Title: Assessing the trophic ecology of Southern Ocean Myctophidae: the added value of DNA metabarcoding

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Abstract

Lanternfishes (Myctophidae) are key components of mesopelagic fish communities globally. In the Southern Ocean, incomplete information on myctophid diets limits our understanding of their energetics, interactions and wider ecosystem impact. Traditional microscopic methods of diet analysis have relatively coarse prey resolution and possible taxonomic and observer biases. DNA metabarcode sequencing promises higher taxonomic and temporal resolution, but uncertainty remains in comparing this is with microscopy-based analyses. Here, we applied 18S DNA metabarcode sequencing to stomach contents from twenty *Electrona antarctica* individuals which had previously been examined via microscopic analysis. Across all fish, crustacean and gastropod taxa dominated the prey identified via both methods, with broad agreement between methods on the relative abundance of different prey items. DNA metabarcode sequencing recovered greater taxonomic diversity and resolution, particularly for soft-bodied prey items and small crustaceans. DNA sequencing results also more clearly differentiated diet between individuals collected from different environments. Overall, our findings illustrate how DNA based methods are complementary to, and consistent with, traditional methods and can provide additional, high-resolution data on a range of trophic interactions.

Keywords: Myctophids, DNA metabarcoding, dietary analyses, method comparison, Southern Ocean

1. Introduction

Lanternfishes (Myctophidae) are an abundant and ecologically important component of Southern Ocean pelagic ecosystems (Murphy et al. 2007, Saunders et al. 2019, Duhamel et al. 2014, Liszka et al. 2022). Acoustic estimates of mesopelagic fish biomass in this region are as high as 570 million tonnes, with estimates suggesting that this group of fishes may consume in excess of 150 million tonnes of zooplankton annually (Koz 1995, Dornan et al. 2022). Myctophids are considered central to some Southern Ocean food webs (McCormack et al., 2020) and may act as an alternative trophic pathway between primary production and higher predators in years when Antarctic krill (*Euphausia superba*) are scarce (Murphy et al. 2007). Furthermore, with a broad vertical distribution (0 – 1000 m) and daily vertical migrations, they contribute to the export of carbon from surface to deep waters in the region (Collins et al., 2012, Saunders et al. 2019, Belcher et al. 2019, Saba et al. 2021).

Myctophids are key prey for king penguins, Antarctic fur seals, and some squid and toothfish species (Olsson & North, 1997, Collins et al. 2007, Cherel et al. 2007). However, the diet of myctophids themselves and their wider influence on Southern Ocean pelagic food-webs is less clear. For example, myctophids are typically considered to be major consumers of crustacean zooplankton, including copepods, euphausiids and amphipods (Clarke et al. 2020, Pakhomov & Perissinoto 1996) but regional, seasonal, and ontogenetic variation in diet (Saunders 2014, 2015a, 2015b, Riaz et al. 2020) complicates inferences on energy budget and predation impact (Shreeve et al. 2009). Of particular debate has been the role of myctophids as consumers of Antarctic krill, a keystone species in the Southern Ocean and the target of a growing commercial fishery (Meyer & Kawaguchi 2022). Saunders et al. (2019) concluded that some larger myctophid species are central consumers of Antarctic krill, whereas Pakhomov et al. (1996) found that Antarctic krill were relatively rare diet items for myctophids, and did not play an important role in their energetics. Given the abundance of myctophids in the Southern Ocean, and their centrality within the food web, efforts to resolve myctophid trophic interactions will have important implications for understanding food web dynamics, resource partitioning, and potential impacts of environmental change on ecosystem structure and function.

Traditional methods for analysing the in-situ diet of small fish, currently the main source of data for myctophid diets, have their limitations. When identifying prey via microscopic identification of stomach contents, a large proportion of the stomach contents can consist of

unidentifiable or partially digested tissue (Baker et al. 2014, Amundsen & Sánchez-Hernández 2019) which makes identification to lower taxonomic levels challenging. Microscopic identification of stomach contents is also limited when detecting soft-bodied prey, such as ctenophores, cnidarians, chaetognaths and salps (Amundsen & Sánchez-Hernández 2019, Novotny et al. 2022). Moreover, there are uncertainties over the gut retention times of different stomach contents and whether all prey have comparable gut passage times (Baker et al. 2014, Amundsen & Sánchez-Hernández 2019). Finally, these analyses require specialised taxonomic expertise, and results are not always reproducible across observers (Berry et al. 2015). Other diet analysis methods, including biomarker analyses such as stable isotopes and fatty acids provide complementary insights, but are limited in their ability to resolve prey type, and typically average diet signals over weeks to months, which can be challenging when considering feeding in a highly temporally variable environment, such as the polar regions (Schmidt et al. 2006).

DNA metabarcode sequencing of stomach contents offers a complementary approach to conventional, morphological diet analyses. Strengths of DNA metabarcode sequencing as a diet analysis approach include high prey type resolution, high temporal resolution, and a more broadly applicable skill set required (de Sousa et al. 2019). Prey type resolution varies with the choice of gene marker used and the specific prey involved, but can be as high as species or even sub-species level (Clarke et al. 2017). Temporal resolution in any stomach contents study is a function of digestion and gut evacuation rates. In many small marine predators, DNA has been shown to be digested much more quickly than the hard parts used in microscopic analyses, thus providing a shorter snapshot of diet at a given time and location (Troedsson et al. 2009). DNA analysis of stomach contents requires specialised skills in molecular approaches, but unlike the regionally specific expertise required for morphological analyses, these skills are similar across ecosystems and taxa, and overlap with the skills applied in other DNA-based fields such as medical research. This may be particularly advantageous for research centres which are still developing their programs in polar science and may not have an existing legacy of taxonomic expertise. Nevertheless, DNA sequencing for diet analysis is not without challenges. There remains uncertainty in the quantification of the sequencing reads, due to potentially variable digestion rates, different DNA marker:biomass ratios, and differences in PCR amplification efficiency across differing prey types (Deagle et al. 2018). Furthermore, present methods based on DNA sequencing do not provide information on the size, stage (eggs, larvae, adults) or part (whole animal, single

limb, or exuvia) of the consumed prey (Reese et al. 2014). The relationships between prey items within a stomach are also not captured by DNA metabarcode sequencing; prey or parasites within consumed prey items are detected similarly to directly consumed prey, although sequence reads from these are expected to be much lower in abundance, and network and correlational analyses can start to tease these interactions apart given large enough data sets (de Vargas et al. 2015). Lastly, laboratory reagents, equipment, and DNA sequencing itself remain significant costs, although the current trends of decreasing costs for all of these are expected to continue (Steinke et al. 2022, van der Reis et al. 2022).

