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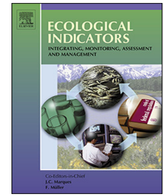

Original Articles

Minimising the limitations of using dietary analysis to assess foodweb changes by combining multiple techniques

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ABSTRACT

Dietary studies of marine predators offer an immediate signal of foodweb changes occurring at lower trophic levels, and therefore are often used to assess the ecosystem status of marine systems. Conventionally, these studies are based on morphological analysis of prey remains in stomach contents, involving invasive and destructive techniques to collect samples. More recently, the number of dietary studies based on less invasive biochemical and molecular approaches has dramatically increased. However, all three methods, morphological, biochemical and molecular, have well-documented limitations for resolving taxonomy, temporal variation or biomass composition. In this study, we minimise these limitations by considering multiple techniques in combination. As a case study, we report the target prey species and diet composition of a marine predator that has been used to assess annual change in managed fishing areas for several decades, the macaroni penguin *Eudyptes chrysolophus*. We use biochemical (stable isotope) and molecular (DNA) analysis of faecal samples collected across the different phases of a single breeding season, and compare the resolved diet to a 26-year dataset of stomach contents collected from a closely located colony (0.25 km apart) that exploits identical foraging grounds. Molecular analysis increased the known target prey species for this highly monitored population by 31%, including a fish species of commercial importance. Biochemical analysis detected subtle changes in the proportion of fish and krill in the diet, demonstrating promising opportunities for using a combined molecular and biochemical method to assess inter-annual foodweb changes at lower trophic levels. The combined approach offers a less invasive sampling methodology, compared to morphological analysis, and provides more information regarding prey species diversity and the overall trophic signature of the diet. Further studies are required to examine the feasibility of using this approach for long-term dietary studies of different marine predator species.

1. Introduction

Dietary monitoring techniques have been used extensively to examine the predator-prey interactions of species that have an otherwise unobservable lifestyle. For species that exist at the top of relatively short food chains, diet is also often used as a proxy for ecosystem status and the availability of target prey species. Such studies are typically based on morphological identification of prey remains in scats or stomach contents (Tollit and Thompson, 1996; Waluda et al., 2012). However, the number of studies based on molecular techniques, such as DNA sequencing (Jarman et al., 2004; Deagle et al., 2005; Jarman et al., 2013; McInnes et al., 2016), or biochemical markers, such as stable isotope analysis (SIA) and fatty acid signature analysis (Hobson et al., 1997; Iverson et al., 2004), has also increased over the past decade. Unfortunately, there are several well-documented limitations associated with using each of these three techniques to assess diet.

Prey species identified by morphological analysis are likely to be biased towards those with hard parts that survive digestion (Gales, 1988). Furthermore, studies with repeat sampling are needed to examine temporal variation with this technique. The sampling schedule of morphological studies based on stomach contents is often restricted in order to minimise any direct effects of invasive sample collection, and indirect effects to offspring that rely on regular provisioning. In contrast, molecular DNA sequencing of faeces provides a high resolution

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Table 1
The breeding cycle of macaroni penguins at Bird Island, South Georgia, with maximum foraging distance from the colony (mean ± SD) for each phase (Horswill et al., 2016a) and the number of stomach and faecal samples collected during the 2011/12 breeding season. Faecal samples processed by stable isotope analysis (SIA) and DNA analysis with bony fish and krill primers; parentheses indicate number of samples that amplified. Asterisk denotes samples that were removed from the analysis because DNA was largely absent indicating that SIA may not reflect the dietary signature.

Breeding phase	Month	Max. foraging distance (km)	Stomach contents samples	No. faecal samples		
				SIA	DNA fish	DNA krill
Start of breeding season						
Incubation	November-December	411 ± 87		8	9 (3)	9 (2)
Brood	December-January	23 ± 19		9	12 (9)	12 (7)
Crèche	January-February	22 ± 35	40	12	11 (1)	11 (1)
Pre-moult trip	February	358 ± 40		*11**	11 (1)	11 (1)
Moult	March					
End of breeding season						

snap shot of prey species diversity (Deagle et al., 2007; Jarman et al., 2013), based on samples that are considerably less invasive to collect. Therefore, this approach can be implemented at shorter sampling intervals. The disadvantage of this approach is that diet composition cannot be reliably resolved compared to morphological analysis (Deagle et al., 2010), and the ability to identify sequenced prey is dependent on the availability of reference sequences in public repositories, such as Genbank. Finally, biochemical markers in animal tissues, such as stable isotope ratios and fatty acid signatures, do not provide taxonomic data but can be used to resolve a diet's trophic signature through time. Temporal changes can be examined within a single sampling occasion by collecting tissues that possess different turnover rates, or alternatively, by conducting longitudinal studies of the same tissue or sample type. However, studies that compare different tissues or sample types need to consider diet-tissue fractionation factors, and also establish isotopic inventories of prey in the study area (Hobson et al., 1994).

Central place foragers need to integrate foodweb changes over restricted foraging ranges. This constraint in further intensified for species, such as seabirds and seals, that exist within relatively short food chains. Consequently, the diets of seabirds offer an immediate signal of foodweb changes occurring at lower trophic levels, and are often used to assess the ecosystem status of marine systems (Parsons et al., 2008). For example, the population of macaroni penguins *Eudyptes chrysolophus* on Bird Island, South Georgia, has been used to assess annual change in managed fishing areas for several decades (CCAMLR, 2004). Macaroni penguins are one of the most important avian marine consumers in the sub-Antarctic region, reported to consume more prey than any other seabird species (Brooke, 2004). They are entirely pelagic during the winter months, foraging away from the continental shelf zone of South Georgia (Ratcliffe et al., 2014), however they operate under tight central-place constraints during the breeding season in order to provision the chick at the colony on a daily basis. Antarctic krill *Euphausia superba* are a key food resource during this time, such that fluctuations in krill density can substantially impact the diet, activity budgets, and levels of breeding investment employed by this species (Croxall et al., 1999; Horswill et al., 2017; Waluda et al., 2012).

