Received: 16 January 2024 | Revised: 8 April 2024 | Accepted: 30 April 2024

DOI: 10.1002/edn3.561

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ORIGINAL ARTICLE

Detecting the invisible through DNA metabarcoding: The role of gelatinous taxa in the diet of two demersal Antarctic key stone fish species (Notothenioidei)

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Funding information
Dirección Nacional del Antartico Instituto Antártico Argentino, Grant/Award Number: PICTA 0100; Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Grant/Award Number: VH-NG-1400; Deutsche Forschungsgemeinschaft, Grant/Award Number: HA 7627/3-1 and LE 2323/11-1; Fondo para la Investigación Científica y Tecnológica, Grant/Award Number: PICT 2018-03310 Res.401/19

Abstract

Gelatinous zooplankton (GZP), i.e., ctenophores, cnidarian medusae, chaetognaths, appendicularians and salps, are considered climate change winners. This becomes particularly obvious in the Southern Ocean, which has undergone a significant shift from a krill-based to a salp-based ecosystem over the last decades. A better knowledge on the role of gelatinous invertebrates as prey is needed to predict the impact of such a gelatinous shift. Until recently, GZP was considered as a "trophic dead end". However, their true importance in diets has remained unresolved due to the rapid digestion of their watery and soft tissues in predators' stomachs. In this study, we want to validate the paradigm shift from GZP being considered as "survival food" to be considered a "regular" prey item for two demersal fish species (*Notothenia rossii* and *N. coriiceps*) of Potter Cove, South Shetland Islands, using a multimarker (COI and 18S) metabarcoding approach. We found that GZP taxa commonly occurred in the diets of both species, represented by pelagic tunicates (appendicularians, salps), cnidarians, chaetognaths and ctenophores. Salps were the most abundant prey group, preyed upon by each individual of both species, reaching 98.7% relative read abundance for 18S. We recovered a wide range of different taxa in their diets, from primary producers to highly abundant invertebrates, thus the two nototheniid species can be regarded as "natural samplers" of the ecosystem in study. Finally, we want to point out the importance of multimarker metabarcoding approaches for broad ecological assessments, given the differential amplification and sequencing success of different markers for specific groups and the unequal taxonomic coverage of the reference databases. The output of each marker was highly complementary, since an important prey item such as salps, was only detected with 18S, while other taxa (e.g., Arthropoda) were represented with a higher taxonomic resolution with COI.

KEYWORDS
Antarctica, diet, gelatinous zooplankton, metabarcoding, Nototheniidae

1 | INTRODUCTION

Gelatinous zooplankton (GZP), including ctenophores, cnidarian medusae and pelagic tunicates (e.g., salps), are reputed to be climate change winners and an effort to address their potential recent increase in numbers in many marine ecosystems is ongoing (Condon et al., 2012; Lilley et al., 2011; Lucas et al., 2014; Lynam et al., 2010). This shift is particularly obvious in the Southern Ocean, where a poleward range contraction and decrease in the abundance of krill, concomitant with an increase and poleward shift of salp populations have been reported for the Scotia Arc and Antarctic Peninsula area, all changes associated with climate change factors (Atkinson et al., 2004, 2017, 2019; Bernard et al., 2012; Fuentes et al., 2016; Loeb et al., 1997; Loeb & Santora, 2012; Moline et al., 2004 among others). Considering that GZP constitute a large fraction of the pelagic biomass, particularly when occurring in very high densities in the shape of large blooms, they might have an even more central role in the near future pelagic ecosystems (Purcell, 2012; Richardson et al., 2009). Hence, their availability and role as prey may similarly increase and therefore needs to be better understood. Until recently, GZP were considered to be a "trophic dead end" in the pelagic food web, contributing in a negligible manner to the transfer of energy to higher trophic levels (Hays et al., 2018; Robinson et al., 2014; Sommer et al., 2002). This can be explained because of their watery and soft tissues, which are rapidly digested and therefore often "invisible" in predators' stomachs for traditional microscopy (Amundsen & Sánchez-Hernández, 2019; Brodeur et al., 2021).