Direct comparisons between microscopic identification and DNA metabarcode sequencing of stomach contents can provide valuable improvements to our understanding of the strengths and limitations of each approach, and are key to comparing between studies applying different approaches. Comparing across methods can also inform broader ecological interpretations and guide future best practices as different approaches may be best for different research questions (Amundsen & Sánchez-Hernández 2019). There is a growing body of such direct comparisons, but there remains uncertainty due both to physiological and ecological differences between fish species, and variation in the molecular methods applied. Direct comparisons have so far largely focused on commercially important species and model systems and have not yet been reported for the ecologically important myctophid fishes. Berry et al. (2015) compared metabarcoding with morphological analysis when investigating the diets of eight commercially exploited fish species. The metabarcoding approach revealed a greater diversity of prey items, and provided higher taxonomic resolution of these prey items. Additionally, the metabarcoding approach recovered soft-bodied prey – including cartilaginous fishes, salps, and cephalopods - more frequently than morphological identification. Jakubavičiūtė et al. (2017) investigated the diet of sticklebacks and found that morphological and metabarcoding approaches were broadly similar. Again, the DNA metabarcode sequencing approach showed a higher diversity and higher taxonomic resolution than morphological identification, though in this case some species were missed by the DNA metabarcode sequencing approach, likely due to primer mismatch. Similar results of broadly comparable diet with increasing taxonomic resolution have been observed in method comparisons with European hake and polar cod (Gül et al. 2023, Maes et al. 2023). Several of the studies published to date however do not include direct individual level comparisons (Gül et al. 2023, Berry et al. 2015), or only compare presence/absences (Maes et al. 2023) leaving

some additional uncertainty around the ways in which method choice affects the detection and quantification of different prey types.

In this study we applied 18S DNA metabarcode sequencing to stomach contents of twenty individual Antarctic lanternfish, *Electrona antarctica*, and compared the results with morphological analyses of the same stomachs (Collins et al., 2020; Saunders et al. 2014). We evaluate the use of this metabarcoding approach for investigating myctophid diet, and explore its utility in addressing ecological questions by comparing myctophid feeding across environments that are spatially and temporally distinct.

2. Methods

2.1. Field collection

Electrona antarctica, a species of myctophid with a distribution largely restricted to waters south of the Antarctic Polar Front, were collected in the Scotia Sea on three cruises of RRS *James Clark Ross*: JR161 (October-December 2006), JR177 (January - February 2008), and JR200 (March - April 2009). Sampling stations were distributed between the seasonal ice-edge and the Antarctic Polar Front (Fig. 1). Net sampling and haul catch processing methods are detailed in Collins et al. (2012). In summary, mesopelagic fish communities were sampled with a 25 m^2 rectangular midwater trawl net (RMT25) towed at 2.5 knots over discrete depth zones, specifically surface to 200 m, 200–400 m, 400–700 m and 700–1000 m. Water temperature was recorded in real time using a custom-built net monitoring system. *Electrona antarctica* were identified using published guides (Gon and Heemstra 1990; Hulley 1990) and standard length (SL) was measured to the nearest mm. Stomachs were dissected at sea and frozen (-20^{o}C) until analysis. As detailed in Shreeve et al. (2009) and Saunders et al. (2014), stomach contents were thawed prior to being sorted and identified to the lowest taxonomic level possible under microscopy. Note that the fish reported here represent only a subset of those analysed in Saunders et al. (2014), and that all individuals analysed were adults. Any items that were completely undigested were considered to represent trawl feeding and were excluded from subsequent analyses. Trawl feeding is rarely observed in myctophids; this data filtration step reflects that the microscopy protocols are standardized across a wide variety of fish species. Following microscopic analyses, complete stomach contents were preserved in 96% ethanol until molecular analyses. Both high concentrations of

ethanol and freezing have been shown to be effective at preserving DNA for diet studies in marine animals (Passmore et al. 2006).

A sub-sample (n=20) of the *E. antarctica* individuals were analyzed here with molecular approaches, and compared with existing microscopy data (Saunders et al. 2014). All fish analyzed were collected at night, in the upper 700 m, and were identified as female with the exception of one male from cruise JR161. Half of the fish (n=10) analysed were from net hauls with mean SST (over the trawl duration) below 0°C ("Antarctic" group) and the remaining half (n=10) from net hauls with mean SST between 3-5°C ("Sub-Antarctic" group) (Table 1, S1).

2.2. DNA extraction, PCR amplification and high-throughput sequencing of 18S ribosomal DNA

Excess ethanol was removed from each sample by centrifuging and manual pipetting to limit carry-over into the DNA extraction process. The entire stomach contents of each fish was analysed, with no attempt to pick out intact prey items. TNES lysis buffer (2 mL) was added to each sample's stomach contents, which was subsequently homogenised using the TissueRuptor II (Qiagen GmbH, Hilden, Germany). DNA was extracted from 200 µL of homogenate using the DNeasy Blood and Tissue kit, as per manufacturer's directions (Qiagen GmbH, Hilden, Germany).