One of the metrics used to assess changes in the South Georgia management subarea of the Southern Ocean is the diet of macaroni penguin chicks. This metric is based on samples of stomach contents collected annually from breeding adults during late chick-rearing (CCAMLR, 2004; Wilson, 1984). However, the population of macaroni penguins at Bird Island is known to forage in different areas during each phase of the breeding season (Horswill et al., 2016a), and dietary studies of macaroni penguins breeding in the Indian Ocean based on morphological and DNA analysis, report that target prey species can vary across the chick-rearing phases (brood-guard and crèche; Deagle et al., 2007). Consequently, the samples of stomach contents used to assess local foodweb dynamics at Bird Island may under-represent seasonal changes that occur prior to sampling, as well as different species targeted across the wider breeding season.

The limitations associated with using morphological, molecular or biochemical techniques to assess diet are considerable when taken in isolation. However, by combining results from molecular and biochemical methods it may be possible to minimise the drawbacks associated with morphological studies, i.e. low resolution of soft-bodied prey and restricted sampling intervals. The different sets of expertise required to collect, process and analyse these data means that studies combining molecular and biochemical methods are lacking. Typically, studies combine morphological identification with one of the other techniques, and use one approach to ground-truth (Alonso et al., 2014; Connan et al., 2017) or complement the other (Bearhop et al., 2001; Jeanniard-du-Dot et al 2017). In this study, we compare the population-level diet resolved from the long-term dataset of stomach contents, with that described by combining DNA and stable isotope analysis of faecal samples collected across the different phases of a single breeding season. The two sample sets were collected from colonies situated 0.25 km apart that share identical foraging grounds during the breeding season (Trathan et al., 2006). We discuss the feasibility of using the combined approach for assessing the status of marine ecosystems.

2. Methods

2.1. Study site and sample collection

Samples were collected from macaroni penguins at the Fairy Point and Goldcrest Point colonies on Bird Island, South Georgia. The two study colonies are located 0.25 km apart and share a coastal inlet for access to the sea. Individuals from these colonies exploit the same foraging habitats during the breeding season (Trathan et al., 2006), and have a synchronous breeding schedule, lasting from courting in October to chick-fledging and adult moult in March/April (Table 1). Samples of stomach contents were collected at the Goldcrest Point colony as part of a long-term monitoring program (Waluda et al., 2012), whilst faecal samples were collected from individuals at the Fairy Point colony as part of a tracking study (Horswill et al., 2016a).

Fresh faecal samples were collected from the Fairy Point colony during each phase of the 2011/2012 breeding season (see Table 1 for sample sizes). Sampled birds all carried unique PIT-tag identifiers, thus precluding repeat sampling of the same individual within a phase. Samples were collected from birds that were monitored as part of an independent study, and therefore sampling followed the activity schedule of those birds. Sampling during the chick-rearing phases (brood-guard and crèche) was conducted over several days in the middle of each phase (Table 1). Macaroni penguins typically follow a diurnal activity pattern during brood-guard and crèche, leaving the colony at dawn and returning before dusk (Williams, 1995). Therefore samples collected during these phases were assumed to be collected within 1 day

of an individual returning to the colony, and to be representative of foraging trips conducted during that phase. In contrast, birds spend several weeks in the colony following the extended (~2 week) incubation and pre-moult foraging trips, and do not return to sea during this time. Fresh samples collected during these phases were from birds that had spent an unknown period of time in the colony. Faecal samples were sealed immediately in a 1.5 ml microcentrifuge tube (Eppendorf, Hambery) to minimise nitrogen volatilization (Jianjun et al., 2009). All samples were frozen and stored at −20 °C until prepared for analysis. Faeces were taken to represent diet within *c.* 3 days of collection (Bird et al., 2008).

To provide a direct comparison between techniques, we include stomach contents samples collected from the Goldcrest Point Colony during the 2011/2012 breeding season. The prey species identified in stomach samples collected annually between 1989 and 2010 (Waluda et al., 2012) are also included to provide a wider taxonomic comparison of the resolved diet. Stomach lavage methods were employed in agreement with Wilson (1984), regulated by the Cambridge University/British Antarctic Survey Animal Ethics Committee and the UK Home Office. Sampling was conducted during crèche in order to minimise any impact to the provisioning schedule of the chicks. Crèche occurs during late chick rearing when both parent birds conduct near diurnal foraging trips and the chicks of the colony "crèche" together during the day (Williams, 1995). Birds were sampled over a four week period (in 2011/2012: 28th January to 20th February) with ten adult birds sampled on one day each week as they return to the colony (Table 1). Repeat sampling is unlikely due to the size of the Gold-crest point Colony (~40 K individuals in 2011/2012).

2.2. Sample preparation

Each stomach contents sample was drained through 2 sieves (3.35 and 250 μm). Crustaceans were identified to species where possible, and otoliths were used to identify fish species (Reid and Arnould, 1996). All years of stomach contents data were pooled and taken to represent prey captured during crèche. Likewise, faecal samples collected during the 2011/2012 breeding season were pooled by breeding phase, and taken to represent the prey captured during that respective phase. The stable isotope values of faeces samples collected during each breeding phase were compared in order to infer dietary changes over the breeding season without a requirement to collect prey samples.

All individual penguin faecal samples were less than 1.5 ml. Samples were halved to allow DNA and stable isotope analysis of the same sample. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), following the manufacturer's standard protocol. DNA extracts were stored at −20 °C for downstream analysis. PCR amplifications were conducted using a 16S primer that was specific to krill (Euphausiidae) to amplify ~169 bp DNA fragments (Deagle et al., 2007). A 12S primer that was specific to bony fish (Osteichthyes) was also used to amplify ~300 bp DNA fragments (supp. info. T1; Jarman unpublished in Medeiros Mirra, 2010). Amplifications were performed separately for each gene fragment and faecal sample using the Multiplex PCR Kit (Qiagen). Each 20 μl reaction contained 1× Multiplex PCR Master Mix, 0.2 μM of each primer, 0.1 mg/ml of BSA (New England Biolabs) and 2 μl of the DNA template. Identifiers of ten bp (MID codes) were attached to the ends of the primers during the PCR in order to provide each breeding phase with a unique tag. Thermal cycling conditions were as follows: 95 °C for 15 min, 35 cycles (94 °C for 30 s followed by the primer specific annealing temperature for 90 s followed by 72 °C for 90 s), concluding with 72 °C for 10 min. A minimum of three negative controls (the extraction control, plus at least two distilled water blanks) were included in each set of PCR amplifications. PCR products were separated by electrophoresis in 1.5% agarose gels and visualised by staining with ethidium bromide. PCR products were purified using AMPure® XP Beads. To prepare the resulting amplifications for pyrosequencing, the DNA concentration of the individual PCR products was measured using Qubit and the samples from each breeding phase and prey type were pooled according to their concentration, so that each breeding phase pool contained an equal contribution from the individual birds. Samples were further pooled for pyrosequencing a krill library and a fish library, and quality control was performed using the Agilent Bioanalyzer 2100. The multiplexed pools (10 μl at 50 ng/μl) were then sent to Eurofins MWG Operon for amplicon sequencing with Roche GS-FLX Titanium series chemistry (454).

