environmental Regulation, and accessed via https://uniwa.sharepoint.com/teams/EXT-WAMSIWestportReportsandData/Shared%20Documents/Forms/AllItems.aspx?id=%2Fteams%2FEXT%2DWAMSIWestportReportsandData%2FShared%20Documents%2FHistorical%5FData%5FCockburn%5FSound%2FWAMSI%5FWestport%5Fproject%5Fuse%5FONLY%2FDWER%2FCockburn%20Sound%20Mooring%20data&viewid=c4258036%2D8243%2D46aa%2Db02e%2D6bde95ff5f4d,.

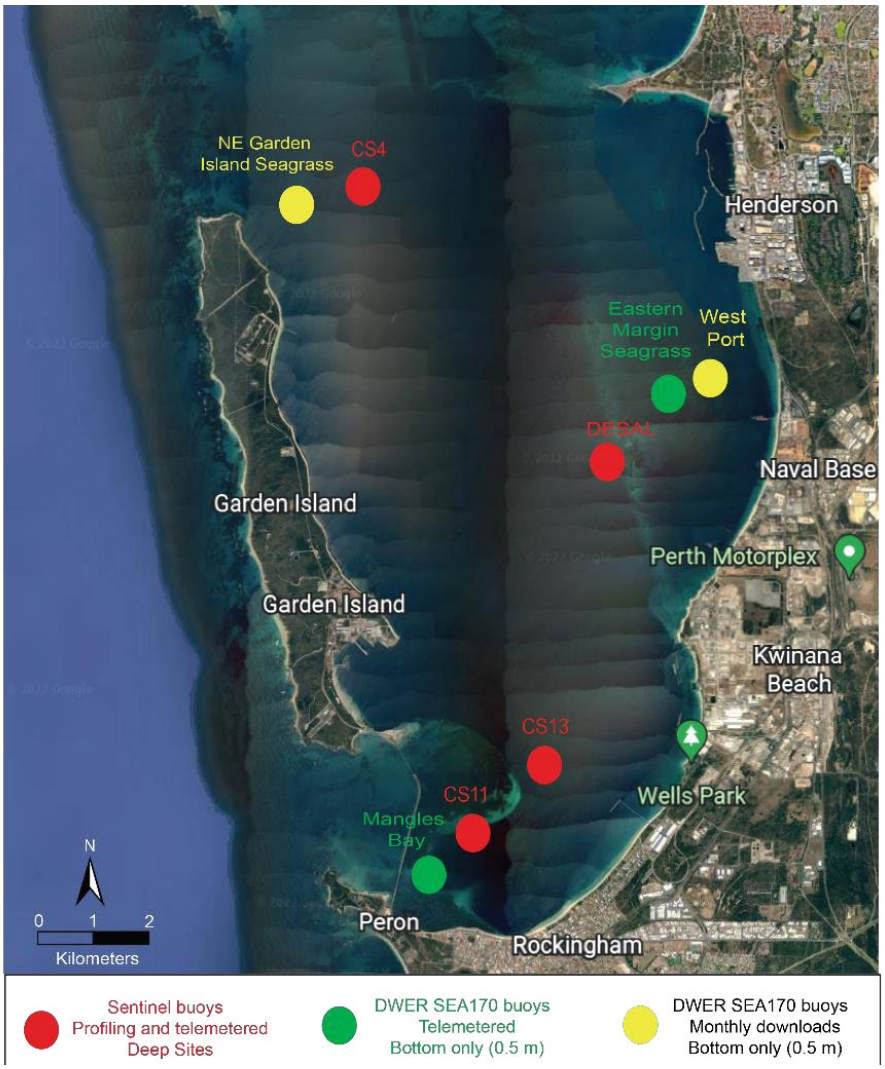

Figure A1. Location of the DWER buoys, CS11, CS13 and Mangles Bay, from which water quality variables were obtained in 2021 – 2023.

Hill numbers

Hill numbers are a family of diversity measures that have different values of q which determine the sensitivity of the diversity measure to common or rare species. All Hill numbers are expressed as the effective number of species, or fish families for our data (i.e. the number of equally abundant elements necessary to produce a given value of the diversity; Jost 2006, Jost 2007). As such, all three diversity measures are directly comparable, and as dominance within an assemblage increases, so too does the difference between these three numbers.

Diversity profiles

The slope of the profile reflects the evenness of the relative abundances of fish genera/families in the diet of each assemblage, from completely even to highly uneven (Gotelli & Chao 2013), and if the profiles do not cross, one assemblage has a more diverse prey composition than the other (Chao & Jost 2015).