Amplification of the V7-V9 region of the 18S ribosomal DNA gene was performed for DNA extracts from each sample in 50 µL polymerase chain reactions (PCRs). 18S is the preferred marker gene for this type of study because it contains highly conserved primer sites, which allows for primers to amplify the DNA of nearly all eukaryotes, at the same time it targets relatively short highly variable regions between these conserved sites, which minimize the impacts of digestion, and there are extensive, curated reference databases available (Cleary et al. 2012, Deagle et al. 2014, Berry et al. 2015). Each PCR reaction contained 25 µL of GoTaq Green Master Mix (Promega, Madison, WI, United States), 2.5 µL each of sample-sequence identification tagged forward and reverse primers (Gast et al. 2004), 10 µL of nuclease free water and 10 µL of extracted DNA template at a concentration of 10 ng μl^{-1}. Thermocycling consisted of an initial polymerase activation and DNA denaturation at 94°C for 30 seconds, followed by 35 cycles of 94°C for 30 s, 58°C for 45s, 72°C for 30s with

a final extension at 72°C for 10 minutes. Resulting PCR products were purified using paramagnetic beads as per Rohland and Reich (2012) and quantified using the Qubit 4 Fluorometer (Waltham, MA, United States).

Amplicons from all samples were pooled together in equimolar ratios prior to sequencing. Sequencing was performed on Illumina MiSeq with Reagent Nano Kit v2 (San Diego, CA, United States) and 250 base pair paired-end reads at the Biochemistry DNA Sequencing Facility (University of Cambridge Department of Biochemistry, Cambridge, United Kingdom).

2.3. Bioinformatic analysis

Bioinformatic analyses were conducted in Qiime2 version 2022.2.0 (Bolyen et al. 2019). Sequences were separated into samples using the in-line barcodes and trimmed to remove primers using cutadapt version 2022.2.0 (Martin 2011). Any reads which did not contain the expected forward primer sequence in the expected position were discarded. Reads were de-noised, forward and reverse reads were merged, and chimeras were removed using the dada2 version 2022.2.0 implementation in Qiime2 (Callahan et al. 2016). The main output of this quality filtering was the assignment of reads to "Amplicon Sequence Variants" (ASVs), each of which represents a unique sequence inferred from the denoising algorithm. For details of the specific parameters used at each step of the Qiime2 pipeline, see the data processing script in supplementary information.

Taxonomy was assigned to each ASV, using a pre-trained machine learning classifier compiled from the Silva-138 99% identity database, implemented in the "scikit-learn" Python library (Bokulich et al. 2018). The lowest taxonomic level to which each ASV could be classified with high confidence was also obtained from manual BLASTN searches against the NCBI "nt" database for all ASVs with a total read abundance above fifty. Based on these two sources of taxonomic information, each ASV was classified as: "Myctophid" (sequences identified as *E. antarctica*, or classified only to a higher taxonomic level which contains *E. antarctica*, such as "myctophidae" or "teleost"), "other fish" (sequences identified with high confidence to fish other than *E. antarctica*), "potential parasite or symbiont" (any organism known to form parasitic or symbiotic relationships, respectively with myctophids or known

myctophid prey items), "prey" (any organism not in the previous categories, known to be present in marine habitats and of a size consumable by myctophids) and "terrestrial contaminant" (any organism resident exclusively on land). ASVs represented by less than 50 reads were excluded from further analysis, given that highly abundant sequences are more likely to represent ecologically-meaningful observations, and the lowest abundance ASVs are most likely to contain sequencing errors or to reflect trace contaminants (Tercel & Cuff 2022).

2.4. Comparative analyses

Multivariate data analyses were applied to both microscopic and molecular data sets in parallel, to compare the diet assemblages between methods and to assess the impacts of method choice on ecological interpretations. We derived metrics of relative read abundance and relative prey abundance. Specifically, relative read abundance was calculated as the number of sequence reads attributed to a given broad taxonomic grouping (e.g. algae) per sample divided by the total number of sequence reads per sample. Similarly, relative prey abundance for the microscopy observations was calculated as the count of prey items microscopically assigned to a given broad taxonomic grouping per myctophid stomach sample divided by the total count of prey items per sample. This approach has been shown to provide the most accurate representations of true diet and is less prone to overweighting the importance of rare diet items or trace contaminants then presence/absence approaches (Deagle et al. 2019).

Bray-Curtis dissimilarities between each fish were calculated as the basis of a dendrogram using the linkage and dendrogram functions in MatLab R2022b (Bray & Curtis 1957). Bray-Curtis dissimilarities were calculated in parallel in R and used to produce Non-metric Multi-dimensional Scaling (NMDS) plots and conduct ANOSIM testing using the "vegan" package (Oksanen et al. 2020). NMDS visualizations summarise intraspecific sample variability in relation to Sea Surface Temperature (SST), Depth Zone, Myctophid size and Cruise Year. We reported the ANOSIM R statistic and a significance value of 0.05 to test if there is a statistically significant difference in molecularly-identified diet composition between Sub-Antarctic and Antarctic groups. All summary and inferential statistical analyses were conducted in R version 4.0.4 (R Core Team, 2021). Figures were produced using the "ggplot" R package (Wickham, 2016).

3. Results

3.1. Microscopic analysis

The diet identified microscopically could be grouped into four broad taxonomic groups:Amphipoda, Calanoida, Euphausiacea and Pteropoda. Additionally, one stomach contained only unidentified crustacea (Fig. 2). This is consistent with broader patterns of *E. antarctica* diet composition recovered from microscopic analysis by Saunders et al. (2014) and Shreeve et al. (2009). Total prey count of each stomach varied between 1-21 items (mean = 5.7 ±4.9) and total prey mass varied between 0.001-0.233g (mean = 0.067 ±0.066). Summary of prey count and prey mass per sample station is given in Table 1.

