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DNA-based diet analysis of mesopelagic fish from the southern Kerguelen Axis

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ABSTRACT

Mesopelagic fish form an important link between zooplankton and higher trophic levels in Southern Ocean food webs, however their diets are poorly known. Most of the dietary information available comes from morphological analysis of stomach contents and to a lesser extent fatty acid and stable isotopes. DNA sequencing could substantially improve our knowledge of mesopelagic fish diets, but has not previously been applied. We used high-throughput DNA sequencing (HTS) of the 18S ribosomal DNA and mitochondrial cytochrome oxidase I (COI) to characterise stomach contents of four myctophid and one bathylagid species collected at the southern extension of the Kerguelen Plateau (southern Kerguelen Axis), one of the most productive regions in the Indian sector of the Southern Ocean. Diets of the four myctophid species were dominated by amphipods, euphausiids and copepods, whereas radiolarians and siphonophores contributed a much greater proportion of HTS reads for *Bathylagus* sp. Analysis of mitochondrial COI showed that all species preyed on *Thysanoessa macrura*, but *Euphausia superba* was only detected in the stomach contents of myctophids. Size-based shifts in diet were apparent, with larger individuals of both bathylagid and myctophid species more likely to consume euphausiids, but we found little evidence for regional differences in diet composition for each species over the survey area. The presence of DNA from coelenterates and other gelatinous prey in the stomach contents of all five species suggests the importance of these taxa in the diet of Southern Ocean mesopelagics has been underestimated to date. Our study demonstrates the use of DNA-based diet assessment to determine the role of mesopelagic fish and their trophic position in the Southern Ocean and inform the development of ecosystem models.

1. Introduction

Mesopelagic fish occupy the upper 1000 m of the world's oceans and are an important component of global oceanic ecosystems, linking macrozooplankton to higher predators such as larger fish, squid, birds and mammals. Despite their importance, there are major gaps in our knowledge of the trophodynamics of mesopelagic assemblages, including gelatinous zooplankton, squids, fishes, and the trophic interactions among these groups, both globally and specifically in the Southern Ocean (Xavier et al., 2016; Young et al., 2015). As a result, mesopelagic assemblages represent a key area of uncertainty in current ecosystem modelling efforts (Hill et al., 2012, 2006; Murphy et al., 2012) and development of conservation and management strategies for the Southern Ocean (Constable et al., 2014; Hofmann, 2016).

In the Southern Ocean, the mesopelagic fish assemblage is dominated (in terms of biomass and abundance) by the family Myctophidae, as well as the family Bathylagidae in the lower mesopelagic zone (Duhamel et al., 2014). Myctophid biomass in the Southern Ocean has been estimated to be 70–130 million tonnes (Lubimova et al., 1987) and they are a major food source for penguins, seals and toothfish (e.g. Deagle et al., 2007; Goldsworthy et al., 2002; Green et al., 1989). Bathylagids, in particular *Bathylagus antarcticus*, also represent a substantial proportion of Southern Ocean mesopelagic biomass (Collins et al., 2012; Duhamel et al., 2014; Lancraft et al., 1991) and are known prey for Patagonian toothfish (*Dissostichus eleginoides*) and squid (Goldsworthy et al., 2002; Phillips et al., 2001).

Southern Ocean myctophid species have been described as opportunistic feeders, preying mainly on crustaceans such as copepods,

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amphipods and euphausiids (Hopkins and Torres, 1989; Lancraft et al., 1991; Lea et al., 2002; Pusch et al., 2004; Shreeve et al., 2009). The diet of *B. antarcticus* is more diverse, including euphausiids, copepods, amphipods, ostracods, as well as gelatinous prey such as salps, coelenterates, polychaetes, and tunicates (Geiger et al., 2000; Hopkins and Torres, 1989; Lancraft et al., 1991). Size is also thought to affect the diet and trophic position of Southern Ocean mesopelagics (Cherel et al., 2010), with euphausiids such as *Euphausia superba* only eaten by larger individuals of some species (Hopkins and Torres, 1989; Shreeve et al., 2009).

Previous diet studies of Southern Ocean mesopelagic fish have used morphology to identify prey items from stomach contents (e.g. Gaskett et al., 2001; Hopkins and Torres, 1989; Pusch et al., 2004; Shreeve et al., 2009). Despite yielding detailed taxonomic and quantitative dietary data, morphology-based approaches are biased towards prey hard structures because of their retention in stomachs and ease of identification; easily-digested cartilaginous and soft-bodied organisms (e.g. jellyfish and salps) are not identified and under-estimated (Arai, 2005; Sweetman et al., 2014). There is increasing appreciation of the number of fish taxa that prey on gelatinous zooplankton (e.g. Diaz Briz et al., 2017), and diet studies using high-throughput DNA sequencing (HTS) have revealed the presence of gelatinous and other soft-bodied prey in the diet of a range of marine predators (Berry et al., 2015; Hirai et al., 2017; Jarman et al., 2013; McInnes et al., 2017; O'Rorke et al., 2012).

