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Comparative visual and DNA-based diet assessment extends the prey spectrum of polar cod *Boreogadus saida*

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ABSTRACT: The Arctic marine ecosystem is changing fast due to climate change, emphasizing the need for solid ecological baselines and monitoring. The polar cod *Boreogadus saida* functions as a key species in the Arctic marine food web. We investigated the stomach contents of polar cod from the northern Barents Sea using DNA metabarcoding with the mitochondrial cytochrome *c* oxidase I gene in parallel with classical visual analysis. Arctic amphipods and krill dominated the diet in both methods. Yet, metabarcoding allowed for the identification of digested and unidentifiable prey and provided higher taxonomic resolution, revealing new and undiscovered prey items of polar cod in the area. Furthermore, molecular results suggest a higher importance of barnacles and fish (presumably eggs and pelagic larvae) in the diet than previously recorded. Parasites and, in 6 cases, other prey items were only visually identified, demonstrating the complementary nature of both approaches. The presence of temperate and boreal prey species such as northern krill and (early life stages of) European flounder and European plaice illustrates the advection of boreal taxa into the polar region or may be indicative of ongoing borealisation in the Barents Sea. We show that a combination of visual analysis and metabarcoding provides complementary and semi-quantitative dietary information and integrative insights to monitor changing marine food webs.

KEY WORDS: DNA metabarcoding · Borealisation · Arctic ecosystem · Arctic cod · Prey composition · Barents Sea · Stomach contents · Trophic ecology

1. INTRODUCTION

Environmental changes are putting an increasing pressure on the Arctic ecosystem and its endemic fauna (Chan et al. 2019). Changes in habitat characteristics such as increased seawater temperature and reduced sea ice coverage influence the distribution of marine organisms (Poloczanska et al. 2013). Consequently, the Arctic is predicted to have the largest species turnover, with numerous local extinctions and invasions (Cheung et al. 2009). In the Barents Sea, clear evidence of northward range expansions of marine species and associated ecosystem changes (called borealisation or Atlantification) is accumulating (Fossheim et al. 2015, Kortsch et al. 2015). The zooplankton community is shifting towards boreal taxa with a decrease in lipid-rich Arctic *Calanus glacialis* and an increase in smaller Atlantic *C. finmarchicus* (Aarflot et al. 2018, Dalpadado et al. 2020). Large predatory boreal fish species such as Atlantic cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* are expanding their distribution ranges

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northward, leading to a changing food web that increasingly resembles that of sub-Arctic ecosystems (Renaud et al. 2012, Fossheim et al. 2015, Frainer et al. 2017). The changes in prey composition and abundance and the increased competition with and predation by boreal species are expected to disrupt the trophic interactions among organisms in the Arctic marine ecosystem (Pecuchet et al. 2020). Improving our understanding of the Arctic marine food web and its response to potential changes in environmental conditions is necessary to foster ecosystem-based management of Arctic marine regions, for which detailed baseline information as well as spatio-temporal monitoring are key elements.

The polar cod *Boreogadus saida* (Lepechin, 1744; Gadidae), an abundant marine fish in both open and ice-covered waters of the Arctic Ocean (Benoit et al. 2008, David et al. 2016), plays a key role in the Arctic marine ecosystem. As a primary forage species, it channels nearly 75% of zooplankton production and sea ice algae-derived carbon to marine mammals and seabirds (Welch et al. 1992, Hop & Gjøsæter 2013, Kohlbach et al. 2017). Good knowledge of the polar cod diet is necessary to assess its ecological function and might be used to monitor changes in prey availability. Furthermore, the diet of key species might provide valuable information for investigating ecological aspects such as behaviour, energy intake and habitat use (Chipps & Garvey 2007, Braga et al. 2012). Polar cod is a largely opportunistic pelagic feeder (Ajiad & Gjøsæter 1990, Christiansen et al. 2012). Prey composition of similar-sized individuals varies among regions and along depth gradients, indicating regional differences in prey availability (Gray et al. 2016, Majewski et al. 2016). Because larger fish are capable of eating larger prey, the diet of polar cod becomes more complex with increasing body size (Gray et al. 2016). Besides variability depending on region, depth stratum and body size, polar cod diet varies interannually (Craig et al. 1982, Gray et al. 2016). Generally, previous findings suggest that the main food items of polar cod include 3 groups of crustaceans: amphipods, copepods and krill (Lønne & Gulliksen 1989, Ajiad & Gjøsæter 1990, Orlova et al. 2009, Kohlbach et al. 2017). It is unknown how recent changes in the Arctic zooplankton communities (Møller & Nielsen 2020) are affecting the polar cod diet.

Traditionally, the most commonly used method for fish diet analysis is visual identification of the stomach contents. Advantages of this approach are the quantification of prey items, the ability to determine the age of (some) prey items and the low cost of scientific equipment, whereas drawbacks are the requirement of expert taxonomic knowledge and high labour intensity (Graham et al. 2014, Jakubavičiūtė et al. 2017). In addition, digested prey items are often not accurately identified (Hyslop 1980). Without consideration of the digestion level, the stomach contents may not accurately reflect species composition, abundance and size of the prey consumed (Scholz et al. 1991, Buckland et al. 2017). Moreover, species-specific digestion rates may influence the perceived prey composition in the stomach (Sutela & Huusko 2000), and classifying remains as unidentifiable is highly subjective (Baker et al. 2014). Unidentifiable prey items have been reported in most polar cod diet studies (Ajiad & Gjøsæter 1990, Renaud et al. 2012, Cusa et al. 2019), up to nearly 22% of the total dry weight of polar cod stomach contents (Lønne & Gulliksen 1989). A promising method to overcome some of the limitations of visual prey identification is molecular identification, such as DNA metabarcoding. This method uses short genetic fragments and high-throughput sequencing for high-resolution taxonomic identification of prey items, including digested ones (Taberlet et al. 2012, Clarke et al. 2017). Disadvantages of metabarcoding in diet studies are its semi-quantitative nature where the number of ingested animals and biomass per stomach are unknown, the lack of knowledge about size or developmental stage of the prey, the potential to detect secondary prey and a limitation in taxonomic scope to known reference sequences (Amundsen & Sánchez-Hernández 2019). However, the frequency of occurrence (FOO) of prey items per sampling station or area can be calculated based on the presence/absence of prey taxa in metabarcoding data and thus provides a good measure to compare with results from visual analysis (Jakubavičiūtė et al. 2017, Bachiller et al. 2020). In addition, the total number of prey taxa and the taxonomic resolution of both methods are comparable with each other as well as with literature data. Such a comparative framework is expected to largely compensate for the inherent biases of both methods and hence provide a more accurate and comprehensive picture of the diet.