3.2. Molecular analysis

Two stomachs failed to generate sufficient quality DNA amplicons for sequencing, giving a final sample size of eighteen individuals for the DNA metabarcoding analysis. Following de-noising and filtering, the final metabarcoding data set included 934,177 sequence reads, which were assigned to a total of 301 unique ASVs. The number of reads per sample ranged from 10,103 to 96,623 (mean reads per sample = 51,898). From the overall reads, 3,366 were removed due to their assignment to low abundance ASVs (222 or 74% of ASVs had < 50 total reads across all samples). Further to this, we also removed 602,513 sequences representing i. myctophid material (584,132 reads attributed to myctophid-assigned ASVs), ii. potential terrestrial contaminants (319 reads attributed to land plant ASVs), iii. parasitic or symbiotic organisms (761 reads attributed to fungal ASVs, 17,141 reads assigned to parasitic ciliate ASVs) and iv. unidentified opisthokont and placental organisms (61 reads attributed to opisthokont ASVs and 99 reads attributed to placental ASVs) leaving 46 unique ASVs across the 10 taxonomic categories listed in Table 2 for further analyses. The most numerous groups were crustaceans, gastropods, and non-myctophid fish (Fig. S1, Table S2), within which several ASVs could be identified to genus level including the genera *Euphausia* and *Thysanoessa* (class Malacostraca), *Calanus* and *Candacia* (class Copepoda), and *Limacina* (class Gastropoda). Grouping these ASVs in a similar way to the microscopy results, DNA metabarcoding identified 19 broad taxonomic

groups across all stomach samples which included the four groups identified via microscopy (Fig. 2).

3.3. *Comparative analyses*

Microscopic analysis found that stomachs contained, on average, 2 prey taxa (max = 3). These same taxa often dominated read abundance from the DNA sequencing analysis (Fig. 2) but the diversity of taxa greatly increased (mean taxa = 6, max = 9). The taxonomic groups present in the metabarcoding but absent from the microscopy analyses mostly consisted of soft-bodied species such as rotifers and cnidaria (Fig. 2).

When assessing variability in diet composition as ascertained with molecular tools across the two environments (Antarctic <0°C vs. Sub-Antarctic 3-5°C SST), we found a greater proportion of euphausiids and pteropods within the diet of samples from Antarctic temperatures than from Sub-Antarctic temperatures (Fig. 3; mean pteropod relative read abundance Antarctic group = 35.1 ± 30.1%, Sub-Antarctic group = 0.81 ± 1.1%; mean euphausiid relative read abundance Antarctic group = 31.1 ± 35.7%, Sub-Antarctic group = 23.7 ± 35.8%). Eight of the ten individuals collected from the Antarctic environment contained at least 15% pteropod sequences, while none of those from the Sub-Antarctic environment contained more than 5%. There was also a greater abundance of amphipods in the diet of individuals collected from the Sub-Antarctic environment than those collected further south (Fig. 3). These dietary differences were sufficient to distinguish individuals sampled from Antarctic and Sub-Antarctic waters using molecular data (Fig. 4). Although we note a similar change in the dominant prey taxa recovered by microscopy methods (Fig. 2), the same metric based on microscopy results did not successfully separate all individuals by temperature (Fig. 4).

Moreover, NMDS plots of the metabarcoding results show distinct clustering of diet composition between temperature groups (Fig. 5, ANOSIM R = 0.52, ANOSIM significance value<0.001) and with research cruise/year (ANOSIM R = 0.64, ANOSIM significance value<0.001). We find greater overlap in diet composition across depth zones (0-200 m and 400-700 m; ANOSIM R = 0.13, ANOSIM significance value = 0.06) and body size (<70 mm and ≥70 mm; ANOSIM R = 0.1732, ANOSIM significance value = 0.02).

4. Discussion

DNA metabarcode sequencing yielded an overall similar picture of myctophid diets to that obtained via microscopic observations, but resolved additional detail. Frequently observed prey groups from both methods included copepods, amphipods, euphausiids, and pteropods. This is in broad agreement to our existing understanding of the predominant prey of *E. antarctica* using microscopy (Pakhomov et al. 1996, Shreeve et al. 2009, Saunders et al., 2014, Riaz et al. 2020) and fatty acids (Stowasser et al. 2009, Connan et al. 2020).

While the dominant prey groups were similar across methods, a greater diversity of prey species was recovered with molecular data. This was partly due to higher taxonomic resolution of prey, a common result when using metabarcoding methods to infer diet of marine species (Berry et al. 2015, Jakubavičiūtė et al. 2017,). The higher resolving power of molecular data was particularly striking for specimen 161-106-2_2; microscopic results classified the stomach contents as "unidentified crustacean", while molecular data resolved this material into seven different taxonomic groups (Fig. 2). *Candacia* spp. copepods are one of the prey items which were able to be classified more finely by molecular approaches (as *Candacia* spp.), than they were by microscopy (as Calanoid copepods). *Candacia* spp. are relatively small copepods, smaller than the genera typically considered to be important prey for myctophids, making this an interesting addition to our understanding of the trophic roles of these fish (Saunders et al. 2019).

Greater prey diversity from molecular methods was also due to the detection of soft-bodied prey, which are often missed and are particularly poorly resolved by microscopy-based analyses, as we find in our samples. Salps and tunicates have been found via microscopic analysis in the diets of a handful of myctophid species from the sub-Antarctic (Pakhomov et al. 1996) and Scotia Sea (Shreeve et al. 2009), yet previous work has shown that molecular approaches are more successful in the detection of these prey types (Urban et al. 2022). Indeed, Clarke et al. (2020) recovered sequences of siphonophores and cnidarians from DNA metabarcoding of mesopelagic fish stomach contents in the Indian Ocean sector of the Southern Ocean, although these were relatively low abundance in their myctophid samples, and primarily observed amongst *Bathylagus* sp. Our molecular data detected sequences of Cnidaria, salps, ciliates, and rotifers, although none were major dietary components. The importance of gelatinous zooplankton in the diet of pelagic fishes is becoming increasingly recognised (Diaz Briz et al. 2017, Clarke et al. 2020, Novotny et al.

2022), and our findings provide further evidence of metabarcoding being an effective tool in this regard.