Rarefaction and extrapolation curves

Both rarefaction and extrapolation standardise unequal sample sizes to an equal size based on a reference sample. Statistical comparisons of biodiversity indices can then be performed between the equal-sized samples (Gotelli & Chao 2013), and significant differences are obtained if the 95% confidence intervals do not overlap (Chao et al. 2014). Because the certainty of the extrapolation decreases if it is extended beyond double the reference sample size (Colwell et al. 2012), we did not extrapolate beyond this limit. As such, we chose the maximum abundance of all the fish genera/families across all the assemblages as a reference sample.

Table A3 The mean (±SD) value of C:N ratio before and after lipid extraction in each of the potential penguin prey species collected in November 2021, May 2022, November 2022 and May 2023 from Cockburn Sound.

Genus and species	Common Name	C:N				C:N lipid removed			
		Nov 2021	May 2022	Nov 2022	May 2023	Nov 2021	May 2022	Nov 2022	May 2023
Aldrichetta forsteri	Yelloweye mullet			3.21 (0.07)				3.34 (0.07)	
Atherinomorus vaigiensis	Common hardyhead	3.28 (0.05)	3.27 (0.03)	3.10 (0.03)		3.21 (0.01)	3.19 (0.03)	3.23 (0.04)	
Engraulis australis	Anchovy	3.34 (0.05)	3.31 (0)		3.25 (0.03)	3.34 (0.10)	3.29 (0.01)		3.29 (0.02)
Hyperlophus vittatus	Sandy sprat	3.31 (0.04)	3.25 (0.04)	3.23 (0.11)		3.36 (0.09)	3.29(0.07)	3.39 (0.16)	
Hyporhamphus melanochir	Garfish		3.23 (0.02)	3.17 (0.07)			3.26 (0.09)	3.34 (0.08)	
Leptatherina prebyteroides	Silverfish			3.09 (0.01)				3.25 (0.05)	
Mugil cephalus	Sea mullet	3.27 (0.04)		3.14 (0.02)		3.41 (0.05)		3.28 (0.06)	
Parequula melbournensis	Silverbelly	3.31 (0.02)	3.26 (0.03)	3.17 (0.04)	3.16 (0.04)	3.28 (0.05)	3.17 (0.10)	3.30 (0.10)	3.21 (0.02)
Spratelloides robustus	Blue sprat	3.36 (0.06)	3.30 (0.09)	3.27 (0.09)		3.39 (0.06)	3.45 (0.13)	3.43 (0.06)	
Pentapodus vitta	Western butterfish				3.18 (0.09)				3.24 (0.04
Pseudocarax wrighti	Skipjack trevally				3.17 (0.05)				3.36 (0.10)
Trachurus novaezelandiae	Yellowtail scad	3.28 (0.04)	3.30 (0.04)		3.16 (0.03)	3.36 (0.11)	3.29 (0.08)		3.28 (0.03)
Upeneus australiae	Australian goatfish				3.20 (0.03)				3.27 (0.04)

8.2 Appendix 2 Beach surveys

Table A4. Dead birds noted during beach surveys conducted in area along the eastern margin of Cockburn Sound, WA, from 1 February 2022- 31 January 2024.

Area	Year	Month	N	Bird type
1	2022	11	1	Rock dove
2	2022	3	1	Darter
2	2022	4	1	Silver gull
2	2022	7	1	Silver gull
2	2022	10	2	Juvenile silver gull, Unknown species
2	2022	12	1	Pied cormorant
3	2023	7	1	Rock dove
3	2023	9	3	3 x Rock doves
4	2022	3	1	Silver gull
4	2022	5	4	Rock dove
4	2022	6	16	11 x Rock dove, 3 x silver gull, 2 unknown
4	2022	8	1	Pied cormorant
4	2023	7	2	Osprey, Crested pigeon
4	2023	9	3	Silver gull, 2 x cormorants
5	2023	6	1	Rock dove
6	2023	3	1	Rock dove
6	2023	5	1	Little pied cormorant
7	2022	3	1	Pied cormorant
10	2022	2	1	Crested tern
10	2022	5	1	Tern or gull
10	2022	8	1	Little shearwater
10	2022	10	1	Rock dove
10	2023	3	1	Rock dove
10	2023	5	3	Grey teal, 2 x Rock doves
10	2023	8	1	Little pied cormorant