Here, we present an analysis of the summer diet of polar cod in the northern Barents Sea off the Svalbard Archipelago using visual stomach analysis and subsequent metabarcoding with stomach contents from the same specimen. We hypothesise that morphological and molecular diet analyses will reveal similar prey spectra in terms of richness and diversity, but we expect a higher taxonomic resolution (i.e. more species-level prey assignments) when using

Table 1. Sampling details of polar cod *Boreogadus saida* collected during expedition PS106/2 in 2017. The total number (n) of fish used for the visual (V) and molecular (M) method (including biological replicates and duplicates) and the subset of stomachs where both methods were used on the same specimen (comparison, C) are given per station. The number between brackets in the molecular method represents the actual number of samples used in the final dataset after quality filtering. Lat: latitude; Lon: longitude; DD: decimal degrees; TL: total length

Stn	Date	Time (h)	Lat (DD)	Lon (DD)	Bottom depth (m)	Mean TL (cm) (min.–max.)	n	n (V)	n (M)	n (C)
89-1	July 16	07:09	78.50	25.11	165	12.26 (8.60–14.60)	26	19	34 (20)	11
90-1	July 16	09:57	78.69	24.49	132	17.01 (15.40–18.20)	10	5	7 (5)	1
91-1	July 16	13:37	78.70	23.28	121	13.45 (10.50–15.90)	10	6	10 (9)	5
92-1	July 17	06:24	78.69	24.50	132	15.25 (13.40–19.90)	10	6	10 (9)	5
93-1	July 17	08:43	78.50	25.11	165	12.82 (10.50–14.10)	9	6	12 (7)	5

metabarcoding particularly due to the identification of digested soft-bodied animals. We compare the results from both methods with an extensive literature review of the polar cod diet in the Barents Sea. Lastly, we provide guidelines on how to optimally integrate visual and molecular diet analysis.

2. MATERIALS AND METHODS

2.1. Sampling

Polar cod were collected in July 2017 during expedition PS106/2 of the icebreaker RV 'Polarstern' in the shallow waters of the Barents Sea using a bottom trawl (Table 1) (Macke & Flores 2018). For this study, 65 fish were collected from the catch at 5 stations (Fig. 1) and preserved frozen at −20°C. Per station, a proportion of the total number of fish were used solely for DNA metabarcoding (n = 23), while 42 fish were used for visual prey item analysis, after which the stomach contents were preserved in 96% ethanol for subsequent molecular analysis (n = 34). We used this setup to test whether prior visual analysis has a detrimental effect on metabarcoding results. Samples were processed in a separate room to prevent within-laboratory contamination.

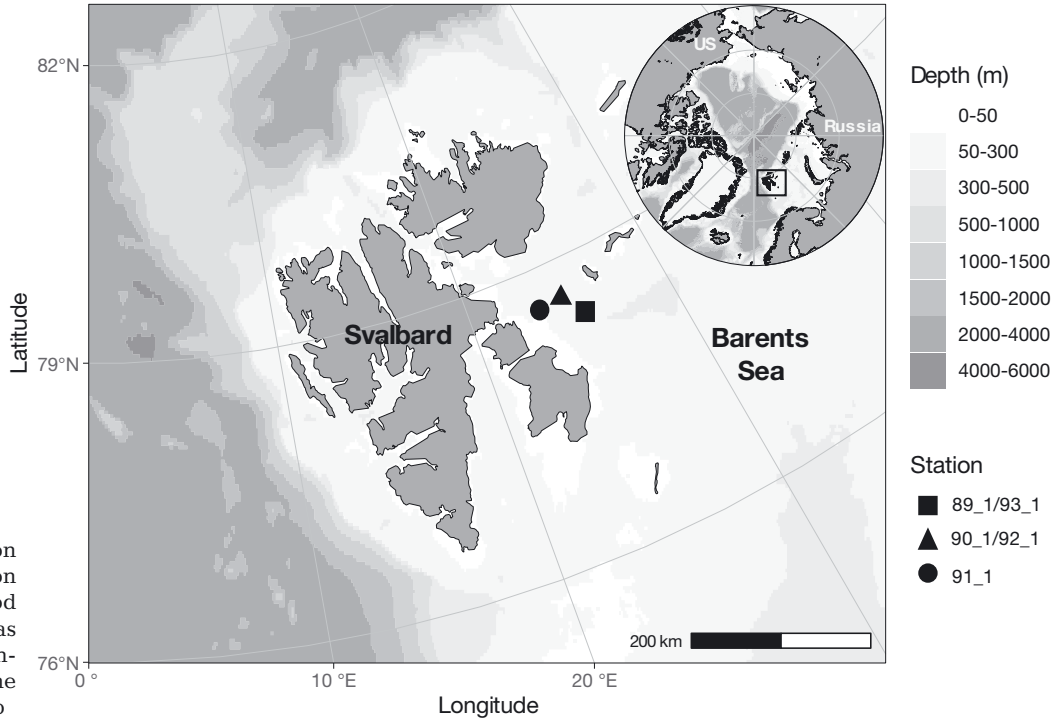

Fig. 1. Study region with sampling station codes where polar cod *Boreogadus saida* was collected in the northern Barents Sea off the Svalbard Archipelago

2.2. Visual identification

Stomachs were extracted from 42 thawed fish. The stomachs were cut open, and the contents were rinsed into a Bogorov counting chamber using 70% ethanol to delay DNA degradation. Digestion rate was estimated on a scale from 1 (hardly digested) to 4 (heavily digested) (Kock 1980). Recognizable food items in the stomach contents were identified to the lowest taxonomic level possible and enumerated using a Discovery V8 stereomicroscope (Zeiss). The biomass of prey items was reconstructed based on length–dry mass regression models to obtain an additional measure of the contribution of prey items to the diet (e.g. to avoid overemphasis of the importance of large numbers of small prey items) (Hyslop 1980). Because of the inability to identify prey items to species level and/or to directly measure prey items due to digestion, or the lack of available regression models for certain species or body parts, it was not always possible to obtain the most precise estimate of reconstructed biomass from the stomach contents directly. In this case, length measurement of prey species from the environment and/or regression models for the species or closest relative that were found in the literature were used to make sure that biomass estimates resemble the actual biomass as closely as possible and at least captured the relative differences in the contributions of different-sized prey.

Whenever possible, the size of the prey items or body parts was measured using an AxioCam HRc with AxioVision40 v.4.8.2.0 software (Zeiss). Krill were enumerated by counting the number of eyes and dividing the final count by 2. Complete bodies were added to this number. The reconstructed biomass (dry mass) was estimated by multiplying the number of individuals counted in a stomach by a dry mass estimate. The average dry mass of copepods was estimated using the regression model for *Calanus glacialis* from Ashjian et al. (2003), based on the average measured prosome length of specimens in the stomach contents, which was 3.0 mm (n = 24). The reconstructed biomass for amphipods was estimated by multiplying the number of gammarid amphipods with a dry weight estimate and adding the number of hyperiid amphipods multiplied with a dry weight estimate. In 1 stomach, we were able to determine the length of the only gammarid amphipod in the stomach by direct measurement. The average dry mass of gammarid amphipods (0.42 mg) was based on a regression model with length and dry mass measurements performed on individuals of *Apherusa glacialis* caught during the same expedition (PS106/2). The average dry mass of hyperiid amphipods and krill was based on average dry weight measurements of *Themisto libellula* and *Thysanoessa inermis* measured on individuals caught during expedition PS106/2 (n = 21 and n = 15, respectively; Schaafsma et al. 2022). The only reference to decapod size in polar cod stomachs was given in Ajiad & Gjøsæter (1990), with individuals ranging from 25 to 60 mm. The median value of 47.5 mm was used to calculate the dry weight based on a length–weight regression from Robinson et al. (2010) and a dry weight of 25% wet weight (Ricciardi & Bourget 1998). The index of relative importance (IRI) per prey item was calculated as:

$$\mathrm{IRI}_i = (A_i + B_i) \times F_i$$

where A is the abundance of prey item i in percentages, B is the biomass of prey item i in percentages and F is the FOO of prey item i in percentages, calculated as the number of fish stomachs in which the prey item was present divided by the total number of fish stomachs with food analysed, times 100 (Pinkas et al. 1971).