The aim of this study was to use high-throughput DNA sequencing to characterise stomach contents of five common and widespread Southern Ocean mesopelagic species, including four dominant myctophids and one bathylagid collected from the southern extension of the Kerguelen plateau (Duhamel et al., 2014; Trebilco et al., in press). This work is part of a larger research program to characterise ecosystems in this region, one of the most productive in the Indian sector of the Southern Ocean (Blain et al., 2007), and, to the best of our knowledge, is the first application of DNA-based diet analysis to mesopelagic fish. We tested whether diet composition varied between species, between northern and southern regions of the survey area, and between individuals of different size. The sensitivity of high-throughput sequencing raises the possibility of secondary predation influencing the results, namely detecting taxa consumed by dietary items of the focal species (e.g. Sakaguchi et al., 2017). For example, it is possible that diatoms consumed by krill could be detected in the stomach contents of mesopelagic fish. We therefore also applied high-throughput sequencing to individual diet items to explore whether secondary predation was likely to influence the results.

2. Materials and methods

2.1. Sample collection and DNA extraction

Samples were collected on board the *RSV Aurora Australis* during cruise V3 from 22 January to 17 February 2016. Fish for diet analysis were collected from four sampling stations along a single transect: two at the southern end in Princess Elizabeth Trough (#16 and 17) and two at the northern end over BANZARE Bank (#22 and 23, Fig. 1). At each station, mesopelagic fish communities were sampled using stratified midwater trawls using an IYGPT net (International Young Gadoid Pelagic Trawl) equipped with a MIDOC (Midwater Open-Close) multiple cod-end device towed at 1.5–3 knots (see Trebilco et al., in press for additional details). Although trawls were conducted at different times of day (day: #16, sunrise: #17 and 22, sunset: #23), fish used in this study were caught in the first cod-end (2 mm mesh) which sampled the full extent of the mesopelagic zone (0–1000 m), and should minimise the effects of diel vertical migration. Fish were stored frozen at −20 °C.

In the lab, 195 fish representing four myctophids and one bathylagid species (Table 1) were thawed, measured (standard length, SL) and identified to species level prior to removing the stomach contents.

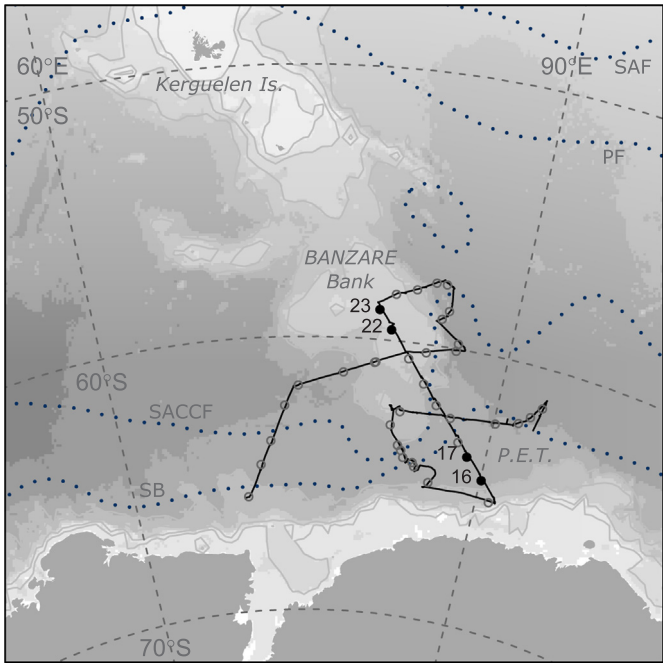

Fig. 1. Midwater trawl locations used in this study of Southern Ocean mesopelagic fish diet. The mean locations of the principal fronts (following Orsi et al., 1995) are shown as dotted lines. SAF – Subantarctic Front, PF – Polar Front, SACCF – southern Antarctic Circumpolar Current front, SB – Southern Boundary of the ACC, P.E.T. – Princess Elizabeth Trough.

Stomach contents were homogenised in Lysis Buffer in lysis matrix A tubes for 40 s on a FastPrep-24 5 G (MP Biomedicals, Santa Ana, CA USA). DNA was extracted from 205 µL of homogenate using the NucleoSpin® Tissue system (Macherey-Nagel GmbH & Co. KG, 52355 Düren, Germany) at the Australian Genome Research Facility (AGRF, Adelaide, Australia; http://www.agrf.org.au). Purified DNA was quantified via UV absorbance (NanoDrop ND-8000 Spectrophotometer; Thermo Fisher Scientific, Waltham, MA USA).

To explore the potential for secondary predation to influence our results, 17 individual prey items, including copepods, amphipods and euphausiids isolated from additional fish stomachs for a separate study, were rinsed in 70% ethanol and processed as per stomach content samples.

2.2. PCR amplification and high-throughput sequencing

2.2.1. 18S ribosomal DNA for diet analysis

DNA was diluted to 5 ng/µL and the V9 region of the 18S ribosomal DNA (ca. 100 bp) PCR-amplified from each sample and four 'no template' controls using modified eukaryotic primers (Jarman et al., 2013, Supplementary Table 1). PCR amplifications were performed in two rounds, the first to amplify the target locus and add sample-specific 6 bp multiplex-identifier (MID) tags (forward and reverse primer) and Illumina sequencing primers, the second to add sequencing adapters and additional 10 bp MIDs as per Clarke et al. (2017). Each PCR reaction mix contained 0.1 µM each of forward and reverse primer, 1 µg/µL bovine serum albumin (BSA), 1 x EvaGreen (Biotium, Hayward, CA, USA), 0.2 U Phusion DNA polymerase in 1 x Phusion Master Mix (New England Biolabs, Ipswich, MA, USA) and 1 µL DNA extract in a total reaction volume of 10 µL. PCR thermal cycling conditions were initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 5 s, 67 °C for 20 s and 72 °C for 20 s, with a final extension at 72 °C for 5 min. PCR products were diluted 1:10 and Illumina sequencing adapters added in a second round of PCR using the same thermal cycling conditions as the first round, except the number of cycles was reduced

Table 1
Number of fish of each species in each trawl used for DNA-based diet analysis. Size ranges are standard lengths.