8a	2023	5	3	Cockatoo
BP1 south	2022	2	2	2 x Pied cormorants
BP1 south	2022	5	3	1 x Rock dove, 2 x unknown
BP1 south	2022	6	2	Little black cormorant, Silver gull
BP1 south	2022	9	1	Rock dove
BP1 south	2022	12	1	Unknown
BP1 south	2023	1	1	Unknown
BP1 south	2023	2	3	Pied cormorant, 2 x Silver gull
BP1 south	2023	3	5	Pied cormorant x 4 (2 decapitated), 2 Silver gull
BP1 south	2023	4	1	Silver gull
BP1 south	2023	5	3	Little tern, Silver gull, Unknown
BP1 south	2023	6	6	Fairy Tern x 2, Bridled tern, Silver gull x 2, Unknown
BP1 south	2023	7	1	Rock dove
BP1 south	2023	8	3	Bridled tern, 2 x unknown
BP1 south	2023	9	1	Fairy tern
BP1 south	2024	2	1	Little pied cormorant
BP2 north	2022	2	2	Pied cormorant, silver gull
BP2 north	2022	4	2	Pied cormorant, unknown
BP2 north	2022	8	1	Little shearwater
BP2 north	2022	9	1	Pied cormorant
BP2 north	2022	10	1	Little tern
BP2 north	2023	5	3	Little tern, Sooty tern, Pied cormorant
Jetty 2-3	2023	4	15	Silver gulls (signs of injury with redness and blood under the wings. Storm of up to 56km/h winds)

Table A5. Dead non-avian fauna noted during beach surveys conducted in area along the eastern margin of Cockburn Sound, WA, from 1 February 2022 - 31 January 2024.

Area	Month	Year	N	Fauna type	N	Fauna type	N	Fauna type	N	Fauna type	N	Fauna type
3	2	2023	32	jellyfish	1	sea star						
3	3	2023	28	jellyfish	1	sea star	1	blue manna crab				
3	4	2023	2	jellyfish	1	Boxfish	1	blowfish				
3	5	2023	3	jellyfish								
3	6	2023	1	jellyfish	3	cowfish						
3	7	2023	2	pilchards or mullets	1	cowfish	1	blowfish				
3	8	2023	10	jellyfish	5	blowfish	1	boxfish	2	rats	1	cowfish
3	8 (cont'd)	2023	1	triggerfish	1	Globe fish (juvenile)	1	damselfish				
3	10	2023	1	jellyfish								
3	12	2023	1	jellyfish								
3	1	2024	1	sea star								
4	12	2022	6	blue manna crab								
4	1	2023	11	clear jellyfish	1	jellyfish	1	sea hare	2	blowfish	2	6-arm sea stars
4	2	2023	>1000[a]	jellyfish								
4	3	2023	127[b]	jellyfish								
4	4	2023	1	blowfish	2	boxfish						
4	5	2023	1	blue manna crab								
4	6	2023	5	jellyfish	4	blowfish						
4	7	2023	2	cowfish	1	blowfish	9	blue bottles				
4	8	2023	1	jellyfish	1	cowfish						
4	9	2023	1	blowfish								
4	10	2023	6	blue manna crabs								
4	12	2023	8	blowfish								

4	1	2024	30	jellyfish							
5	1	2023	3	jellyfish	1	sea star	1	blue manna crab			
5	2	2023	103	jellyfish	1	blowfish	1	blue manna crab			
5	3	2023	>55	jellyfish	1	blue manna crab					
5	4	2023	1	rat							
5	5	2023	1	blowfish							
5	6	2023	4	cowfish	1	blowfish					
5	7	2023	3	cowfish	1	blowfish	1	sea hare	1	triggerfish	1	bluebottle
5	8	2023	1	eel	1	elongated fish, possibly Largehead Hairtail/ribbonfish					
5	10	2023	1	blue manna crab							
5	12	2023	3	jellyfish	2	blowfish	1	blue manna crab			
5	1	2024	51	jellyfish	1	blue manna crab	1	crab, unknown species			
6	3	2023	13	blowfish	1	rat					
6	4	2023	2	blowfish							
6	5	2023	2	blowfish	1	rat					
8	6	2023	1	rat							
9	3	2022	1	Blue-tongue lizard							

[a]Counted on 2 separate occasions, 350+ counted on one day, and at least 700 counted 6 days later

[b]120 counted on one day, and 2 and 5 jellyfish counted on 2 subsequent days

8.3 Appendix 3 Volunteers response to surveys

"Thank you for the opportunity to be part of the penguin walk team!

Overall, the walks were a great excuse to go for a walk along the beach with no other objective than to see what was on the beach.

Days where the water was crystal clear was the high light for me. I can look at that all day!