2.3. DNA metabarcoding

DNA was extracted from 34 stomach contents after visual inspection and 23 additional intact stomachs using the NucleoSpin® Tissue kit following the standard protocol for human or animal tissue and cultured cell purification according to the manufacturer's instructions (Macherey-Nagel). Small-volume stomach contents were used entirely for DNA extraction. Larger-volume stomachs were first homogenised using a mortar and pestle, and a subsample was taken before the DNA extraction. In 18 large-volume stomachs, additional DNA extractions were performed on replicate subsamples (from 16 stomachs) and triplicate subsamples (from 2 stomachs) to assess the effect of subsampling. The PCR mix for cytochrome *c* oxidase subunit I (COI) amplification contained 1 µl template DNA, 12.5 µl MyTaq® HS 2× Mix (Bioline), 10.5 µl nuclease-free H_2O (Sigma-Aldrich) and 1 µl primer mix (20 µM of each primer) using the following primers: mlCOIintF (5′-GGW ACW GGW TGA ACW GTW TAY CCY CC-3′) and jgHCO2198 (5′-TAI ACY TCI GGR TGI CCR AAR AAY CA-3′) (Leray et al. 2013). Touchdown PCR conditions consisted of 10 s initial denaturation at 95°C, 30 s of annealing at 62°C and 60 s elongation at 72°C for 16 cycles, with annealing temperature dropping every cycle by 1°C, followed by 25 cycles with

annealing temperature at 46°C. Two negative controls were included in all PCR runs to detect cross-contamination. PCR amplicons were cleaned using CleanPCR beads (CleanNA, GC Biotech) following the manufacturer's instructions with a bead:template ratio of 0.8:1. The PCR mix for the indexing PCR contained 9 µl template DNA, 10 µl MyTaq® HS 2× Mix and 0.5 µl forward and 0.5 µl reverse indexing primer. PCR conditions consisted of an initial denaturation of 1 min at 95°C; followed by 15 cycles of denaturation for 15 s at 95°C, 15 s of annealing at 51°C and 10 s of extension at 72°C; finishing with a final extension of 5 min at 72°C. The PCR amplicons were cleaned using the CleanPCR beads and then quantified using the Quant-iT Picogreen® kit (Thermo Fisher). PCR products with at least 20 ng DNA were pooled and paired-end sequenced with a MiSeq 3v 600-cycle kit on a MiSeq Sequencing System (Illumina) at the Genomics Core of the KU Leuven (Belgium).

2.4. Filtering and taxonomy assignment

Raw reads were demultiplexed using the bcl2fastq v.2.16 tool integrated in the Illumina platform. The data were processed in R v.3.5.3 (R Core Team 2019) using R package DADA2 v.1.16 (Callahan et al. 2016), which creates amplicon sequence variants (ASVs), a higher-resolution alternative to operational taxonomic units (OTUs). ASVs resolve differences in sequence variants up to 1 single nucleotide. Raw forward and reverse reads were filtered and trimmed with the filterAndTrim function using the following parameters: maxEE = 1 (maximum number of expected errors allowed in a read), maxN = 0 (removes reads with ambiguous nucleotides), trimLeft = 30 (removes first 30 bp of each read) and truncLen = 290250. Filtered and trimmed reads were merged using the mergePairs function. Chimeric sequences were removed with the removeBimeraDenovo function. The following criteria were used for taxonomic assignment in the Barcode of Life Data System (BOLD; Ratnasingham & Hebert 2007) v.4: (1) a taxon was assigned if the barcode matched a single locally occurring taxon in the databases with ≥97% sequence similarity level; and (2) if the barcode matched more than 1 taxon with ≥97% sequence similarity level, a taxon was assigned at the genus level. We excluded species that were identified as highly likely within-laboratory contaminations (i.e. study species from the home laboratory with Southern Hemisphere and North Sea distribution).

2.5. Data analysis

Taxa seen at least twice in at least 1% of the sequenced samples were retained. Samples with less than 20 reads in total were discarded from the final dataset following recommendations by McMurdie & Holmes (2013). Sequence reads were analysed based on FOO and relative read abundance (RRA). FOO was estimated as the percentage of stomachs in which a taxon was present. To simplify interpretation of the data, food items were classified into 10 taxonomic groups to compare both methods based on the presence of food items or taxa. To investigate whether metabarcoding results are affected by prior microscopic visual analysis of the stomach contents or not, we performed the Wilcoxon rank-sum test (Mann-Whitney) to test if the species richness (observed numbers of ASVs), Shannon-Wiener index and Simpson and Chao1 diversity indices (Spellerberg & Fedor 2003, Gotelli & Colwell 2011) differed between samples used with or without prior visual handling using the R package phyloseq (McMurdie & Holmes 2013). The Shannon-Wiener index combines species richness and their relative abundances and helps to compare diversity between communities. The Simpson diversity index indicates species dominance by considering the number of species and their relative abundances. The Chao1 diversity index represents the number of observed ASVs and an estimate of the number of unobserved ASVs. To test whether there was an effect of DNA degradation during visual identification on the DNA samples, we tested if the observed diversity indices were significantly different between samples with or without prior identification using the Wilcoxon rank-sum test and Bonferroni correction.