Species	Size range (mm)	n Station 16	Station 17	Station 22	Station 23	Total
Bathylagidae						
Bathylagus sp.	61–176	13	16	10	14	53
Myctophidae						
Electrona antarctica	45–90	18	18	11	11	58
Gymnoscopelus braueri	70–130	5	5	12	4	26
G. nicholsi	120–170	2	4	3	3	12
G. opisthopterus	104–166	8	–	–	–	8
Totals		46	43	36	32	157

to 10 with an annealing temperature of 55 °C. Second round PCR products were pooled then purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). The concentration of the library was quantified using the Qubit dsDNA BR assay on a QUBIT 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The library was diluted to 2 nM and paired-end sequencing reads generated on a MiSeq (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (2 × 150 bp paired-end).

2.2.2. COI for fish identification

Fish species identifications were confirmed by high-throughput sequencing 313 bp of the cytochrome oxidase c subunit I (COI) barcoding region (Leray et al., 2013) from the same DNA extracts. The region was PCR-amplified using 35 cycles with an annealing temperature of 46 °C as described in Clarke et al. (2017). Paired-end sequencing of amplicons was performed on a MiSeq using MiSeq Reagent Nano Kit v2 (2 × 250 bp). Samples assigned to fish species other than the five target species were excluded from the analysis.

2.3. Data analysis

Sequencing reads were deconvoluted based on 10 bp MIDs on the MiSeq. Fastq reads were merged using the -fastq_mergepairs command in USEARCH v8.0.1623 (Edgar, 2010). Merged reads were sorted by "internal" 6 bp MID tags, and locus-specific primers trimmed with custom R scripts using the ShortRead package (Morgan et al., 2009), with only reads containing perfect matches to the expected MIDs and primers retained. Reads for all samples were dereplicated and global singletons discarded (-derep_fulllength –minuniquesize 2), and clustered into OTUs with the UPARSE algorithm (Edgar, 2013) at 97% identity using the "-cluster_otus" command. Potentially chimeric reads were also discarded during this step. Reads for each sample were then assigned to OTUs (-usearch_global –id 0.97), and an OTU table generated using a custom R script.

For the 18S V9 data, OTUs were assigned taxonomy at order level where possible using MEGAN version 5.10.5 (Huson et al., 2011) based on 50 hits per OTU generated by BLASTN searches against the NCBI "nt" database excluding environmental sequences (downloaded June 2016). An OTU table of prey items was generated by removing OTUs representing parasites, terrestrial contaminants and fish based on the MEGAN taxonomy, as well as those not assigned to phylum or below. Samples with less than 1000 reads assigned to prey items were also removed from the analysis.

Differences in diet composition among species, regions (north vs. south) and trawls were explored using Bray–Curtis dissimilarity in QIIME v1.8.0 (beta_diversity_through_plots.py, Caporaso et al., 2010) based on either a rarefied OTU table or a rarefied 'order table' (taxa pooled at order level), with strength and significance of groupings assessed using the Adonis method (compare_categories.py, 999 permutations).

We calculated the frequency of occurrence of different taxa as prey by summing the reads for OTUs assigned to the same taxon for each sample. Taxa contributing more than 1% of the sequencing reads for each sample were deemed to be prey items. We tested whether the likelihood of detecting certain prey items changed with fish size using binomial logistic regression in R (function 'glm', R Core Team, 2017). Separate models were fit for individual prey taxa, with standard length of each given individual fish (predator) as the predictor and the presence/absence of the relevant prey taxon (> 1% diet reads) as the response. Results are reported as odds ratios, namely the constant effect of the predictor (standard length) on the probability that a given prey taxon is consumed, with an odds ratio > 1 indicating a positive effect (i.e. larger fish are more likely to consume the prey item), < 1 indicating a negative effect (i.e. larger fish are less likely to consume the prey item), and 1.0 being no effect.

Although the COI marker was poorly suited to diet analysis in this study (see Results), we did obtain diet reads with the COI marker for 160 samples. However, the number of diet reads per sample was typically much lower than for 18S. Rather than relying on a percentage cut-off, we deemed a prey item present for the COI marker if represented by 10 or more reads. Relative read abundance was not used for the COI data.

3. Results

3.1. Diet analysis

A total of 157 samples were considered in the 18S diet dataset (excluding 35 fish stomach samples with less than 1000 sequences from potential prey and three identified as non-target species based on COI sequences, see below, Table 1). The number of sequences recovered per sample ranged from 1020 to 43,923 (mean ± SD = 17,000 ± 11,000 reads) and these were assigned to 742 potential prey item OTUs representing 70 taxa (OTU table and FASTA file available in Supplementary material).

Sequencing four 'no template' PCR controls produced 49–23,746 reads per control. However, these reads were assigned to likely terrestrial contaminants (fungi, land plants and insects) and did not include any potential diet items.