The resulting sequences were de-multiplexed and low quality sequences were filtered using the Geneious platform (www.geneious.com, Kearse et al., 2012). Sequences were separated by MID codes, and then primers and MID codes were removed. Sequences were then clustered into groups of nearly identical sequences using CD-HIT-EST (Huang et al., 2010), setting the clustering threshold to 99.5% for the ~300 bp fish fragment and 99.0% for the ~169 bp krill fragment (i.e., allowing for ~2 nucleotide differences among members of a cluster). The longest sequence from each cluster was selected and compared to the NCBI non-redundant nucleotide database "nt" (downloaded November 2017) using BLASTn "megablast" (*E*-value $< 1e-25$) (Altschul et al., 1997). For each query sequence, the top hit was retained and alignment statistics were recorded to assess the quality of each hit. Species level classifications were made when reference sequences achieved greater than 99% identity to the query, and local congeneric species were present in the database. Sequences that did not meet this criteria were classified to the genus level (Deagle et al., 2010).

For stable isotope analysis, faecal samples were freeze dried for 12–24 h and homogenised using a ball mill (TissueLyser II, Qiagen, Manchester, UK). Isotope values of carbon ($\delta^{13}C$) were used to detect shifts in foraging habitat (Cherel and Hobson, 2007; France, 1995), and isotope values of nitrogen ($\delta^{15}N$) were used to assess the relative trophic level of the target prey (Schoeninger and DeNiro, 1984; Vanderklift and Ponsard, 2003). Isotopic measurements were determined by continuous-flow isotope ratio mass spectrometry, undertaken at the Godwin Laboratory, University of Cambridge (UK). All analyses were performed using an automated elemental analyzer (Costech ECS 4010, Milan) coupled in continuous-flow mode to an isotope-ratio-monitoring mass-spectrometer (Thermo Fisher Scientific Delta V mass spectrometer, Bremen). Single subsamples of 0.7 mg aliquots per faecal sample were analysed in tin capsules. Isotopic results are reported as δ values ($\delta^{13}C$ and $\delta^{15}N$) on the VPDB scale for carbon and the AIR scale for nitrogen, using the calculation:

$$\delta X = [(R_{sample}/R_{standard})]$$

where X is ^{15}N or ^{13}C, and R is the corresponding ratio $^{15}N/^{14}N$ or $^{13}C/^{12}C$. Repeated measurements on international and laboratory standards (Caffeine [IAEA, Austria]; in-house standards of Alanine, Nylon and bovine liver) showed that measurement precision of both $\delta^{13}C$ and $\delta^{15}N$ was estimated to be ≤0.2‰. All values presented are means ± 1 SD.

2.3. Statistical analysis

Clustering within the stable isotope data that could not be attributed to the available covariate information (i.e. sex and breeding phase), prevented the use of linear modelling approaches for analysing this dataset. Following Horswill et al. (2016a), finite Gaussian (FG) mixture models were used to objectively assign individual samples to groups based on the combined $\delta^{13}C$ and $\delta^{15}N$ data. These models were fitted in program R using the statistical package *mclust* (Fraley et al., 2014). The number of groups within each tissue type was determined using the Bayesian Information Criterion (BIC). The groups, hereon referred to as classes, were described as Gaussian kernels, each with its own variance-covariance structure.

Table 2
Taxonomic assignment of 12S fish and 16S krill sequences amplified from macaroni penguin faecal samples with % identity to the BLAST query. Fish (number of otoliths identified) and krill species (Y = present) identified from stomach contents samples collected during crèche are also shown. All samples were collected during the 2011/12 breeding season from Bird Island, South Georgia.

	Stomach	Proportion of total sequences by identified species or genera				
Organism	Crèche	Inc.	Brood	Crèche	Pre-moult	% identity of match
Fish (Osteichthyes)						
Brauer's lanternfish (*Gymnoscopelus braueri*)	1					
Painted notie (*Lepidonotothen larseni*)[a]	1					
Mackerel icefish (*Champsocephalus gunnari*)[a]	1	0.004	0.004	0.012	0.075	> 99
Nichol's lanternfish (*Gymnoscopelus nicholsi*)		0.003				> 99
Blackfin icefish (*Chaenocephalus aceratus*)				0.032		> 99
Blackfin icefish[b]		0.006	0.003	0.124		≤99
Lanternfish (*Krefftichthys* sp.)[a]	14	0.019	1×10^{-4}	0.003		≤99
Cod icefish (*Dissostichus* sp.)		0.731	0.269	0.416		≤99
Cod icefish (*Notothenia* sp.)		0.008	1×10^{-4}			≤99
Antarctic dragonfish (*Parachaenichthys* sp.)				0.036		≤99
Krill (Euphausiidae)						
Antarctic krill (*Euphausia superba*)[a]	Y	0.010	0.043	0.030	0.459	> 99
Thysanoessa sp.[a]	Y	0.004	0.012	0.010	0.066	> 99
T. macrura[a]	Y		0.004	0.096		> 99
E. valentini					0.001	100
Pygmy krill (*E. frigida*)[a]	Y		0.002			> 99
Thysanoessa sp.[a]	Y	0.209	0.608	0.133	0.007	≤99
Euphausia sp.	Y	0.006	0.054	0.019	0.393	≤99
Total number of sequences		789	7570	1143	1665	

[a] Species identified in the diet of macaroni penguins based on stomach samples collected during crèche annually from 1989 to 2010 (Waluda et al., 2012).
[b] Species identified with < 99% identity but to a genus that has a single species.