3. RESULTS

3.1. Diet composition using microscopic analysis of stomach contents

A total of 16 taxa (1 class, 2 subclasses, 4 orders, 3 families, 3 genera and 3 species) from 3 different phyla were identified based on morphological traits. The stomach contents were numerically dominated by euphausiids (krill) in 48% of the stomachs investigated and occasionally by copepods (9.5%) and amphipods (4.8%). Reconstruction of the mass of the food items (excluding parasites) showed that krill also dominated the stomach contents in terms of

Table 2. (a) Summary of stomach contents of polar cod *Boreogadus saida* based on visual identification per station. n: total number of individuals; TL: total length; SCW: stomach content weight; RFI: recognizable food items in the stomach. (b) Stomach content information per station and for the total number of investigated fish stomachs. Data include details on morphological measurements for krill, amphipods, decapods, copepods and parasites (limited to the mean no. of RFI); mean estimated reconstructed biomass; frequency of occurrence expressed as per cent (%FOO); and index of relative importance (IRI). For station codes, see Table 1. NA: not available

(a)

Stn	n	TL (mean ± SD; cm)	TL range (min.–max.; cm)	Weight (mean ± SD; g)	SCW (mean ± SD; g)	Mean degree of digestion	No. of RFI (mean ± SD)
89-1	19	12.35 ± 1.68	8.60–14.60	13.42 ± 5.39	0.14 ± 0.18	3.81	0.79 ± 1.36
90-1	5	16.70 ± 1.13	15.40–18.20	29.96 ± 4.32	1.92 ± 1.08	2.50	91.10 ± 173.4
91-1	6	13.80 ± 2.15	10.50–15.90	18.39 ± 7.04	0.39 ± 0.32	2.83	11.67 ± 9.93
92-1	6	15.73 ± 2.18	14.00–19.90	NA	2.36 ± 1.36	2.83	13.33 ± 4.41
93-1	6	12.87 ± 1.47	10.50–14.10	14.96 ± 4.89	0.18 ± 0.13	3.00	2.33 ± 1.21
Total	42	13.27 ± 2.18	8.60–18.20	16.73 ± 7.91	0.64 ± 0.99	3.23	15.11 ± 61.43

(b)

Stn	Krill	Amphipods	Decapods	Copepods	Parasites
Mean no. of RFI (n ind.$^{-1}$)					
89-1	0.63 ± 1.12	0.11 ± 0.32	0	0.05 ± 0.23	0.79 ± 1.23
90-1	9.60 ± 8.29	0.60 ± 0.89	0.90 ± 1.34	80.00 ± 178.89	1.60 ± 2.07
91-1	2.68 ± 2.80	1.83 ± 3.13	0	7.17 ± 7.25	0.67 ± 0.82
92-1	10.00 ± 2.3	3.33 ± 3.93	0	0	0.50 ± 1.22
93-1	1.83 ± 1.33	0.50 ± 0.55	0	0	0.67 ± 1.63
Total	3.50 ± 4.92	0.93 ± 2.13	0.11 ± 0.51	10.57 ± 61.66	0.81 ± 1.33
Mean estimated reconstructed biomass (mg ind.$^{-1}$)					
89-1	15.71 ± 27.76	2.28 ± 6.84	0	0.02 ± 0.10	NA
90-1	238.80 ± 206.32	13.01 ± 19.40	347.91 ± 518.63	34.04 ± 76.11	NA
91-1	66.33 ± 69.77	36.22 ± 59.43	0	3.05 ± 3.08	NA
92-1	248.75 ± 58.86	65.21 ± 68.91	0	0	NA
93-1	45.60 ± 33.06	10.85 ± 11.88	0	0	NA
Total	87.06 ± 122.26	18.62 ± 39.79	41.42 ± 198.10	4.49 ± 26.24	NA
%FOO					
89-1	35.30	11.80	0	5.90	52.90
90-1	100.00	40.00	40.00	20.00	60.00
91-1	66.70	50.00	0	83.30	50.00
92-1	100.00	83.30	0	0	16.70
93-1	83.30	50.00	0	0	16.70
Total	65.00	37.50	5.00	17.50	45.00
IRI					
89-1	5901.27	305.96	0	39.95	NA
90-1	5483.25	122.90	2235.36	1882.59	NA
91-1	5711.39	2500.65	0	5359.68	NA
92-1	15 422.96	3814.20	0	0	NA
93-1	13 279.94	2032.04	0	0	NA
Total	5238.78	691.12	140.15	1276.51	NA

biomass, except for at Stn 90-1 (Table 2). Two individuals at this station contained large decapod remains, which impacted the reconstructed weight distribution. Most krill parts were unidentifiable, but those that could be classified belonged to *Thysanoessa inermis* or *T. longicaudata* (1 stomach). Most amphipods were hyperiids (24% of the stomachs), which in 2 stomachs were identified to the genus *Themisto* and in 1 stomach to the species *Themisto libellula*. In 2 stomachs, amphipods were identified as gammarids, of which 1 belonged to the family Oedicerotidae. Most fish stomachs from Stn 91-1, and 1 fish stomach from Stn 90-1, contained relatively high numbers of copepods, although their contribution to the total biomass was relatively small. These copepods mainly consisted of *Calanus* spp. The importance of prey items in the diet of polar cod varied per station according to the IRI (Table 2). Two

stomachs from Stn 89-1 were empty. Differences in the number of recognizable food items were found between stations as well as in the degree of digestion and mean stomach content weight (Table 2; Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m698p139_supp.pdf). Four individuals from Stn 89-1 and 1 from Stn 93-1 contained pieces of what looked like fish skin tissue (Fig. S2). Trematode parasites occurred regularly in stomachs from all stations (Table 2). One nematode parasite was observed in a fish caught at Stn 93-1.

3.2. DNA metabarcoding of stomach contents

Illumina MiSeq sequencing provided 2 011 892 paired-end reads and 709 ASVs of the COI gene. Of those, 48 868 and 375 951 reads were discarded because of possible within-laboratory contamination and no database matches, respectively. Additionally, *Boreogadus saida* reads were excluded from the full dataset (1 169 492 reads), as this is likely host-specific DNA. Although cannibalism is possible (Bain & Sekerak 1978), we were not able to distinguish between polar cod DNA as prey or as host. *Limanda* sp. and *Merlangius* sp. ASVs were detected in 1 negative control and subsequently removed from the entire dataset (29 394 reads). One sample was removed due to high levels of indisputable within-laboratory contamination (>75% reads). Seven samples were removed due to the low number of total reads (<20 reads).

3.3. Remarks on molecular taxonomic assignment

Misidentified, mislabelled or otherwise erroneous sequences or specimen details are present in BOLD (Christiansen et al. 2018), which may prevent correct taxonomic identification even if the sequence is present in the data. Here, we used tree-based classification and common sense to improve taxonomic identification in cases where BOLD did not provide unambiguous identification likely due to doubtful database entries. *T. inermis* barcodes, even with a 100% identity match, were not distinguishable from a single barcoded specimen (AB-2009) classified as *Thysanoessa* sp. in BOLD. We suggest reclassifying the unidentified *Thysanoessa* specimen as *T. inermis*. Barcodes belonging to the genus *Pleuronectes* had 4 options for the species-level identification (*Pleuronectes platessa*, *Pleuronectes* sp. SH-2018, *Pleuronectes* sp. and *Platichthys flesus*). Tree-based classification assigned all barcodes to the species level of *P. platessa*. Individuals belonging to the genus *Balanus* could not be classified at the species level. Two *Liparis* species (*L. liparis* and *L. bathyarcticus*) were assigned to the species level after tree-based identification in BOLD. Two species were classified in BOLD by their formerly accepted names: the temperate amphipod *Deflexilodes tenuirostratus* (first classified as *Monoculodes tenuirostratus*) and the chaetognath *Parasagitta elegans* (first assigned as *Sagitta elegans*).