There was a large amount of rubbish washed up on the beach, which is sad, but I am not sure how to address this. Seems to be a systemic thing, the amount of rubbish entering our waterways." *Andrew*

"My time assisting with Dr. Belinda Cannell's research on little penguins was a unique and memorable experience. The walks were an enjoyable way to learn new survey skills while also a great opportunity to get out into nature and contribute to meaningful research.

It was also very eye-opening during the year to witness first-hand the number of marine debris (such as bait bags, rope, crates) and plastics (water bottles, caps, hard plastics) being deposited on our beach's. In one week, I removed a total of 20kg from the three beaches in my survey area. I was also able to witness first-hand the sheer amount of coastal erosion present at Well's Park, with the beach profile changing drastically across months and especially so after stormy swells.

I greatly enjoyed assisted Dr Belinda Cannell with this research survey and would be happy to assist her again in the future." *Cody*

"I enjoyed the beach surveys as any time spent on the beach is enjoyable, especially at Point Peron where there is always something interesting to watch, birds, fish, dolphins etc.

What is really sad is the amount of rubbish that I have been collecting. I have been doing this for over 20 years, since my daughter and I found a Pied cormorant on the Rockingham foreshore wrapped in fishing line and with multiple fishing hooks embedded in its body, sadly it didn't survive despite the vet's best efforts.

The worst of it continues to be fishing paraphernalia as it is the most harmful to wildlife. School holidays increase my workload considerably with clothes, toys and food and drink containers left behind. In winter, it is ropes and broken bits of cray pots. I have also noted an increase in single use drink containers since a caravan selling coffee and cup cakes has set up business at Point Peron. I know that I am not the only person cleaning up the beaches in this area which is encouraging." *Dominique*

"I enjoy beach walking which is why l volunteered though l wasn't familiar with the area l chose. The beach is mostly flat, rarely any weed and no rocks for things to snag on. l walked along the shoreline and returned on the sand closer to the low dune. The last northern 200 mts allowed dogs and was the most popular area as it backed on to a large picnic area.

Rubbish was the worst thing, mostly small pieces of plastic, children's beach toys, drink bottles and possibly abandoned doggie bags. A day after Jan 26th the shire uses a tractor to remove any accumulated litter from that event.

If the wind was NE there was often an extensive film of dust on the water from the grain loading facility.

In August '22 a large cabin cruiser broke its mooring ending up on the beach. It hadn't seen a hard stand in a long while, the variety of marine growth was very interesting, lots of mussels (photo attached). It was removed a few days later.

In December '23 a Sperm Whale beached itself on a sandbar about 60 mts offshore from the survey beach. It had been seen further north in previous days and there had been speculation that it wasn't well. It was a very sad sight to see it early the next morning after it had been euthanased. I didn't take photos, although its huge shape was obvious there was little above the water. It was towed to Henderson then to be removed, buried for 5 years before the WA Museum receives the skeleton.

Apart from stingrays patrolling the shallows l often saw birds foraging close in or on the shore. Here is my list:

Pied Oystercatcher
Silver Gull
Crested Tern
Caspian Tern
Fairy Tern
Roseate Tern Mar-June
Pied Cormorant
Little Pied Cormorant
Darter
Australian Gannet
Welcome Swallow
Osprey
Nankeen Kestrel over dune
Black-shouldered Kite over dune" *Pauline*

One volunteer recycled the rope they collected into hanging planter holders, whilst another volunteer collected rubbish, and gave any containers for change collected to various charities.

"Unfortunately, one of the main things I noticed during the surveys was the amount of rubbish similar to what has been previously reported washed up on the beach." *Simon*

One volunteer, a high school student, conducted the surveys for a school requirement to participate in a community project:

"For my year nine rite journey we needed to participate in a community project, in which I was given the opportunity to participate in Dr Belinda Cannell's Beached Penguin Survey in Cockburn Sound. During the months June, July and August I would walk along the beach one day week for approximately 30min to one hour looking for dead penguins, other injured or dead seabirds and any environmental changes, along with recording weather conditions.

While doing the beached penguin survey, I decided I would also include doing a beach cleanup, picking up all the rubbish that I saw. I chose to do the rubbish cleanup to help prevent injury or death to marine life. The first time I cleaned up the beach I collected two full garbage bags of rubbish and by the end of August I had collected a total of 18kg of Rubbish. This included rope, hard and soft plastics of all sorts, fishing line, face masks and large washed-up items (not included in the 18kg) of broken cray pots and wood (Figure A2).

Overall, I am very happy with how my project went and that I got the opportunity to take part in the survey to help collect data on little penguins in Cockburn Sound.

By Isla, Year 9 South Coast Baptist College 2023”

Figure A2. Photos from beach surveys conducted and rubbish collected by Isla

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