2.4. Comparison of techniques

The list of target prey species identified in the samples of stomach contents was qualitatively compared with the list of species returned from the DNA analysis. This comparison was undertaken using the stomach contents samples collected in the same year as the faecal samples (2011/2012), as well as the list of species identified through long-term stomach contents analysis published by Waluda et al. (2012). To examine temporal changes in diet composition, the list of species returned from the DNA analysis of faecal samples collected in each breeding phase were compared to the isotopic values returned from these samples.

3. Results

The final data set for the 2011/2012 breeding season comprised 40 samples of stomach contents, 29 isotopic analyses of faecal samples, 14 faecal samples analysed by pyrosequencing DNA with fish primers (33% of samples successfully amplified) and 11 faecal samples from pyrosequencing DNA with krill primers (26% of samples successfully amplified) (Table 1). BLAST searches of the fish and krill DNA datasets revealed that 15% of the filtered reads achieved greater than 99% similarity to the query (Table 2). Two fish reads (from the incubation phase), and one krill read (from the brood-guard phase) were unassigned. Successful amplifications of DNA were limited for samples representing crèche and pre-moult foraging trips (Table 1). Low DNA recovery from the pre-moult samples may indicate that collection was primarily from birds that had been in the colony for more than 3 days, and already excreted the majority of prey items. Stable isotope analysis of these faecal samples is likely to generate anomalous nitrogen isotopic values due to high concentrations of ammonia and low content of prey samples, such that they are not comparable with other faecal analyses. Therefore, these samples were removed from further analysis. Unsuccessful amplification of DNA from faecal samples collected during crèche is unlikely to be due to prolonged time in the colony because individuals follow a near diurnal foraging strategy (Williams, 1995), therefore the stable isotope samples for this phase were retained (Table 2).

3.1. Diversity in prey species recovered by genetic analysis in relation to breeding phase

In total, seven distinct genera of fish were recovered from the DNA analysis of faecal samples. This included three species achieving high identity scores. The number of fish genera identified was six for incubation, and five for brood-guard and crèche (Table 2). Samples from incubation and crèche returned one unique fish species each; Nichol's lanternfish *Gymnoscopelus nicholsi* and Antarctic dragonfish *Parachaenichthys* sp., respectively. Blackfin icefish *Chaenocephalus aceratus* was only identified with high accuracy during crèche, however sequences achieving a lower match were also recovered from the incubation and brood-guard samples. Because blackfin icefish belongs to a single species genera, we conclude that this species was also present in the diet of macaroni penguins during these other phases (Table 2).

The number of fish genera identified during incubation (n = 6) was not replicated in the single sample that amplified from the pre-moult foraging trip (n = 1), when birds forage at a similar distance from the colony (Table 1, Horswill et al., 2016). In contrast, the number of krill sequences recovered from the single pre-moult trip (n = 1540) was much higher than the total number recovered from the three incubation trips that returned DNA (n = 181). The number of krill species sequenced was similar for all phases; with the exception of Pygmy krill *Euphausia frigida*, which was only present in brood-guard samples, and *Euphausia valentini*, which was represented by a single sequence in the pre-moult samples. *Euphausia valentini* was absent from the wider 26-year dataset of stomach contents collected during crèche (Waluda et al., 2012). Whilst it is common practice to discount species identified from a single sequence, *Euphausia valentini* occurs within the foraging range of the incubation foraging trips (Ward et al., 1990), and was therefore retained in the study.

3.2. Diversity in prey species during the crèche phase recovered by stomach sampling and DNA analysis

The stomach contents samples collected during the 2011/2012 crèche phase returned four species of krill. These species largely matched the results of the DNA analysis, although in the DNA samples, pygmy krill *Euphausia frigida* appeared in brood-guard, but not in crèche phase. Four species of fish were also recovered from the 2011/2012 samples of stomach contents, albeit with single otoliths for 3 of these. One of these species, Brauer's lanternfish *Gymnoscopelus braueri*, was not identified in the long-term study of stomach contents (Waluda et al., 2012), increasing the total number of fish species identified in this morphological dataset to twelve. Two species identified through morphological analysis in 2011/2012 were not identified in the DNA samples collected from the same crèche phase: Brauer's lanternfish and painted notie *Lepidonotothen larseni* (Table 2).

DNA analysis of faecal samples collected during the crèche phase of the breeding season returned three genera of fish that were absent from the 2011/2012 stomach contents samples. These genera were also absent from the wider 26-year dataset of stomach contents, including: two species of cod icefish *Dissostichus* sp. and *Notothenia* sp.; and blackfin icefish *Champsocephalus aceratus* (Table 2). The BLASTn results for *Dissostichus* sp. did not achieve 99% identity to the query, however all sequences were most closely matched with Patagonian toothfish *Dissostichus eleginoides*. The dataset of stomach contents collected between 1989 and 2010 included an additional eight species of fish that were not identified in the 2011/2012 samples by either DNA sequencing or morphological analysis. These were: Antarctica lanternfish *Electrona Antarctica*; electron Subantarctic lanternfish *Electrona carlsbergi;* gaptooth lanternfish *Protomyctophum choriodon*; South Georgia icefish *Pseudochaenichthys georgianus*; slender escolar *Paradiplospinus gracilis*; smalleye moray cod *Muraenolepis microps*; and Bolin's lanternfish *Protomyctophum bolini*. Antarctic dragonfish *Parachaenichthys georgianus* was not identified in the DNA analysis but sequences of *Parachaenichthys* sp. were identified with a lower identity match in samples collected during crèche. These sequences were consistently matched to *Parachaenichthys charcoti*. At the time of publication, reference sequences of 12S for many of these additional species were missing from Genbank. Antarctica lanternfish, electron Subantarctic lanternfish and Bolin's lanternfish are present as partial sequences (< 360 bp). Smalleye moray cod was the only species with a full sequence available.