Permutational Adonis tests using Bray-Curtis dissimilarities amongst samples showed the relative read abundance of prey items differed significantly amongst the five fish species (R^2 = 0.212 for OTUs, 0.264 for orders, $P < 0.001$ for both), with crustaceans (amphipod, copepod and euphausiid) representing 90% or more of sequences on average for the four myctophids, whereas gelatinous prey items represented more than 65% of reads for *Bathylagus* sp. (Fig. 2a). Siphonophores and Collodaria (radiolarians) each contributed on average ca. 20% or more of reads for *Bathylagus* sp. Diet composition also differed amongst the four myctophid species (R^2 = 0.147 for OTUs, 0.177 for orders, $P < 0.001$), but this was driven by the dominance of euphausiids in *G. opisthopterus* samples (Fig. 2a), with R^2 values showing that less than 6% of variance was explained by myctophid species when *G. opisthopterus* was excluded (R^2 = 0.057 for OTUs, P = 0.01, R^2 = 0.046 for orders, $P = 0.053$).

A total of 44 taxa represented more than 1% of diet reads per

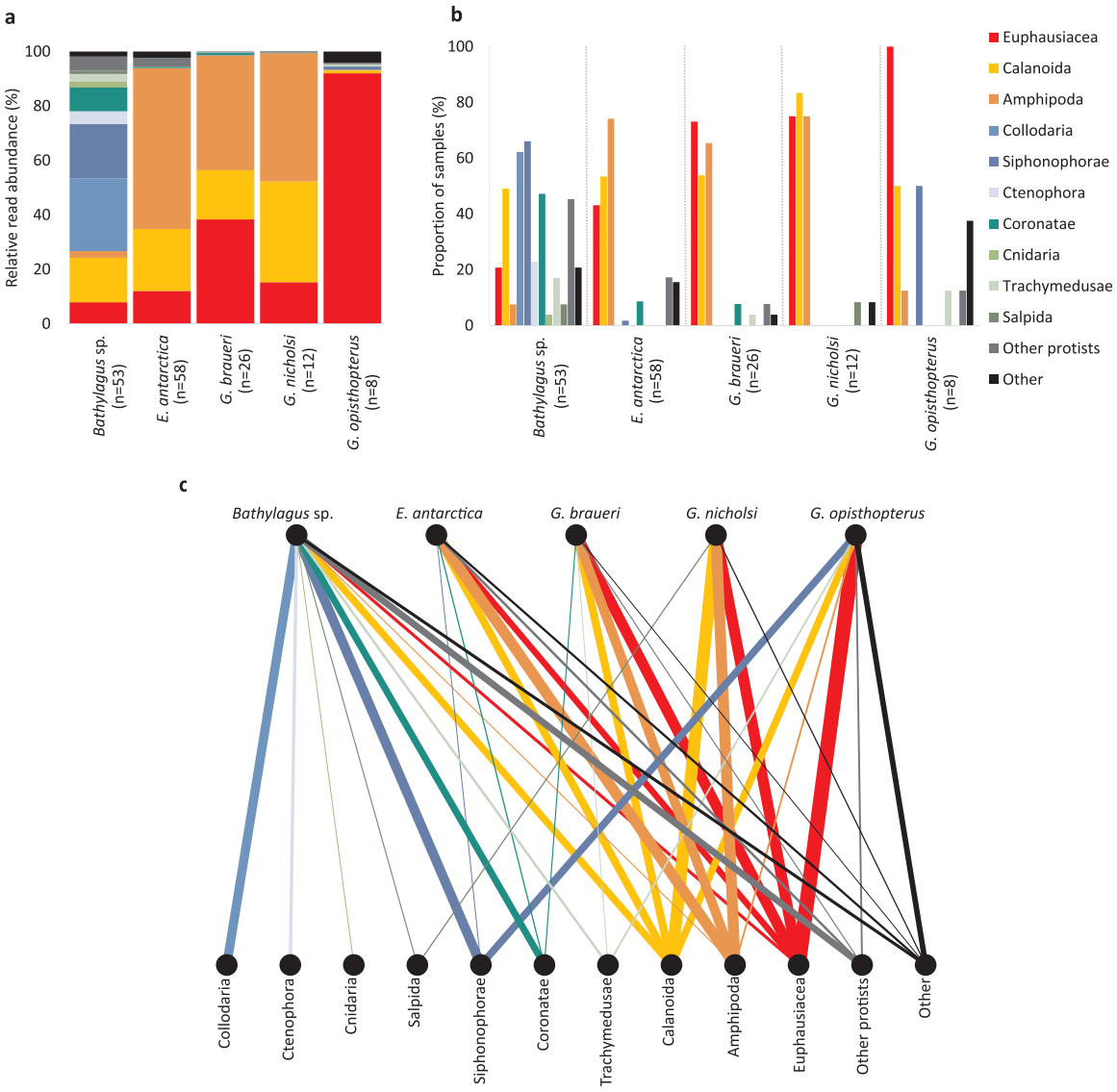

Fig. 2. Prey items (classified to order) recovered in each fish species. (a) Mean relative read abundance and (b) frequency of occurrence. (c) Representation of prey preferences using DNA-based diet analysis. The width of each link is proportional to the frequency of prey item occurrence in each fish species. Prey items were deemed present in a sample if they comprised > 1% total reads obtained. Prey items that represented > 50% of the reads in any sample are shown, remaining taxa are pooled as 'Other protists' or 'Other'.

sample, with between 1 and 14 taxa detected at this level per sample (median 2, mean ± SD = 3 ± 2 taxa). Amphipods, calanoid copepods and euphausiids were typically the most common prey items detected for all myctophid species (Fig. 2b and c). In contrast, *Bathylagus* sp. samples were more likely to contain siphonophores and Collodaria DNA, with each present in more than half the samples for this species. Collodaria was not detected as a prey item for any myctophid species.