3.4. Molecular replication of subsamples

One subsample represented the entire stomach contents in most samples. In larger-volume stomachs, biological replicates (15 sets of 2 subsamples) and triplicates (2 sets of 3 subsamples) were taken to test the representativeness of subsamples. The taxon composition between subsamples was compared after applying 2 filtering criteria: filter taxa recorded twice in at least (1) 1% of the samples and (2) 5% of the samples. In both datasets, the taxon composition between subsamples was identical for 3 of 17 replicate sets (Table S1). When retaining taxa seen twice in at least 1% of the samples, 5 of 14 sets had 1 or 2 missing taxa. When retaining taxa seen twice in at least 5% of the samples, 4 of 14 sets had 1 or 2 missing taxa. Missing taxa often contained less than 20 reads. When discarding amplicon sequence variants with less than 20 reads, 6 sets had identical taxonomic composition, and an additional 8 sets had 1 or 2 taxonomic mismatches for the 1 and 5% datasets. Incomplete taxon composition between subsamples likely resulted from insufficient homogenisation of the stomach contents. Because most missing taxa were represented by a low number of reads and to avoid introducing bias in large-volume stomachs without biological replicates, only the first replicate and second triplicate were arbitrarily kept for subsequent dietary analyses. A total of 50 samples remained for subsequent analyses.

3.5. Diet composition using DNA metabarcoding of stomach contents

After retaining taxa observed twice in at least 1% of the samples, the final dataset contained 38 species belonging to 7 phyla, 10 classes, 15 orders, 27 families and 35 genera, identified by 216 unique ASVs of

Table 3. (a) Summary of the stomach contents of polar cod *Boreogadus saida* based on metabarcoding per station. n: total number of individuals; TL: total length. (b) Stomach contents per station and for the total number of investigated fish stomachs. %RRA: relative read abundance expressed as per cent; %FOO: frequency of occurrence expressed as per cent. For station codes, see Table 1

(a)

Stn	n	TL (mean ± SD; cm)	TL range (min.–max.; cm)	Weight (mean ± SD; g)
89-1	21	12.36 ± 1.80	9.00–14.60	13.44 ± 5.44
90-1	5	17.38 ± 0.34	16.80–17.70	34.70 ± 6.01
91-1	9	13.78 ± 1.59	11.60–15.90	18.05 ± 5.53
92-1	8	15.41 ± 2.13	13.40–19.90	24.30 ± 5.81
93-1	7	12.79 ± 1.34	10.50–14.10	15.27 ± 4.45
Total	50	13.69 ± 2.32	9.00–19.90	18.39 ± 8.49

(b)

Stn	Krill	Amphi-pods	Deca-pods	Cope-pods	Fish	Barna-cles	Chaeto gnaths	Ptero-pods	Poly-chaetes	Iso-pods	Dendro-chirotida	Hydro-zoans
%RRA												
89-1	8.4	38.1	8.5	9.6	26.9	7.6	0.6	0.03	0.2	0	<0.01	<0.01
90-1	34.8	22.2	3.4	4.1	6.3	28.0	0.5	0.5	0.2	0	0	0
91-1	22.2	53.0	11.1	9.9	3.2	0.5	0	0	0	0.01	0	0
92-1	26.5	22.9	9.0	22.3	6.6	11.9	0.7	<0.01	0	0	0	0
93-1	40.6	44.8	14.3	0.08	0.3	0.02	0	0.01	0	0	0	0
Total	20.9	37.7	9.4	9.6	13.6	8.0	0.4	0.1	0.1	<0.01	<0.0	<0.01
%FOO												
89-1	61.9	85.7	38.1	33.3	66.7	14.3	9.5	14.3	4.8	0	4.8	4.8
90-1	100	100	60	40	80	100	20	20	20	0	0	0
91-1	77.8	88.9	22.2	77.8	44.4	44.4	0	0	0	11.1	0	0
92-1	75	87.5	37.5	50	50	50	25	12.5	0	0	0	0
93-1	57.1	100	57.1	28.6	42.9	14.3	0	14.3	0	0	0	0
Total	70	90	40	54	58	34	10	12	4	2	2	2

the COI gene (Table S2). In the more stringent dataset wherein taxa seen twice in at least 5% of the taxa were kept, 3 phyla, 5 classes, 8 orders, 13 families, 16 genera and 17 species were found based on 52 unique ASVs. In both datasets, the same 11 species were detected with more than 1% relative abundance (*Apherusa glacialis*, *C. glacialis*, *C. hyperboreus*, *Eualus gaimardii*, *Gammarus wilkitzkii*, *L. fabricii*, *Onisimus litoralis*, *P. platessa*, *Sabinea septemcarinata*, *Semibalanus balanoides*, *T. inermis*). We continued our dietary analyses with the least stringent dataset (retaining taxa observed twice in at least 1% of the samples) and report all taxa (including taxa with <1% RRA) in Table S2.

Based on all ASVs, polar cod prey on a broad species spectrum, of which amphipods (37.7% RRA) were the dominant food item, followed by krill (20.9%), fish (Perciformes, Pleuronectiformes and Scorpaeniformes; 13.6%) and decapods (9.4%; Table 3). In terms of FOO, all stations had high numbers of amphipods and krill and relatively high values of copepods, decapods, fish and barnacles (Table 3). Six other taxonomic groups (chaetognaths, pteropods, polychaetes, isopods, sea cucumber order Dendrochirotida and hydrozoans) were detected at some stations (Table 3). At the species level, the most common prey item in terms of RRA was the amphipod *A. glacialis* (27.2%), followed by the krill *T. inermis* (20.3%), the gelatinous snailfish *L. fabricii* (11.3%) and the decapod *E. gaimardii* (8.1%; Table S2). Besides *A. glacialis*, 12 other amphipod species were identified as prey, of which 7 were not yet documented as polar cod prey in the Barents Sea literature (Table S2). Stns 89-1 and 90-1 differed in observed ASV diversity ($p = 0.045$) and in Chao1 ($p = 0.045$), Shannon ($p = 0.006$) and Simpson ($p = 0.018$) diversity indices. No differences in these alpha diversity measures were present between the other stations (Fig. S3). A total of 16 species and 1 genus were retrieved in 1 stomach, and 14 of those were represented by less than 20 reads (Table S3). The total number of different consumed prey species was weakly positively correlated with the size of polar cod ($R^2 = 0.09$, $p = 0.04$), indicating an increased diversity of the diet composition with size (Fig. S4)

3.6. Comparison of visual identification versus DNA metabarcoding

In both methods, amphipods and krill were the dominating food items in terms of FOO, followed by copepods (Fig. 2A). Metabarcoding results suggest higher FOO of amphipods, copepods, decapods and fish than visual identification. Moreover, barnacles, chaetognaths and pteropods were not detected visually but were detected molecularly in 34, 10 and 12% of the stomachs, respectively. Based on occurrence data (i.e. presence/absence), 56 (43 excluding parasites) and 90 mismatches were observed between the visual and metabarcoding methods, respectively (Table 4). Metabarcoding failed to detect parasites (13 cases) and sometimes krill, copepods, fish and amphipods (2 cases each) that were visually observed. In 1 of these cases, krill was visually identified as *T. inermis*. In total, at least 9 times more species- and genus-level assignments were made using metabarcoding compared to visual identification applied on the same stomachs (Fig. 2B). More specifically, 3 taxa were visually identified to the species level (*T. inermis*, *T. longicaudata*, *T. libellula*), whereas 28 additional taxa were identified to the species level based on DNA found in the same stomachs. Furthermore, in most stomachs with unidentified krill remains, metabarcoding confirmed the presence of *T. inermis*, and in stomachs with further unidentified gammarid amphipods, DNA analysis confirmed the presence of multiple species (*A. glacialis*, *G. wilkitzkii* and *O. litoralis*). Differences in the number of microscopically recognizable food items among stations seemed to be related to the degree of digestion. No correlation between digestion and the number of detected taxa was found in the differences in diversity indices from metabarcoding (Fig. S3), suggesting that these are not influenced by the degree of digestion of the stomach contents and/or that traces of DNA can still be detected after complete digestion of the food item.