DNA metabarcode sequencing captures all organisms for which DNA for the marker gene analysed is present within the stomach contents. This raises the potential for confounding factors to influence results, such as the inadvertent detection of gut parasites, prey-of-prey, and parasites-of-prey. Relatively few of the sequences identified here fell into these groups – 17,902 reads (1.9% of the total reads) were excluded from analysis as potential parasites (of myctophids or prey). Single-celled organisms which would potentially be present as prey of prey (phytoplankton, ciliates, and cercozoan) were also relatively rare with only 2,306 reads from 19 ASVs (0.7 % of the total reads). This is similar to existing results with targeted analyses of prey in fish stomach contents, which have indicated prey of prey are rare in stomach contents sequencing data, typically making up less than 1% of the total reads (Clarke et al. 2020). Prey of prey thus appears not to be a major confounding factor in myctophid stomach contents sequencing for dietary analysis, but it is still worth viewing with caution unexpected prey items, particularly small protists. On the other hand, the trace amount of reads recovered from prey and parasites within consumed prey could potentially be informative of broader ecological interactions; myctophids serving as a form of biological environmental sampler. Network analyses with the data presented here yielded no interactions of interest, but such analyses with larger data sets in the future hold potential. Metabarcoding diet studies can in some cases be limited by the reference databases available covering relevant prey species. 18S rDNA has one of the most comprehensive databases available, and in the relatively low diversity Southern Ocean this was not a significant limitation as all abundant sequences were well classified, but future expansions of these databases may allow for even higher taxonomic resolution of prey items. Lastly, we here took the conservative approach of excluding all Myctophidae sequences, as they may have originated from the host's stomach tissue. Thus, cannibalism or predation on eggs and larvae of confamilials would have been missed.

Diet differences between individuals sampled in Antarctic and Sub-Antarctic regions were observed in both molecular and microscopy data, but these differences were better resolved using the molecular data (Figs 3 & 4). The presence of pteropods in the diet was a particular differentiating feature, making up a larger proportion of sequence reads for fish in Antarctic waters than in the Sub-Antarctic. Pteropods can be abundant in the Scotia Sea,

sometimes exceeding 35% of the total mesozooplankton abundance, with an estimated contribution to biomass of 32 mg C m^{-2} (Hunt et al. 2008, Bednaršek et al. 2012). Amphipods were more abundant in the Sub-Antarctic samples, although this difference was less striking than that of pteropods. In other Southern Ocean zooplanktivores, amphipods have been found to be particularly important prey in warmer temporal periods (Croxall et al. 1999). Previous analyses of *E. antarctica* diet across a north-south gradient in East Antarctica found no regional differences, which may reflect the weaker ecological gradients in the more bathymetrically homogenous eastern sector (Clarke et al. 2020). Similarly, a comprehensive network analysis of food webs in the East and West sectors of the Southern Ocean found that the euphausiid Antarctic krill were not the biggest contributors to energy flow pathways in the Indian and West Pacific sectors (McCormack et al. 2021). This may partly explain the higher abundance of euphausiids in the diet of *E. antarctica* specimens collected in Antarctic, southern waters surrounding the South Orkney archipelago (this study) and in the Western Antarctic Peninsula region (Pusch et al. 2004) but their relatively low abundance in samples further east (Pakhomov et al. 1996, Gasket et al. 2001).

From the molecular data presented here, significant differences in prey were also found between small and large fish and between the three cruises from which the data were collected. Prey composition is known to vary with myctophid size, both within and between species. This is likely due to a combination of gape size, filtering capacity of the gill rakers and the vertical distribution of the predators and target prey (Shreeve et al. 2009). Cruise timings varied both yearly and seasonally, complicating inferences on the temporal variation in myctophid diet. However, Saunders et al. (2014) also detected seasonal patterns in *E. antarctica* using microscopy methods with a large (>400) number of samples. Copepod prey dominated the diet of small fish and those collected from austral spring and summer (JR161 and JR171) while euphausiids were the dominant prey in larger fish and those collected in austral autumn (JR200). This may reflect seasonal changes in copepod ontogeny, as the older copepod stages become less abundant in surface waters in autumn and winter (Saunders et al. 2014). That molecular tools were able to detect variation in diet from the small subset analysed, combined with the higher taxonomic resolution achieved, gives promise that future applications will offer significant new insights into the trophodynamics of myctophids when applied to a greater number of samples and at finer spatial and temporal scales.

5. Conclusions

DNA metabarcode sequencing shows clear potential for future application to understanding the feeding ecology of myctophid fishes. Prey groups identified were broadly consistent with results from microscopy, but provided additional detail by identifying sub-groups within broader categories such as copepods, and capturing smaller and soft-bodied prey items. Future applications of this approach to larger sample sizes, particularly if collected across larger spatial and/or temporal scales, have the potential to greatly improve our understanding of the environmental drivers of variation in myctophid diets, and thus fill key gaps in our knowledge of the role myctophids play in Southern Ocean ecosystems.

6. Funding

This work was supported by a NERC GW4+ REP award (MV), and the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 887760 (ACC). Samples were provided as part of the ongoing National Capability programme CONSEC run by the Ecosystems team at British Antarctic Survey to examine Southern Ocean ecosystem structure and function. Myctophid sample collection was co-ordinated by MAC as part of this programme.

7. Data Availability Statement

Raw sequence data is available in the NCBI short read archive under BioProject ID PRJNA931893. Morphological gut contents data is available from https://data.bas.ac.uk/full-record.php?id=GB/NERC/BAS/PDC/01235.

8. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Author Contributions

MV: Conceptualization, laboratory analyses, bioinformatics, and writing, JJF: conceptualization, data compilation, supervision, writing and funding, MAC: data collection, conceptualization and supervision, ACC: conceptualization, laboratory analyses, supervision, writing, project administration and funding.