Within the four species sampled from all trawls, we found little evidence for difference in diet composition between trawls or the southern and northern region of the survey area, with the exception of *G. braueri*, where diet differed among trawls (R^2 = 0.32 and 0.33 for OTUs and orders, respectively, P = 0.001) and regions (R^2 = 0.32 for both OTUs and orders, P < 0.01). Individual *G. braueri* from southern trawls had a higher relative abundance of euphausiid reads, which may have been due to the presence of larger *G. braueri* individuals in these trawls (mean ± SD = 107 ± 17 mm and 87 ± 10 mm for southern and northern trawls, respectively, see below).

3.2. COI for fish identification and diet analysis

Sequencing 313 bp of the COI barcoding region produced a clear signal from the fish predator. In all samples > 90% of COI reads from fish were assigned to one fish species. In fact, there were only 19 instances where less than 99% of these reads were assigned to a single fish. These sequences confirmed the morphological species identifications for the majority of individuals. However, three individuals identified by morphology as *Gymnoscopelus* sp. returned COI sequences matching congeneric non-target species (*G. bollini and G. fraseri*); these were excluded from the analysis. Each of the four target myctophid species were assigned to a single OTU based on COI sequences. However, bathylagid samples were assigned to four distinct OTUs; three OTUs were assigned to *Bathylagus antarcticus* and one to *Bathylagus* sp. The two most common *B. antarcticus* OTUs were present in both the north and south of the survey area. Two fish from the southern trawls represented a distinct OTU, and one fish from a northern trawl represented an additional OTU assigned to *Bathylagus* sp.

The COI marker was poorly suited to diet analysis in this study due to the recovery primarily of DNA from the fish predator rather than

Table 2
Frequency of occurrence of prey items for each species based on the COI marker. Prey items were deemed present in a sample if represented by 10 or more reads. Shading shows frequency of occurrence by species (categories: blue < 15%, light pink 15–25%, dark pink 35–50%, red > 50%).

Prey item	*Bathylagus* sp.	*Electrona antarctica*	*Gymnoscopelus braueri*	*G. nicholsi*	*G. opisthopterus*
No. individuals	24	38	24	13	7
Euphausiids					
Thysanoessa macrura	9	17	10	10	2
Euphausia superba	-	1	6	2	7
Euphausiidae	-	4	5	2	-
Amphipoda					
Vibilia	1	4	2	2	-
Themisto gaudichaudii	-	1	3	-	-
Cyllopus lucasii	-	1	-	-	-
Cyllopus magellanicus	-	1	-	-	-
Hyperia macrocephala	-	-	-	1	-
Lanceoloidea	-	1	-	-	-
Amphipod	-	2	-	-	-
Copepoda					
Oncaea sp.	4	-	-	-	-
Rhincalanus gigas	-	2	-	-	-
Calanus 1	-	3	-	1	-
Calanus 2	-	-	-	1	-
Metridia	-	-	1	-	-
Metridia gerlachei	1	-	-	-	-
Paracalanidae	1	-	-	-	-
Calanoida	-	-	1	-	-
Ostracoda					
Alacia belgicae	2	3	4	1	-
Proceroecia brachyaskos	1	-	-	-	-
Ostracod	2	1	1	-	-
Mollusca					
Clio pyramidata	-	10	1	2	1
Mollusc 1	-	-	2	-	-
Mollusc 2	-	-	-	-	1
Cnidaria					
Aurelia (Semaeostomeae)	4	-	-	-	1
Sphaeronectes sp. (Siphonophorae)	2	-	-	-	-
Atolla wyvillei (Coronatae)	1	-	-	-	-
Periphylla periphylla (Coronatae)	1	-	-	-	-
Botrynema brucei (Trachymedusae)	1	-	-	-	-
Trachymedusae	1	-	-	-	1
Ctenophora					
Lobata	5	-	-	-	-
Ctenophora	1	-	-	-	-
Tunicata					
Tunicate 1	1	-	-	-	-
Tunicate 2	1	-	-	-	-

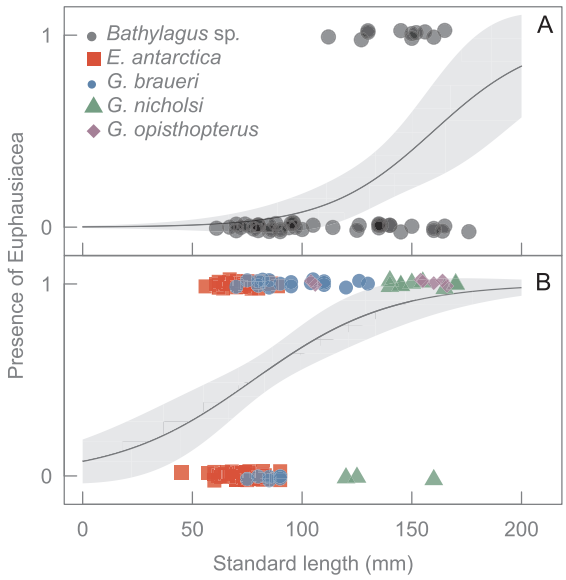

Fig. 3. Relationship between fish size and detection of Euphausiacea (> 1% read abundance) in stomach contents for a) *Bathylagus* sp. and b) myctophids. Fitted curves are logistic regressions for *Bathylagus* sp. and all myctophids, respectively.