3.3. Isotopic values in relation to breeding phase

The finite Gaussian mixture models identified two isotopic classes in the penguin faeces (Fig. 1A-C). Class 1 was characterised by consistently lower δ^{13}C values (*c.* −26.3‰ to −24.2‰) than class 2 (*c.* −24.4‰ to −20.8‰). The difference between the mean δ^{13}C values of the two classes was ~3‰. The majority of individuals appeared within class 1 during the incubation foraging trip, and all individuals were in class 2 during brood-guard and crèche. The observed variation in δ^{13}C values between breeding phases with long and short foraging trips is likely to reflect change in habitat use, with greater ^{13}C enrichment from in-shore food-webs compared with those offshore, a characteristic of marine food-webs in general (France, 1995; Hobson et al., 1994). Both classes had similar maximum δ^{15}N values over the different breeding phases (*c.* 8.4‰), however minimum δ^{15}N values decreased by approximately 1‰ (Fig. 1). Following the incubation and brood-guard foraging trips the minimum δ^{15}N values were 4.2‰ and 4.7‰, respectively, dropping to 3.2‰ during crèche.

4. Discussion

Previous studies on penguins report an increase in the resolution of prey diversity by DNA sequencing faecal samples, compared to morphological identification of hard part remains (Deagle et al., 2007; Jarman et al., 2013). In this study, DNA analysis returned new prey species targeted by macaroni penguins, and a greater number of species compared to the results gained from stomach contents samples collected during same breeding season. However, the number of species [identified] was less than 50% of that present in the longer 26-year study of stomach contents. Some species identified in the long-term study were only found in one or two years, and therefore may be less prevalent in the diet of macaroni penguins more generally. Another explanation is that prey densities fluctuate between years and certain species were in low abundance during the year that DNA samples were collected. Finally, the availability of 12S reference sequences for the target species from Genbank may also contribute towards the diversity of species identified, such that alternative genetic markers (see Alonso et al., 2012; Deagle et al., 2007; Hebert et al., 2003) might provide a higher coverage of sequences.

Stable isotope analysis indicates that the composition of krill and fish in the diet of macaroni penguins remains largely unchanged within

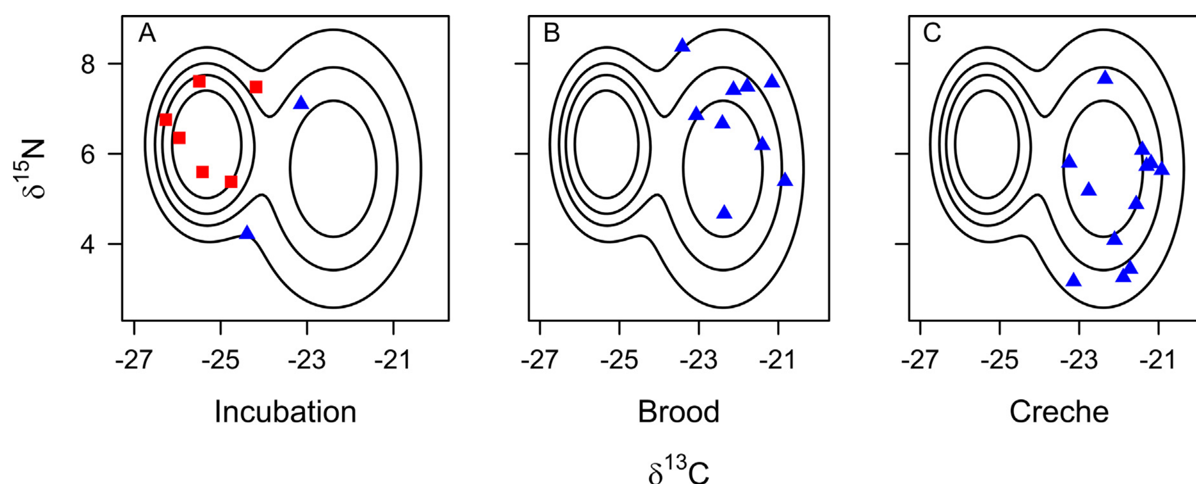

Fig. 1. Stable isotope values of faecal samples collected from macaroni penguins during different phases of the breeding season. Two distinct dietary classes: class 1 (red squares) and class 2 (blue triangles). Data points shown as de-standardised. Density contours shown for total data set at 0.25, 0.5, 0.75 and 0.95. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the breeding season despite birds foraging in different areas and under varying central-place constraints. The consistent δ^{15}N signal across the incubation and brood guard foraging trips suggests that the average tropic level, i.e. the dietary ratio of krill to fish, is consistent between these phases. The minimum observed δ^{15}N values decreased from brood guard to crèche, suggesting a more varied diet within the population during this phase with some individuals potentially increasing their krill consumption. A subtle change in the lower limit of the δ^{15}N values during crèche was also found using tissues with longer turn-over periods (i.e. blood plasma) that are more representative of the average dietary signature for the phase (Horswill et al., 2016a). Furthermore, this phase of the breeding season aligns with the seasonal influx of Antarctic krill into the region (Murphy et al., 2007). The proportion of krill sequences returned from the DNA analysis was higher during incubation and brood-guard, compared to crèche. This may reflect the limited number of successfully amplified samples for crèche, compared to incubation and brood guard, but also highlights the limitations of using DNA sequence prevalence to assess diet composition.