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11. Figures

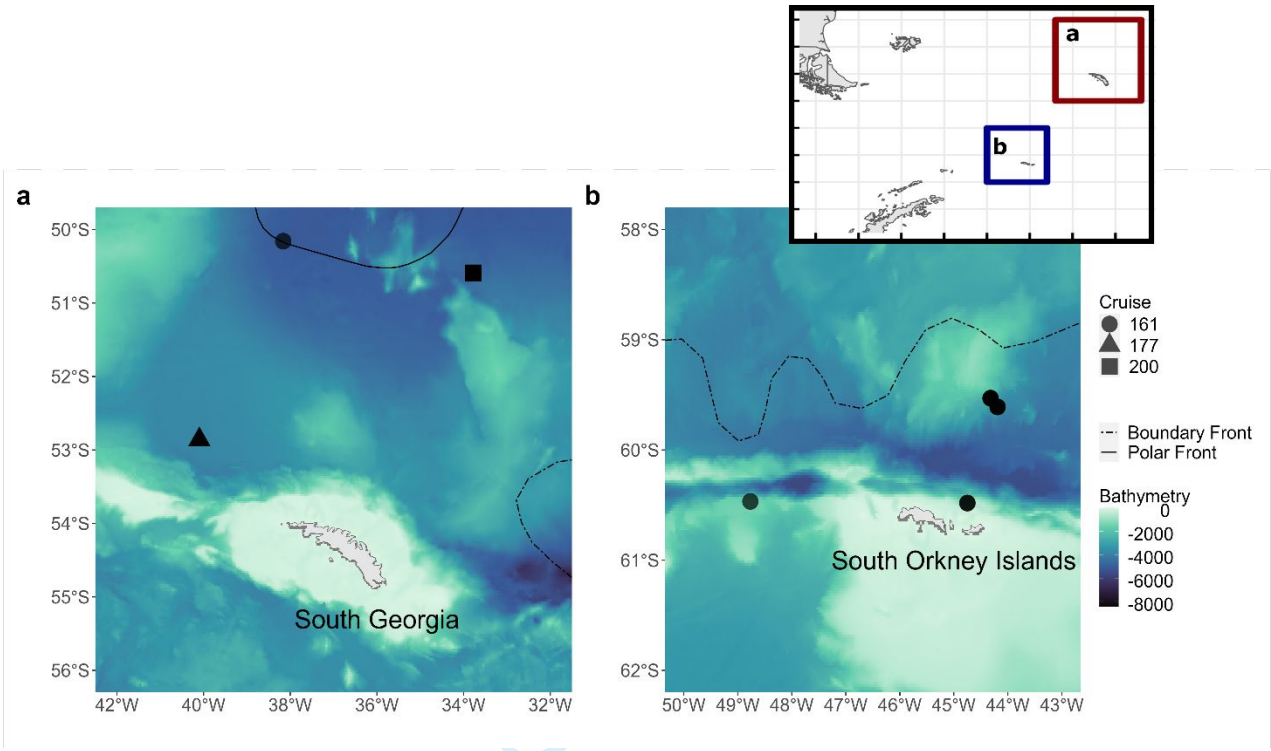

Figure 1: Map of sampling locations and bathymetry around South Georgia (a) and South Orkney Islands (b) with inset showing locations within the wider area of the Scotia Sea and South Atlantic. The main frontal features in the region (Polar Front and the Southern Boundary of the Antarctic Circumpolar Current) are indicated via solid and dashed lines taken from Orsi et al. (1995), bathymetry was sampled from GEBCO (2022), and country boundaries are indicated as per Massicotte and South (2023). Sampling locations are indicated via black markers, with each marker shape representing a different cruise. Note that multiple samples were taken at each location.

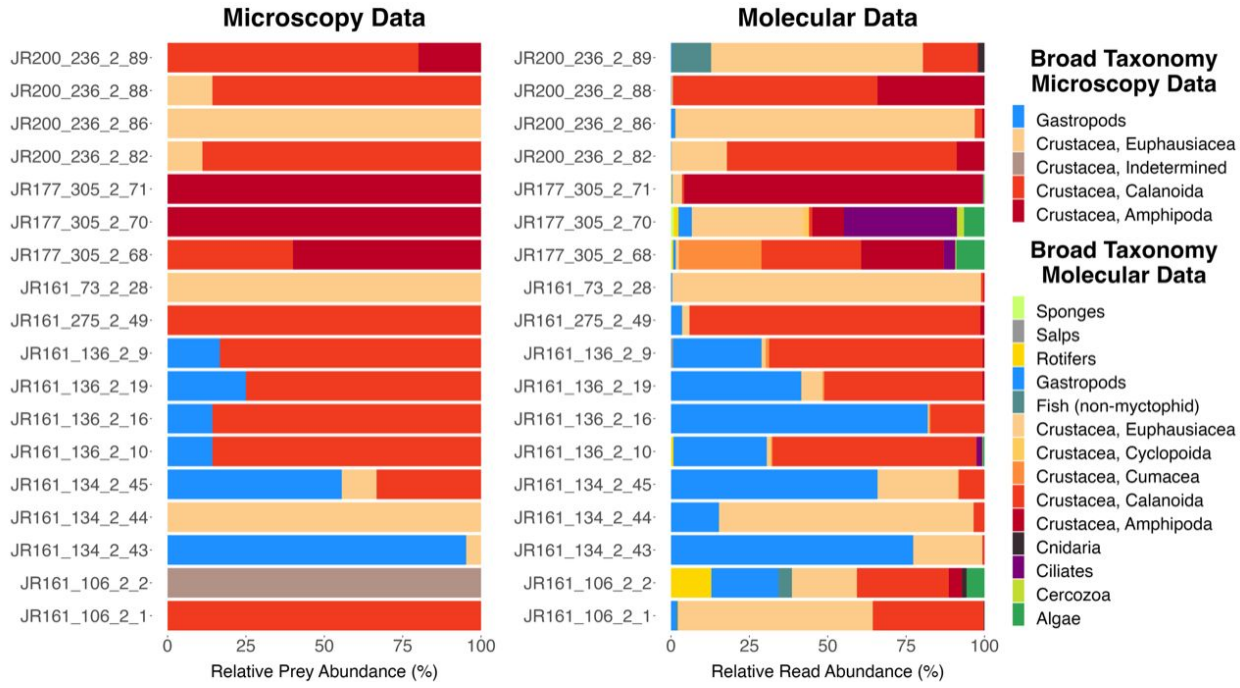

Figure 2: Diet composition of *E. antarctica* as determined by morphological and molecular approaches.