DNA from diet items (85 ± 21% fish reads per sample for COI versus 14 ± 26% for 18S). Both markers can recover DNA from the fish predator, so the difference is likely due to the longer fragment size of COI (313 bp vs. 100 bp for 18S) or amplification biases. We did obtain diet reads with the COI marker for 160 samples and the longer fragment length and greater variability allows species-level taxonomic resolution in many cases (e.g. Clarke et al., 2017). However, the number of diet reads per sample was typically low, with a median of 42 diet reads per sample (mean ± SD = 125 ± 175 reads). Using a detection threshold for prey items of 10 or more reads, 35 prey taxa were detected across 106 samples. Although many non-metazoan taxa identified as common diet items with 18S were not detected with COI (e.g. Collodaria), the additional taxonomic resolution provided further insights into Southern Ocean mesopelagic fish diets. For example, all species were found to prey on *Thysanoessa macrura*, but *Euphausia superba* was only detected in the stomach contents of myctophids (Table 2). Again, Cnidaria and Ctenophore taxa were more frequently detected from *Bathylagus* sp.

3.3. Size structuring of diet

Only *Bathylagus* sp. individuals 110 mm or longer had Euphausiacea (most likely *T. macrura* based on COI) as prey items (Table 2, Fig. 3a). In contrast, both *T. macrura* and *E. superba* were detected as prey items in myctophids smaller than 65 mm (Fig. 3b). However, odds ratios calculated from the 18S frequency of occurrence dataset indicated that the probability of both *Bathylagus* sp. and myctophids eating euphausiids increased by 51% and 38%, respectively, with each 10 mm increase in size (*Bathylagus* sp. odds ratio = 1.51, 95% CI: 1.13–2.02, myctophid odds ratio = 1.38, 95% CI: 1.14–1.67, Fig. 4), with borderline significant relationships for *G. braueri* (odds ratio = 2.00, 95% CI: 0.89–4.51) and *G. nicholsi* (odds ratio = 2.51, 95% CI: 0.77–8.22). For *Bathylagus* sp., the probability of consuming euphausiids across a body size range of 75 to 150 mm increased from 0.03 (95% CI: 0.004–0.18) to 0.40 (95% CI: 0.23–0.61), whereas the probability for a 75 mm and 150 mm myctophiod was 0.48 (95% CI: 0.37–0.59) and 0.91 (95% CI: 0.73–0.98), respectively. We also found a positive relationship between the size of *Bathylagus* sp. individuals and detecting Coronatae as a prey item (odds ratio = 1.28, 95% CI: 1.06–1.54), and a trend toward decreasing predation on Collodaria with increasing size (odds ratio = 0.85, 95% CI: 0.71–1.02).

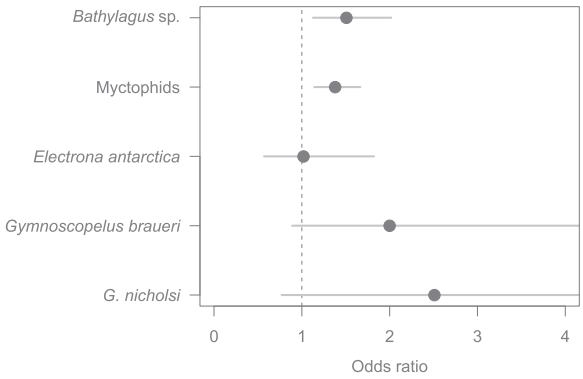

Fig. 4. Odds ratio for the relationship between detection of Euphausiacea in stomach contents and standard length in Southern Ocean mesopelagic species or species groups. An odds ratio > 1 indicates more detections in larger fish and 1.0 (dotted line) shows no effect. Error bars show 95% confidence intervals. The number of *G. opisthopterus* individuals was too low to calculate an odds ratio for this species. The upper end of the x-axis is truncated for clarity.

3.4. Secondary predation

In this analysis, we sequenced 18S V9 ribosomal DNA from whole prey items to determine if material in their stomachs could be detected and if this would influence the fish diet datasets. For the individual copepod, amphipod and euphausiid specimens (focal species) isolated from fish stomachs, the number of reads obtained ranged from 1340 to 34,220 (mean ± SD = 17,300 ± 9700 reads). Sequences from the focal species made up > 50% reads in 16/17 samples, and represented > 99% reads for 9/17 samples (Fig. 5). For the eight samples with < 99% reads from the focal species, other taxa detected were typically not their potential prey. For example, Gnathostomata and Euteleostomi (most likely fish) were > 25% reads for two samples, fungi were the second most abundant taxa for 4/8, and amphipod DNA was the second most abundant taxon for one copepod. However, the relative read abundance of potential diet items for one krill (*Euphausia* 2) was 11%. Plausible diet items were detected in 11/17 samples, with the relative read abundance for individual diet items ranging from 0.1% to 4.4%. Overall, the mean and median percentage of the most abundant dietary item was 0.7% and 0.1%. Potential diet items > 1% were: Alveolata and Dinophyceae (*Paraeuchaeta*), Calanoida (*Thysanoessa* 1), Siphonophorae (*Euphausia* 1), Cryomonadida, Cercozoa and Pteriomorphia (*Euphausia* 2). Each of these taxa except Pteriomorphia were also detected as potential diet items for fish samples. It remains plausible that secondary predation could be detected in our fish diet samples, but the relative abundance of these items would rarely be greater than 1%.