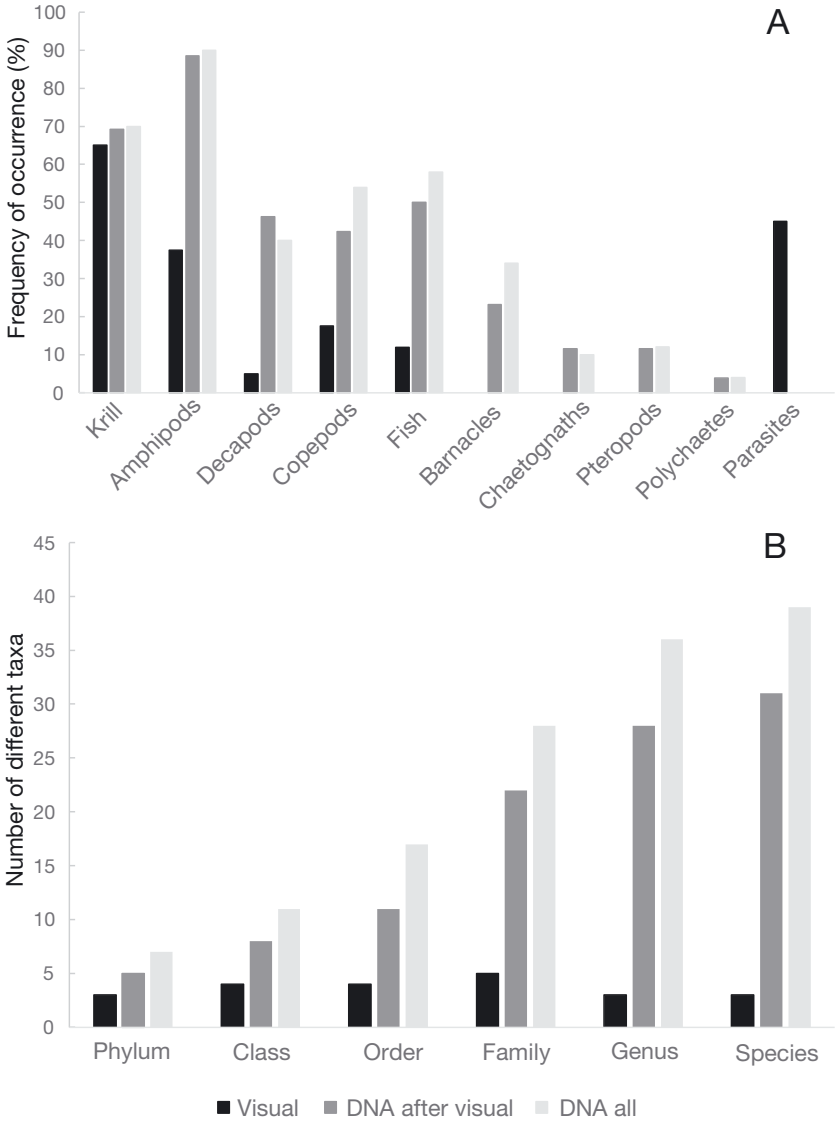

Fig. 2. Comparison of the (A) frequency of occurrence of taxa and (B) number of different taxa identified in polar cod *Boreogadus saida* stomachs using visual identification and metabarcoding. Taxa found with metabarcoding in all stomachs (both pre-visual analysis and intact stomachs) are included

3.7. Effect of pre-analysis visual enumeration on metabarcoding results

There were no significant differences between stomach samples handled for pre-visual analysis and intact stomachs regarding DNA quality, quantity and COI amplification success. The diversity of observed ASVs ($p = 0.005$, Wilcoxon rank-sum test; Fig. 3) and the Chao1 ($p = 0.005$) and Shannon ($p = 0.028$) indices were higher in stomachs without prior visual analysis. However, no difference was detected for the Simpson diversity index ($p = 0.069$), representing the number of species detected.

Table 4. Prey items categorised in 10 taxonomic groups based on presence/absence per method: visual (V) or DNA-based (D) identification. Taxa represented by >20 reads were indicated with X. (X) indicates taxa represented by <20 reads

Stn	Fish ID	Amphi-pods		Chaeto-gnaths		Cope-pods		Deca-pods		Ptero-pods		Fish		Krill		Para-sites		Poly-chaetes		Barna-cles	
		V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D
89	129		(X)				(X)						X	X	(X)	X					
	132	X	X						X				X	X	X						
	133		X				X						(X)		(X)						
	134				X				X						X	X					
	137		(X)										X			X					
	139		X				X						X	X	X						
	141		X			X						X		X	X	X					
	146		X						X		(X)	X			X						(X)
	150		X						X							X					X
	153		(X)						X				X		(X)	X					
	154	X	X						(X)		(X)		X			X					
90	168	X	(X)					X	X		X		X	X	X				X		X
91	109	X				X	X	X[a]	X												
	111	X	X			X	X							X	X						(X)
	112	X[a]	X			X	X						X			X					X
	113	X[a]	X			X								X	X	X					
	114	X	(X)			X	(X)							X	X	X					
92	69	X	X				X						X	X	X						
	70	X	X				X							X	X	X					
	71	X			(X)		X							X							
	73	X	X		X		X	X[a]	X				X	X	X						X
93	119	X	X						(X)					X							
	121		(X)											X	X	X					
	122		X						(X)				X	X	X						
	124	X	X									X	X			X					
	126	X[a]	(X)						X					X	X						
Total	15	23	0	3	6	11	3	12	0	3	3	13	16	18	13	0	0	1	0	6	

[a]Prey items classified as crustaceans but reclassified as amphipods or decapods based on DNA data