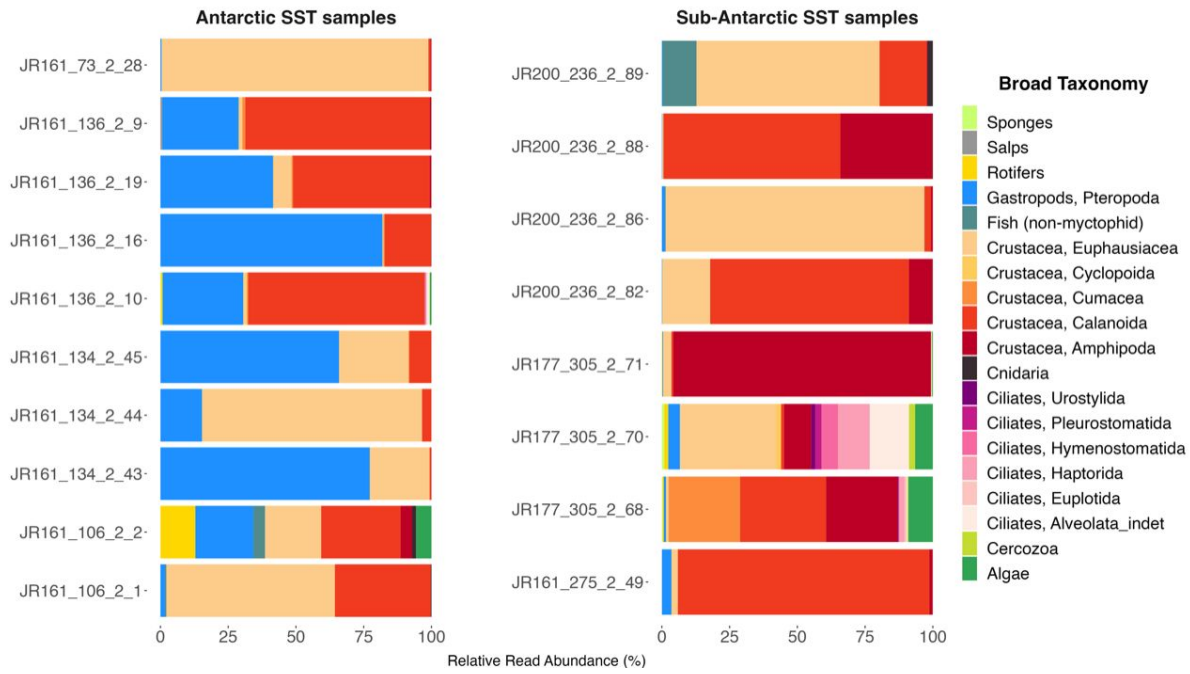

Figure 3: Comparison of diet composition between samples from Antarctic (≤ 0 °C) and Sub-Antarctic (3-5°C) sea surface temperature conditions. Results are based on those from molecular methods.

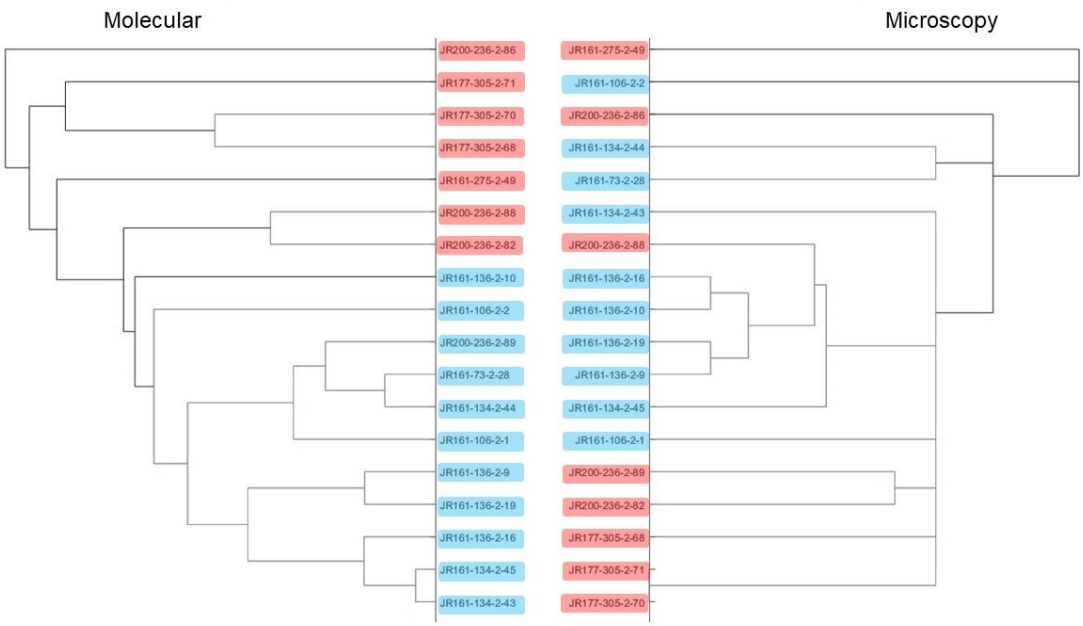

Figure 4: Dendrogram of stomach sample contents from molecular and microscopic methods based on clustering of Bray-Curtis dissimilarities. Red and blue label shading corresponds to the Sub-Antarctic and Antarctic temperature groupings respectively, illustrating the tighter clustering by environment observed in the molecular data.