4. Discussion

4.1. DNA-based diet analysis of mesopelagic fish

Our study is the first to use DNA to characterise the diet of several of the most abundant mesopelagic fish species in the Southern Ocean. Our results confirm that myctophids in the southern Kerguelen Axis region commonly prey on crustaceans (e.g. Hopkins and Torres, 1989; Lancraft et al., 1991; Pusch et al., 2004; Shreeve et al., 2009), but highlights gelatinous prey in the diet of both Southern Ocean bathylagids and myctophids. The significant relationship between fish body size and detection of certain taxa as prey items is relevant to understanding ontogenetic diet shifts in these key Southern Ocean mid-trophic level taxa. Such ontogenetic dietary changes present a challenge to species-focused ecosystem modelling approaches, and provides further incentive for exploring size-based analysis and models to describe Southern Ocean food webs (Murphy et al., 2012).

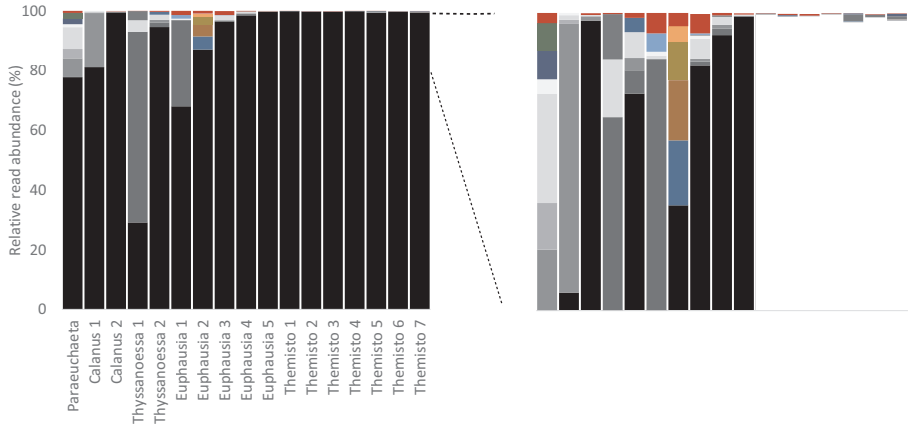

Fig. 5. Proportion of high-throughput sequencing reads from individual copepod, amphipod and euphausiid specimens (focal species) to explore the likelihood of secondary predation influencing the results. Reads were dominated by the focal species (shown in black) and non-prey sequences (grey scale). Sequences that could represent signal from secondary predation (colour) were generally uncommon.

A key finding of our study is the previously undescribed frequency of predation on gelatinous zooplankton, and the diversity of gelatinous prey items for Southern Ocean mesopelagics. We detected gelatinous prey in the stomach contents of all five species (Fig. 2), with DNA-based diet analysis revealing at least two taxonomic orders of gelatinous prey for each myctophid, and seven for *Bathylagus* sp. Many fish diet studies, including studies of Southern Ocean mesopelagics, detect a very limited diversity of gelatinous zooplankton (Shreeve et al., 2009), or group gelatinous prey at very broad taxonomic levels such as coelenterates (Hopkins and Torres, 1989; Sweetman et al., 2014) or simply as 'gelatinous organisms' (Geiger et al., 2000). Given the difficulties of morphologically identifying gelatinous prey in stomach contents due to rapid digestion and short passage time (Arai et al., 2003; Sweetman et al., 2014), DNA-based approaches are likely to continue to highlight the importance of gelatinous zooplankton in Southern Ocean food webs.

The frequency of radiolarian predation by Southern Ocean *Bathylagus* sp. is noteworthy given only two other studies identified radiolarians as prey items of *B. antarcticus* (Hopkins et al., 1993; Hopkins and Torres, 1989). Collodaria (radiolarians) are abundant globally in the bathypelagic zone (Pernice et al., 2016), and have been recorded as prey items of other marine taxa, including congeneric species (Grimes, 1979; O'Rorke et al., 2012; Sweetman et al., 2014). Of the previous studies reporting diet of *Bathylagus antarcticus*, most did not report radiolarians as prey (Gaskett et al., 2001; Geiger et al., 2000; Lancraft et al., 1991), or only infrequently (1/50 individuals, Hopkins et al., 1993). In contrast, we found Collodaria as prey items in more than 60% of *Bathylagus* sp. individuals, highlighting their potential importance as prey for bathylagids.