4. DISCUSSION

4.1. Complementary diet analysis

Both morphological and molecular diet analysis revealed amphipods, krill and copepods as frequent prey items of the polar cod summer diet in the northern Barents Sea. The molecular data provided a higher taxonomic resolution of the prey items (38 taxa at species level) compared to the visual analysis (3 taxa at species level). Amphipods were the most frequent order found by both methods. Yet, the most common prey item detected by metabarcoding, the sea ice amphipod *Apherusa glacialis* (observed in 80% of the stomachs), was not visually detected. This observation indicates prior feeding of the polar cod on ice-associated biota, which were likely already digested by the time of sampling. In fact, a few days before sampling, sea ice retreated from the sampling area as observed on the sea ice portal meereisportal.de (Grosfeld et al. 2016). Metabarcoding might therefore provide dietary information on a potentially longer temporal scale than visual analysis and reveal feeding in different habitats than assumed based on visual analysis only. In addition, metabarcoding enables detection of easily degraded and unrecognizable prey items, such as eggs and pelagic larvae of fish, barnacles and other soft-bodied invertebrates (see Sections 4.2 & 4.3). The visual analysis, however, contributes information on the size, life stage and biomass of prey items, which provides a better indication of the importance of prey items in the diet. The IRI, for example, integrates several methods of quantifying prey items in the stomach and indicated that krill was the most important food item for polar cod in the Barents Sea during the time of study. A combined approach provides a complementary inven-

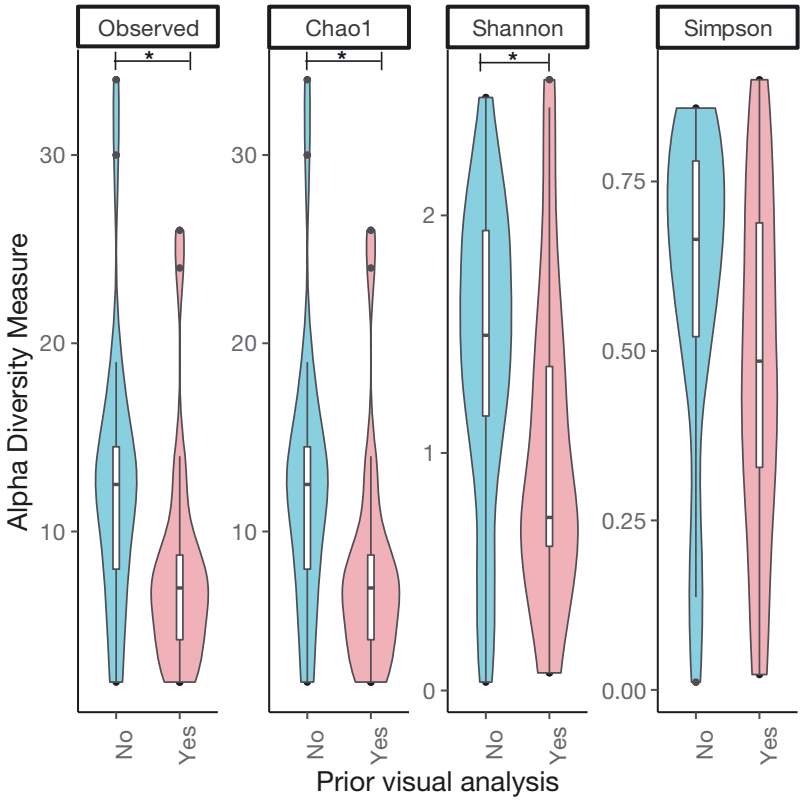

Fig. 3. Violin plot visualising alpha diversity among stomachs used for metabarcoding with and without prior visual analysis. Diversity differed ($p < 0.05$) for observed amplicon sequence variants and Chao1 and Shannon diversity indices, as indicated by asterisks. Boxes indicate interquartile range of 25th to 75th percentiles. Median value is indicated with a line in the box. Whiskers represent minimum and maximum values within 1.5× interquartile range. Dots indicate outliers

tory of the diet, as both methods give information on certain aspects of the diet, while both also have disadvantages. In addition, a combined approach can potentially provide information on different timescales: feeding at the time of sampling and also several days before, as indicated in our study by the presence of hard-bodied prey detected by metabarcoding but not by visual analysis. In other cases, traditional diet analysis may also identify older prey, e.g. through otoliths that remain in the stomach for longer. Future investigations could attempt to identify the exact timescales of both methods. If a longer timescale is needed, stable isotope analysis can be added as a third complementary approach (Kohlbach et al. 2017, Matley et al. 2018, Bachiller et al. 2020). Detailed diet studies using multiple methods may thus provide useful information on temporal changes in diet and habitat use, a more complete overview of the fish diet and, ultimately, the polar cod's ecology and role in the Arctic ecosystem.

4.2. Extending the prey spectrum of polar cod

The most common prey items detected with both methods generally matched with published studies on polar cod diet from the Barents Sea. Calanoid copepods and the hyperiid amphipod *Themisto libellula* are important diet components, whose dominance depends on fish size, season and region (Lønne & Gulliksen 1989, Węsławski & Kuliński 1989, Renaud et al. 2012, Hop & Gjøsæter 2013). Krill is often documented as polar cod prey (Renaud et al. 2012), occasionally as a regionally dominant food item regarding biomass (Ajiad & Gjøsæter 1990, Orlova et al. 2009).

In addition, to confirm these results, we have evidence for a wider prey spectrum than previously anticipated in terms of both prey frequency and new prey species. Polar cod regularly fed on fish, decapods and barnacles, which were detected with metabarcoding. The consumption of fish by polar cod has been previously reported (Ajiad & Gjøsæter 1990, Eriksen et al. 2020), but the FOO in our study (29 of 50 stomachs across all sites) is higher than expected. The gelatinous snailfish *Liparis fabricii* was the most frequent fish prey (in 23 of 50 stomachs). Data from the Canadian Beaufort Sea show that the majority of snailfish larvae are present pelagically during summer (Walkusz et al. 2016), which may explain the high frequency observed here. Decapods, the fifth most common prey taxon in this study, were detected in 40% of the stomachs and are recorded relatively regularly in the stomachs of polar cod (Lønne & Gulliksen 1989, Gray et al. 2016, McNicholl et al. 2018). Species identification, however, is often difficult. Here, *Eualus gaimardii* was the most common decapod species (observed in 36% of the stomachs). Although *E. gaimardii* is a benthic species, this small circumpolar shrimp uses the sea ice as a temporary feeding ground, with sympagic amphipods as the most important prey (Nygård et al. 2007). In addition, there is evidence that *E. gaimardii* fully retracts into brine channels of ice during stable salinity conditions (Nygård et al. 2007). In the East Siberian and Laptev seas, *E. gaimardii* has been documented as an impor-

tant prey item for polar cod (Gorbatenko & Kiyashko 2019). Furthermore, barcodes of barnacles from the genera *Balanus* and *Semibalanus* were observed in almost 40% of the investigated stomachs. Polar cod likely feed on the planktonic larval cirripeds, which spend several weeks in the water column and can be abundant in the area (Crisp 1962, Ehrlich et al. 2020).