The local biomass of Antarctic krill at South Georgia can fluctuate several-fold between years (Fielding et al., 2014), generating changes in the foraging behaviour (Horswill et al., 2017; Trathan et al., 2006) and demographic rates of macaroni penguins (Horswill et al., 2016b, Horswill et al., 2014). During years with adverse prey conditions, the study population is known to increase the proportion of fish in their diet, as well as amphipods, *Themisto gaudichaudii* (Waluda et al., 2012). In this study, we demonstrate that subtle changes in diet composition can be detected within a breeding season, i.e. between brood-guard and crèche, however pilot studies comparing morphological and biochemical results are needed to ascertain how the isotopic signature responds to foodweb changes between years. The δ^{13}C values are likely to indicate a change in foraging distribution, but distinguishing between a diet dominated by different lower trophic-level species, e.g. krill and amphipods, from the δ^{15}N patterning may be more complicated. Some prey samples will also be needed to quantify any annual shift in the environmental baseline signature. The number of studies that use DNA sequencing of faeces to examine the target prey of marine species is considerably lower than the number that employ stable isotope analysis. This method is most limited if the target DNA is present in low amounts and the quality of the samples is poor (Taberlet et al., 1999). Furthermore, the proportion of faecal samples that successfully amplified fish and krill DNA was 33% and 26%, respectively. This is slightly lower than other DNA-based analysis of macaroni penguins diet (44%, Deagle et al., 2007). The achieved levels of successful amplification should be considered when designing field protocols and setting sample sizes. Studies designed to evaluate data collection methods are needed; i.e. to compare DNA sequences recovered from samples of different ages and with varying collection and storage methods. In addition, an extensive reference library of prey species relating to the selected genetic markers is necessary to minimise the number of unassigned and poorly matched sequences.

The krill species recovered from faecal DNA collected during the chick-rearing phases are all found within the inshore foraging range of macaroni penguins during the breeding season (Ward et al., 1990). *E. valentini* was a new prey species identified by the DNA analysis. It was recovered from the DNA sample collected after the pre-moult foraging trip when macaroni penguins travel north-east from the colony to the Antarctic Polar Front (Horswill et al., 2016a). South Georgia is at the southern edge of the *E. valentini* distribution (Ward et al., 1990), which may explain the absence of this species from phases with shorter foraging ranges, as well as the long-term dataset of stomach contents collected during crèche. This is also the first study to report blackfin icefish, Nichol's lanternfish and species of cod icefish in the diet of macaroni penguins. These species were predominantly recovered from faecal DNA collected following the longer incubation foraging trips, however sequences of cod icefish (*Dissostichus* sp.) and blackfin icefish were also abundant in samples representing the brood-guard and crèche foraging trips. The genus *Dissostichus* includes two species, Patagonian toothfish and Antarctic toothfish *Dissostichus mawoni*, however the distribution of Antarctic toothfish does not extend to the continental shelf of South Georgia (Dewitt et al., 1990). Furthermore, all of the sequences were most closely matched with Patagonian toothfish. This species can have adult body sizes greater than 60 cm. Therefore, their presence in the diet of macaroni penguins is likely to reflect penguins targeting larval and early-stage juveniles on the continental shelf. The cohorts of targeted fish may explain why otoliths for these species are absent from the long-term dataset of stomach contents.

Three of the species identified as target prey species of macaroni penguins during the breeding season are currently exploited by managed fisheries at South Georgia. Antarctic krill and mackerel icefish *Champsocephalus gunnari* were previously known, while Patagonian toothfish was not previously considered a target species for penguins (CCAMLR, 2013). Lantern fish, or myctophids, were also targeted as part of an unrestricted fishery that peaked in the late 1980s and early 1990s (Agnew, 2004), but this fishery closed in 2003 (CCAMLR, 2013). The long-line fishery for Patagonian toothfish at South Georgia targets adult fish and operates at minimum fishing depths of 700 m in order to protect juvenile fish (GSGSSI, 2016). Similarly, the mid-water trawl fishery for mackerel icefish limits fishing efforts in areas where hauls contain more than 100 kg, or 10% by number of juvenile fish smaller than 240 mm (CCAMLR, 2015), and the South Georgia krill fishery operates only during winter, moving south with the retreating ice during the summer. Consequently, it seems highly unlikely that these three fisheries will be in direct competition with the penguin populations at South Georgia. However, changes to the spawning stock that in turn influence the density of juvenile fish in the system could generate changes in penguin diet, and there is also the potential for carry-over effects, in which fishing for krill during winter depletes the standing stock available to penguins during the following breeding season (Crossin et al., 2010a,b; Ratcliffe et al., 2015).

Dietary studies based on biochemical and molecular techniques require a wider set of analytical skills, compared to morphological analysis. However, their application is less invasive, both directly to the individual animal and indirectly to the offspring that may experience a reduced rate of provisioning. Using biochemical and molecular methods in combination also allows for shorter re-sampling intervals, thus longitudinal studies within breeding seasons can be conducted, increasing both the taxonomic and temporal resolution of diet composition. Being able to understand within season variability in diet is central to separating whether observed changes in diet reflect a change in target prey availability, or a change in the phenology of the system. Further studies are required to fine-tune the suitability of this approach for assessing inter-annual foodweb changes in the marine system, as well as its application to different species of marine predator.

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Data Accessibility

All data are publicly available from the British Antarctic Survey Polar Data Centre (polardatacentre@bas.ac.uk).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ecolind.2018.06.035.

References

Agnew, D.J., 2004. Fishing South: The History and Management of South Georgia Fisheries. Government of South Georgia and The Penna Press, St Albans.

Alonso, H., Granadeiro, J.P., Paiva, V.H., Dias, A.S., Ramos, J.a., Catry, P., 2012. Parent-offspring dietary segregation of Cory's shearwaters breeding in contrasting environments. Mar. Biol. 159, 1197–1207 10.1007/s00227-012-1900-2.

Alonso, H., Granadeiro, J.P., Waap, S., Xavier, J., Symondson, W.O.C., Ramos, J.A., Catry, P., 2014. An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding. Mol. Ecol. 23, 3719–3733. http://dx.doi.org/10.1111/mec.12785.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25, 3389–3402.

Bearhop, S., Thompson, D.R., Phillips, R.a., Waldron, S., Hamer, K.C., Gray, C.M., Votier, S.C., Ross, B.P., Furness, R.W., 2001. Annual variation in great skua diets: the importance of commercial fisheries and predation on seabirds revealed by combining dietary analyses. Condor 103, 802–809 10.1650/0010-5422(2001) 103[0802:avigsd]2.0.co;2.