A limitation of DNA-based diet analysis is that proportions of DNA sequences from different prey items within a sample provide only a very approximate indication of their relative biomass (Pompanon et al., 2012). Therefore, most of our analyses use indices based on frequency of occurrence, although the overall diets are similar when relative read abundances are considered (Fig. 2). Future DNA-based diet studies could use stomach content mass to give a weighting between samples, potentially highlighting the greater contribution of larger prey items to nutrient cycling and energy flows, or a minimum mass below which diet analysis becomes unreliable. Our frequency of occurrence data can be compared to those from traditional stomach content dietary studies, but just like stable-isotope (Woods et al., in press) or fatty acid studies (Lea et al., 2002), the DNA-based approach provides a unique and complimentary view of the true diet of these fish. Because DNA-based diet analysis can detect highly digested prey, combining these data with stable isotope or fatty acid analyses may be the best means to estimate the contribution of gelatinous and other prey to fish tissue composition (e.g. Hardy et al., 2010).

4.2. Size structuring of diet

Our results add to evidence of ontogenetic dietary shifts and trophic size-structuring in Southern Ocean food webs (Woods et al., in press), with larger bathylagid and myctophid individuals more likely to prey on euphausiids. In addition, smaller (75 mm) individuals of *Bathylagus* sp. were less likely to prey on euphausiids than myctophids of the same size. Although we were not able to control for the possibility of fish continuing to feed after capture in the net, the size-based dietary shifts detected presumably reflect actual feeding preferences. The finer taxonomic resolution provided by the COI marker also showed that *Bathylagus* sp. preyed on *T. macrura* but not the typically larger Antarctic krill (*E. superba*, Table 2). Almost all fish are gape-limited in the size of prey they can consume (Helfman et al., 2009; Karpouzi and Stergiou, 2003). Species-specific diet preferences may reflect the relatively small gape of bathylagids compared to myctophids (Gartner et al., 1997; Walters et al., unpublished data), filtering capacity of gill rakers, or the vertical distribution of predators and prey (Shreeve et al., 2009). It is worth noting that prey items have a range of developmental phases from larvae to adults, and that size of prey cannot be assessed using DNA-based analysis. Factors such as ontogenetic changes to habitat depth (Moteki et al., 2009) or feeding times could also potentially contribute to the observed shifts in diet with size. We also found that larger *Bathylagus* sp. were more likely to prey on Coronatae. The two Coronatae species identified using the COI marker can grow to diameters of 150 and 300 mm, respectively, thus may be more suitable prey for larger bathylagids. In agreement with Hopkins and Torres (1989), we found that Collodaria were more likely to be present in stomach contents of smaller *Bathylagus* sp. individuals. Although gape limitations are recognised for hard-bodied prey, our results suggest that it is important for gelatinous prey as well – something that is difficult if not impossible to detect with traditional morphological gut content analysis.

4.3. Secondary predation

Our secondary predation study suggests that secondary predation could potentially be detected in our fish diet samples, but the relative abundance of these items would rarely be greater than 1%. Detection of 'Other protists' (typically diatoms) as prey items for *G. opisthopterus*, *G. nicholsi* and *E. antarctica* could represent secondary predation given how often these species prey on euphausiids and other taxa likely to

consume diatoms. Indeed, 11/12 detections of non-Collodarian protists in myctophids co-occur with Euphausiacea. Regardless of whether taxa represent primary or secondary prey items, their detection in stomach contents indicates their potential as an energy source for mesopelagic fish species and helps elucidate energy pathways through Southern Ocean food webs.

4.4. Future directions

4.4.1. Spatial extent

Our study expands the area over which the diet of Southern Ocean mesopelagic fish has been characterised, with most previous studies centred in the Scotia Arc or north of the Polar Front (although see Pakhamov et al., 1996). Continued expansion of the spatial coverage of dietary data will be important to develop a general understanding of trophodyamics for this important food web component throughout the Southern Ocean.

4.4.2. Predation on other fish

Although *G. nicholsi* is known to occasionally prey on other fish (Pusch et al., 2004), we could not reliably detect fish in the diet of samples in this study. This is because the 18S marker lacks taxonomic resolution to discriminate between fish species and the COI marker sequences were dominated by sequence reads from the predatory fish. However, the average number of reads per sample was low for the COI marker, hence deeper sequencing would increase the potential to detect predation on other fish. Future studies seeking to use a DNA-based approach to detect fish as prey items should use a marker with species-level resolution, such as COI, and much higher read depth or employ other methods to prevent amplification of DNA from the predatory fish (e.g. Pompanon et al., 2012).

4.4.3. Fish parasites

Although not a focal point of the current study, DNA-based analysis of stomach contents also provides insights into the prevalence and diversity of fish parasites. Parasites were detected with the 18S marker in all five species, including Platyhelminthes, Ellobiopsidae, Apostomatida, Icthyophonida Poecilostomatoida, Syndiniales, and Blastodiniales. Exploring whether different fish species host different parasite assemblages, and relationships between the incidence of parasite DNA and fish size or condition could yield important information on the role of parasites in Southern Ocean ecosystems.

5. Conclusion

This study highlights the utility and complementarity of DNA-based diet analysis to traditional morphology-based approaches for characterising trophic links in mesopelagic fish assemblages that are difficult to study. Critically, this method highlighted the importance and diversity of gelatinous prey items, which are largely missed with morphological analysis. Our dual marker approach also helped avoid misidentification of fish species, including cryptic species. Indeed, the presence of multiple COI OTUs assigned to *Bathylagus* sp. supports the presence of cryptic diversity in Southern Ocean specimens of this genus (Dettai et al., 2011). The results of this study provide important information for developing models of Southern Ocean food webs and isotopic niche analyses for Southern Ocean mesopelagics.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dsr2.2018.09.001.

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