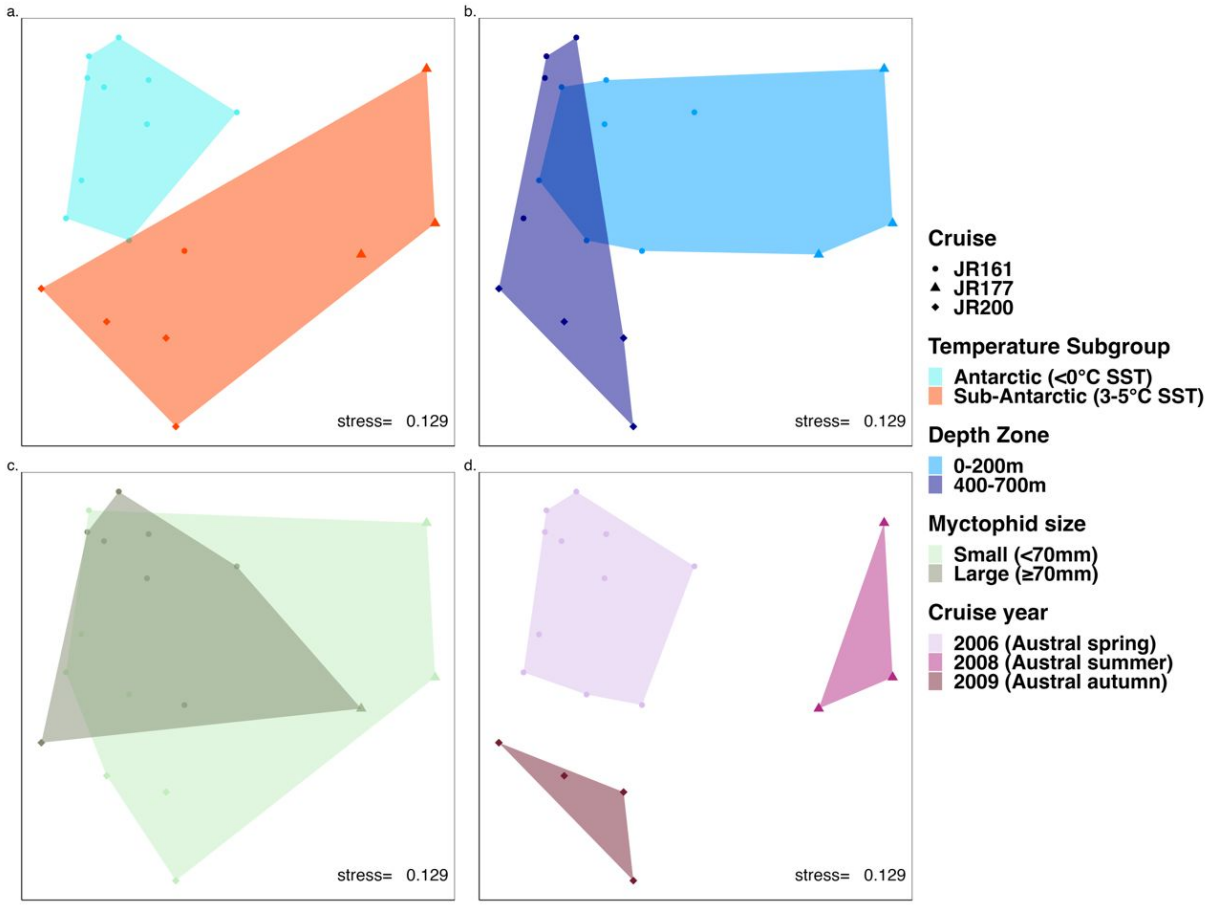

Figure 5: Non-metric multidimensional scaling (NMDS) ordination plot and stress values from a dissimilarity matrix of diet composition across all samples as identified by molecular methods. The NMDS x and y axes represent NMDS dimensions (NMDS1 and NMDS2, respectively). The NMDS coordinate labels represent each stomach sample and colours correspond to (a)temperature , (b)depth zone ,(c) body size of each sample and (d) timing of the research cruise .

12. Tables

Environment	Cruise	Date	Lat (°)	Lon (°)	Water depth (m)	Net Depth (m)	SST (°C)	N. samples	Mean Standard Length (mm)	Mean total prey item count [morphologically determined] (n)	Mean total prey item mass [morphologically determined] (g)
Sub-Ant.	JR200	11/04/2009	-50.58	-33.77	4756	551	3.69	4	83.25	5.5	0.10
Ant.	JR161	09/11/2006	-59.53	-44.31	2589	115	-0.76	4	65	7	0.02
Sub-Ant.	JR161	28/11/2006	-50.15	-38.16	5091	100	3.73	1	36	9	0.044
Ant.	JR161	06/11/2006	-60.48	-44.74	1024	100	-0.78	2	85	1	0.001
Ant.	JR161	01/11/2006	-60.46	-48.76	1631	565	-1.43	1	79	1	0.069
Ant.	JR161	09/11/2006	-59.60	-44.19	3498	550	-0.92	3	67	11.66	0.125
Sub-Ant.	JR177	03/02/2008	-52.85	-40.09	3794	107	3.26	3	71	2.33	0.072

Table 1: Environmental and sample information for each of the seven stations used in this analysis, including the number of *E. antarctica* samples at each station and metrics of mean sample length and prey content per station.

773

	Total ASVs	Total reads	Total samples present (>1 read)	Total samples present (>40 reads)	Dietary origin
Crustacea	18	227103	18	17	Prey
Gastropods	2	93609	18	15	Prey
Cnidaria	2	795	13	2	Prey
Rotifers	1	162	9	1	Prey
Salps	1	57	1	1	Prey
Sponges	1	57	6	0	Prey
Ciliates (free-living)	9	1195	10	3	Prey of prey
Algae	8	1003	9	2	Prey of prey
Cercozoa	2	108	5	1	Prey of prey
Fish (non-myctophid)	2	4209	17	1	Larval fish

774

775 Table 2: The number of reads and ASVs for each taxonomic group detected after filtering and
776 quality control steps. Likely dietary origin (whether prey or prey of prey) also included.

777