Bird, M.I., Tait, E., Wurster, C.M., Furness, R.W., 2008. Stable carbon and nitrogen isotope analysis of avian uric acid. Rapid Commun. Mass Spectrom. 22, 3393–3400.

de Brooke, M., L, 2004. The food consumption of the world's seabirds. Proc. R. Soc. B Suppl. 271, S246–S248. https://doi.org/http://dx.doi.org/10.1098/rsbl.2003.0153.

CCAMLR, 2015. Conservation measure 42-01. Limits on the fishery for Champsocephalus gunnari in Statistical Subarea 48.3 in the 2015/16 and 2016/17 seasons. Commission for the Conservation of Antarctic Marine Living Resources, Hobart.

CCAMLR, 2013. CCAMLR Statistical Bulletin, vol. 25 CCAMLR, Hobart.

CCAMLR, 2004. CCAMLR Ecosystem monitoring program; Standard Methods. CCAMLR, Hobart.

Cherel, Y., Hobson, K.A., 2007. Geographical variation in carbon stable isotope signatures of marine predators: a tool to investigate their foraging areas in the Southern Ocean. Mar. Ecol. Prog. Ser. 329, 281–287. http://dx.doi.org/10.3354/meps329281.

Connan, M., Bonnevie, B.T., Hagen, C., van der Lingen, C.D., McQuaid, C., 2017. Diet specialization in a colonial seabird studied using three complementary dietary techniques: effects of intrinsic and extrinsic factors. Mar. Biol. 164, 171. http://dx.doi.org/10.1007/s00227-017-3201-2.

Crossin, G., Trathan, P.N., Phillips, R.A., Dawson, A., Le Bouard, F., Williams, T.D., 2010a. A carryover effect of migration underlies individual variation in reproductive readiness and extreme egg size dimorphism in macaroni penguins. Am. Nat. 176, 357–366.

Crossin, G., Trathan, P.N., Phillips, R.A., Williams, T., 2010b. Trade-off between migration and reproduction and the physiological basis of egg size dimorphism in macaroni penguins. Integr. Comp. Biol. 50, E36.

Croxall, J.P., Reid, K., Prince, P.A., 1999. Diet, provisioning and productivity responses of marine predators to differences in availability of Antarctic krill. Mar. Ecol. Prog. Ser. 177, 115–131.

Deagle, B.E., Chiaradia, A., McInnes, J.C., Jarman, S.N., 2010. Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? Conserv. Genet. 11, 2039–2048. http://dx.doi.org/10.1007/s10592-010-0096-6.

Deagle, B.E., Gales, N.J., Evans, K., Jarman, S.N., Robinson, S., Trebilco, R., Hindell, M.A., 2007. Studying seabird diet through genetic analysis of faeces: A case study on macaroni penguins (Eudyptes chrysolophus). PLoS One 2. http://dx.doi.org/10.1371/journal.pone.0000831.

Deagle, B.E., Tollit, D.J., Jarman, S.N., Hindell, M.A., Trites, A.W., Gales, N.J., 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Mol. Ecol. 14, 1831–1842. http://dx.doi.org/10.1111/j.1365-294X.2005.02531.x.

Dewitt, H.H., Heemstra, P.C., Gon, O., 1990. Nototheniidae. In: Gon, O., Heemstra, P.C. (Eds.), Fishes of the Southern Ocean. JLB Smith Institute of Ichthyology, Grahamstown, pp. 279–331.

Fielding, S., Watkins, J.L., Trathan, P.N., Enderlein, P., Waluda, C.M., Stowasser, G., Tarling, G.A., Murphy, E.J., 2014. Interannual variability in Antarctic krill (Euphausia superba) density at South Georgia, Southern Ocean: 1997–2013. ICES J. Mar. Sci. 71, 2578–2588. http://dx.doi.org/10.1093/icesjms/fst048.

Fraley, C., Raftery, A.E., Scrucca, L., 2014. Package "mclust" v4.3. CRAN.

France, R.L., 1995. Carbon-13 enrichment in the benthic compared to planktonic algae: food web implications. Mar. Ecol. Prog. Ser. 124, 307–312. http://dx.doi.org/10.3354/meps124307.

Gales, R.P., 1988. The use of otoliths as indicators of little penguin Eudyptula minor diet. Ibis (Lond. 1859) 130, 418–426. http://dx.doi.org/10.1111/j.1474-919X.1988.tb08816.x.

GSGSSI, 2016. Toothfish licensing for the 2016 and 2017 seasons. Information for Applicants. Government of South Georgia & the South Sandwich Islands Office, Stanley.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. R. Soc. B Biol. Sci. 270, 313–321. http://dx.doi.org/10.1098/rspb.2002.2218.

Hobson, K.A., Piatt, J.F.J., Pitocchelli, J., 1994. Using stable isotopes to determine seabird trophic relationships. J. Anim. Ecol. 63, 786–798. http://dx.doi.org/10.2307/5256.

Hobson, K.A., Sease, J.L., Merrick, R.L., Piatt, J.F., 1997. Investigating trophic relationships of pinnipeds in Alaska and Washington using stable isotope ratios of nitrogen and carbon. Mar. Mammal Sci. 13, 114–132.

Horswill, C., Matthiopoulos, J., Green, J.A., Meredith, M.P., Forcada, J., Peat, H.J., Preston, M., Trathan, P.N., Ratcliffe, N., 2014. Survival in macaroni penguins and the relative importance of different drivers: individual traits, predation pressure and environmental variability. J. Anim. Ecol. 83, 1057–1067. http://dx.doi.org/10.1111/1365-2656.12229.

Horswill, C., Matthiopoulos, J., Ratcliffe, N., Green, J.A., Trathan, P.N., McGill, R.A.R., Phillips, R.A., O'Connell, T.C., 2016a. Drivers of intrapopulation variation in resource use in a generalist predator, the macaroni penguin. Mar. Ecol. Prog. Ser. 548, 223–247. http://dx.doi.org/10.3354/meps11626.

Horswill, C., Ratcliffe, N., Green, J.A., Phillips, R.A., Trathan, P.N., Matthiopoulos, J., 2016b. Unravelling the relative roles of top-down and bottom-up forces driving population change in an oceanic predator. Ecology 97, 1919–1928. http://dx.doi.org/10.1002/ecy.1452.