Several species identified with metabarcoding were unknown food items to polar cod. Various amphipod species have never been recorded as prey items before. Prey from the genera *Onisimus* and *Ischyrocerus* have been reported before but not to the species level. In addition, the species *Deflexilodes tenuirostratus* has not been documented in the study region before (Prestrud et al. 2004, McGovern et al. 2018). Several of these identified amphipods were benthic, among which 3 belonged to the Oedicerotidae. The only specimen from the latter family visually observed in the stomach contents was barcoded as *Arrhis phyllonyx*. This species has been observed before in polar cod stomachs in Billefjorden, but in the winter season (Cusa et al. 2019). Furthermore, we document for the first time in the Barents Sea diet the polychaete genus *Spio* as prey. Among the fish species, Arctic staghorn sculpin *Gymnocanthus tricuspis*, gelatinous snailfish *L. fabricii*, striped seasnail *L. liparis*, 2 temperate flatfish and the European plaice *Pleuronectes platessa* have never been identified before in the Barents Sea polar cod diet. European flounder *Platichthys flesus* is uncommon in the northern Barents Sea, but its presence is not disputed (Johannesen et al. 2021). In addition to these temperate fish species, the temperate–boreal northern krill *Meganyctiphanes norvegica*, first observed in polar cod stomachs in 2015 in the northern Barents Sea (Eriksen et al. 2020), was observed in 3 stomachs. The northern krill, increasingly observed in the Arctic Ocean with the enhanced Atlantic water inflow, has previously already been suggested as a useful indicator of change (Buchholz & Buchholz 2010). As rising sea temperatures and changes in circulation patterns in the Barents Sea allow boreal and sub-Arctic species to expand northward into areas previously occupied by Arctic species (Fossheim et al. 2015, Dalpadado et al. 2020), a shift in the prey composition of polar cod is expected. Although several temperate species such as *M. norvegica* occur in the Barents Sea (Dalpadado & Skjoldal 1991, Schmidt 2010), temperate species in the stomachs indicate that the polar cod has the potential to incorporate temperate–boreal species in its diet. Overall, with a relatively constrained spatio-temporal sampling design, we extended the prey spectrum of polar cod considerably and provide indications that integrated diet assessment can be a useful tool to monitor changes in species assemblages and potential consequences for the food web.

4.3. Technical recommendations

Clearly, visual and molecular diet analyses are beneficial for extended complementary insights on fish diet. Nevertheless, we documented some discrepancies between visual and molecular results. The molecular absence of visually confirmed krill, copepods, amphipods, trematodes and nematodes might be explained by the following factors. (1) PCR amplification failure was caused by DNA degradation. Different degradation rates might cause underrepresentation of certain taxa (Paula et al. 2015). However, overall, the effects of DNA degradation bias have been shown to be of minor importance in other studies (Krehenwinkel et al. 2018), and we would expect that visually identifiable remains also contain sufficient DNA for amplification of a small fragment such as COI. (2) A considerable proportion of reads (16.2%) remained unidentified and were discarded due to the absence of any match in public databases. These reads might be valid unidentified or unknown COI genes, for example of parasites for which no reference entries exist yet or false COI reads. Such COI-like reads might be attributed to the amplification of pseudogenes or nuclear copies of non-functional or coding mitochondrial-derived genes (Buhay 2009). (3) In large-volume stomachs, subsampling may have caused the absence of visually identified taxa in molecular results. Except for parasites, which may be affected by PCR amplification bias and/or missing database entries, subsampling effects seem the most likely explanation for molecular false negatives. We therefore emphasise the importance of proper homogenisation for representative results. Larger stomachs could be homogenised using a blender or vortexed with glass beads instead of a mortar (Roussel et al. 2005) to avoid subsampling effects. Another option would be to consistently sequence several replicates per stomach if time and research budget allow for that. In general, it should be kept in mind that metabarcoding can provide evidence for the presence of a species in the stomach contents but not necessarily prove its absence when not detected. Performing visual analysis on

the same stomach contents prior to metabarcoding might also cause bias related to different DNA degradation rates between species. When combining both methods, we therefore recommend keeping the stomach contents on 96% ethanol during morphological identifications in addition to storing in ethanol until DNA extraction. Metabarcoding data here merely represent a snapshot from the diet composition of polar cod, albeit at high taxonomic resolution. The FOO data should be interpreted with care because of the small sample size in certain stations.

Lastly, it is not possible to distinguish secondary prey, i.e. prey of prey or accidental prey consumed during feeding, and cannibalism among polar cod from true prey with metabarcoding (Sheppard et al. 2005). Accordingly, it cannot be ruled out that planktonic larval stages of barnacles, found in over one-third of the stomachs, are secondary prey items. However, there are some strong indications that barnacles are in fact genuine prey species of polar cod. Nauplii of barnacles are characterised with frontal horns that make them unsuitable prey for fish early life stages (eggs and larvae) (Walker 1973, Fossum & Ellertsen 1994) and presumably also smaller organisms like the prey of polar cod. Furthermore, to the best of our knowledge, there are no records of barnacles as part of the calanoid copepod and krill prey spectrum. In contrast, there is evidence of cirripeds, although not common, as part of the polar cod diet (Bouchard & Fortier 2020). The absence of diatoms, an important food source for both calanoid copepods and sea ice-associated amphipods, but also other common copepod prey such as polychaete larvae (almost absent) and ctenophores (Poltermann 2001, Cleary et al. 2017) is a strong indication that secondary prey was not picked up to a large extent by metabarcoding. Regardless, several common prey species of polar cod share some of the prey species of its predator. For example, amphipods, such as *T. libellula* and *T. abyssorum*, feed on *Calanus* copepods (Auel & Werner 2003, Kraft et al. 2013). Yet, the DNA of secondary prey likely represents a minor part of the total ASV reads compared to primary prey, due to higher levels of degradation and much lower biomass (Jakubavičiūtė et al. 2017). Here, 14 species with less than 20 reads were documented, several of which are documented polar cod prey items. Additional dietary studies using both methods in a complementary approach, but on a larger spatio-temporal scale, are necessary to confirm if these species are either secondary accidental food items or an actual part of the diet.

5. CONCLUSION

Our data reveal a diverse summer diet of polar cod in the northern Barents Sea. Krill, amphipods and, to a lesser extent, copepods are frequent prey items of polar cod in concordance with the available literature. Barnacle larvae and fish (eggs and/or larvae) are more important diet components than previously documented. In addition, highly digested sea ice-associated amphipods recovered by metabarcoding suggest prior feeding in ice-covered waters. Unravelling the diet, but also the feeding behaviour and ecology, of key species in marine food webs affected by climate change helps design appropriate ecosystem-based management plans. In remote, often sea ice-covered and thus undersampled areas, it is even more important to use the full suite of available techniques to elucidate trophic relationships. Our results demonstrate that the combination of visual and molecular stomach content analysis provides complementary information regarding quantification, taxonomic resolution and temporal scale. Additionally, we show that the combination of methods can provide powerful and complementary information on partially digested prey, which is needed to monitor rapidly changing marine food webs.

Data availability. The dataset generated and analysed in this study is available from the corresponding author upon reasonable request.

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missions were required. The fish collected are neither endangered nor protected in the coastal waters of the Svalbard Archipelago. Polar cod were sacrificed immediately after sampling.

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