Horswill, C., Trathan, P.N., Ratcliffe, N., 2017. Linking extreme interannual changes in prey availability to foraging behaviour and breeding investment in a marine predator, the macaroni penguin. PLoS One 12, 1–12. http://dx.doi.org/10.1371/journal.pone.0184114.

Huang, Y., Niu, B., Gao, Y., Fu, L., Li, W., 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics 26, 680–682.

Iverson, S.J., Field, C., Don Bowen, W., Blanchard, W., 2004. Quantitative fatty acid signature analysis: a new method of estimating predator diets. Ecol. Monogr. 74, 211–235.

Jarman, S.N., Deagle, B.E., Gales, N.J., 2004. Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. Mol. Ecol. 13 (5), 1313–1322. http://dx.doi.org/10.1111/j.1365-294X.2004.02109.x.

Jarman, S.N., McInnes, J.C., Faux, C., Polanowski, A.M., Marthick, J., Deagle, B.E., Southwell, C., Emmerson, L.M., 2013. Adelie penguin population diet monitoring by analysis of food DNA in scats. PLoS One 8. http://dx.doi.org/10.1371/journal.pone.0082227.

Jeanniard-du-Dot, T., Thomas, A.C., Cherel, Y., Trites, A.W., Guinet, C., 2017. Combining hard-part and DNA analyses of scats with biologging and stable isotopes can reveal different diet compositions and feeding strategies within a fur seal population. Mar. Ecol. Prog. Ser. 584, 1–16.

Jianjun, S., Renbin, Z., Yashu, L., Zhijun, G., Liguang, S., 2009. Simulation of ammonia emissions from Antarctic typical sea animal excreta. Chin. J. Polar Res. 21, 167–176.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649.

McInnes, J.C., Emmerson, L.M., Southwell, C., Faux, C., Jarman, S.N., 2016. Simultaneous DNA-based diet analysis of breeding, non-breeding and chick Adélie penguins. R. Soc. Open Sci. 3, 150443. http://dx.doi.org/10.1098/rsos.150443.

Medeiros Mirra, R.J., 2010. The Migration Strategy, Diet and Foraging Ecology of a Small Seabird in a Changing Environment. University of Cardiff.

Murphy, E.J., Watkins, J.L., Trathan, P.N., Reid, K., Meredith, M.P., Thorpe, S.E., Johnston, N.M., Clarke, A., Tarling, G.A., Collins, M.A., Forcada, J., Shreeve, R.S., Atkinson, A., Korb, R., Whitehouse, M.J., Ward, P., Rodhouse, P.G., Enderlein, P., Hirst, A.G., Martin, A., Hill, S.L., Staniland, I.J., Pond, D.W., Briggs, D.R., Cunningham, N.J., Fleming, A.H., 2007. Spatial and temporal operation of the Scotia Sea ecosystem: a review of large-scale links in a krill centred food web. Philos. Trans. R. Soc. B Biol. Sci. 362, 113–148. http://dx.doi.org/10.1098/rstb.2006.1957.

Parsons, M., Mitchell, I., Butler, A., Ratcliffe, N., Frederiksen, M., Foster, S., Reid, J.B., 2008. Seabirds as indicators of the marine environment. ICES. J. Mar. Sci. J. du Cons. 65, 1520–1526. http://dx.doi.org/10.1093/icesjms/fsn155.

Ratcliffe, N., Crofts, S., Brown, R.M., Baylis, A.M.M., Adlard, S., Horswill, C., Venables, H., Taylor, P., Trathan, P.N., Staniland, I.J., 2014. Love thy neighbour or opposites attract? Patterns of spatial segregation and association among crested penguin populations during winter. J. Biogeogr. 41, 1183–1192. http://dx.doi.org/10.1111/jbi.12279.

Ratcliffe, N., Hill, S.L., Staniland, I.J., Brown, R.M., Adlard, S., Horswill, C., Trathan, P.N., 2015. Do krill fisheries compete with macaroni penguins? Spatial overlap in prey consumption and catches during winter. Divers. Distrib. 21, 1339–1348. http://dx.doi.org/10.1111/ddi.12366.

Reid, K., Arnould, J.P.Y., 1996. The diet of Antarctic fur seals Arctocephalus gazella during the breeding season at South Georgia. Polar Biol. 16, 105–114.

Schoeninger, M.J., DeNiro, M.J., 1984. Nitrogen and carbon isotopic composition of bone collagen from marine and terrestrial animals. Geochim. Cosmochim. Acta 48,

625–639.
Taberlet, P., Waits, L.P., Luikart, G., 1999. Noninvasive genetic sampling: look before you leap. Trends Ecol. Evol. 14, 323–327.
Tollit, D.J., Thompson, P.M., 1996. Seasonal and between-year variations in the diet of harbour seals in the Moray Firth. Scotland. Can. J. Zool. 74, 1110–1121.
Trathan, P.N., Green, C.J., Tanton, J., Peat, H.J., Poncet, J., Morton, A., 2006. Foraging dynamics of macaroni penguins Eudyptes chrysolophus at South Georgia during brood-guard. Mar. Ecol. Prog. Ser. 323, 239–251. http://dx.doi.org/10.3354/meps323239.
Vanderklift, M.A., Ponsard, S., 2003. Sources of variation in consumer-diet δ15N enrichment: a meta-analysis. Oecologia 136, 169–182. http://dx.doi.org/10.1007/s00442-003-1270-z.
Waluda, C.M., Hill, S.L., Peat, H.J., Trathan, P.N., 2012. Diet variability and reproductive performance of macaroni penguins Eudyptes chrysolophus at Bird Island, South Georgia. Mar. Ecol. Prog. Ser. 466, 261–274. http://dx.doi.org/10.3354/meps09930.
Ward, P., Atkinson, A., Peck, J.M., Wood, A.G., 1990. Euphausiid life cycles and distribution around South Georgia. Antart Sci. 2, 43–52.
Williams, T.D., 1995. The penguins; Scheniscidae. Oxford University Press, Oxford.
Wilson, R.P., 1984. An improved stomach pump for penquins and other seabird. J. F. Ornithol. 55, 109–112.