In recent years, the application of new techniques such as DNA metabarcoding and in-situ observations caused a paradigm shift from GZP being considered a trophic dead end or a "survival food" to be a common or even important part of the diet of various animals worldwide, such as seabirds (e.g., McInnes et al., 2017), turtles (e.g., González Carman et al., 2014; Heaslip et al., 2012), and fish (e.g., Ayala et al., 2018; Günther et al., 2021). In the context of the Southern Ocean food web, literature already exists discussing the role of *Salpa thompsoni* as an alternative prey to Antarctic krill (McCormack et al., 2021; Queirós et al., 2024). These findings are striking because many GZP are assumed to have low energy content and, therefore, a limited associated energy benefit to their consumers (Thiebot & McInnes, 2020). However, this view is challenged by the fact that GZP are more rapidly digested and easier captured, particularly when occurring in aggregations or blooms (Diaz Briz et al., 2017; Hays et al., 2018). Some alternative explanations of the consumption of GZP are; targeting energy-rich tissues such as gonads or the frequent occurrence of high numbers of parasites (e.g., amphipod crustaceans) on GZP, the presence of bioactive compounds in their tissues, and their accidental or secondary ingestion (Henschke et al., 2016; Thiebot & McInnes, 2020). DNA metabarcoding uses so-called universal DNA primers and high-throughput sequencing of PCR amplicons in order to identify a broad spectrum of taxa from the stomach content of the species under study (Taberlet et al., 2012). Overall, metabarcoding is a powerful technique that allows to identify a high number of species, including rare, small, damaged and digested as well as cryptic species, independently of the taxonomic expertise of the researcher (Dick et al., 2023; Wangensteen et al., 2018). In this way, when performing broad taxonomic screens, a multimarker metabarcoding approach provides a better coverage of the potential prey spectrum of which the output can be complementary with regard to identification and taxonomic resolution, but also reducing taxonomic biases associated with individual primers or markers (Pappalardo et al., 2021; Van der Reis et al., 2018).

Demersal fish play a key role in the Southern Ocean, and, in terms of species diversity, abundance and biomass, they are dominated by a unique coastal endemic fish group, the Cryonotothenioidea or "Antarctic clade" included in the suborder Notothenioidei (Near et al., 2012, 2015). Although nototheniids lack a swim bladder, they are not confined to the benthic habitat and virtually all species utilize pelagic food resources. Krill feeding is especially common among primarily demersal nototheniids (Foster & Montgomery, 1993; Hollyman et al., 2021; Kock et al., 2012; Kock & Jones, 2005; Stefanov, 2022). In addition to krill, nototheniids prey on other plankton components such as copepods, hyperiid amphipods as well as squids and other fish (summarized in Barrera-Oro, 2002; Barrera-Oro et al., 2019; Moreira et al., 2021, 2023), and consume an array of other taxa including algae (Gröhsler, 1994; McKenna Jr, 1991). Within the Cryonotothenioidea, members of the family Nototheniidae experienced the greatest ecological and morphological diversification of the entire suborder with species occurring in all latitudes of the Southern Ocean. The two nototheniid species *Notothenia rossii* and *Notothenia coriiceps* are sympatric species with a similar ecology in high-Antarctic fjords, living predominantly from 5 to 50 m depths on rocky bottoms covered with macroalgae beds (Barrera-Oro, 2002; Barrera-Oro et al., 2019; Moreira et al., 2023). Although both species are benthic-demersal fish, they have significantly different buoyancies. This divergence in buoyancy is reflected in their distinct morphology, not only in body shape but also in skeletal weight and is associated with differences in activity patterns and diets (Barrera-Oro, 2003; Eastman et al., 2011). Conventional stomach content analyses of these species at Potter Cove, an inshore locality at King George Island/Isla 25 de Mayo, South Shetland Islands, have shown that they are generalist feeders during their ontogeny (Barrera-Oro et al., 2019; Moreira et al., 2023) but differences in prey composition have been registered. While *N. coriiceps* feeds on a wider range of benthic organisms, *N. rossii* is semipelagic, feeding not only on benthos but also on planktonic prey, when available, during the summer months (Barrera-Oro et al., 2019; Casaux et al., 1990; Moreira et al., 2014, 2023). In particular, salps have been reported as occasional or secondary prey for *N. rossii* and *N. coriiceps* only through traditional analysis (Barrera-Oro, 2002, 2003; Barrera-Oro et al., 2019). Thus, considering the limitations of conventional identifications of GPZ in stomach contents, the role of GZP taxa in these fish species' diets still needs to be clarified. Recently, the complexity, structure and function of the food web in Potter Cove was assessed, suggesting that *N. coriiceps* is a keystone species in this ecosystem, given that it is one of the species that showed a high number of trophic links in the food web (Marina et al., 2018). While many trophic connections are known, it is essential to reveal the so

far invisible connections within local food web; this is important to understand consequences of local extinctions or range shifts due to climate change.

In this study we want to test the paradigm shift from GZP being considered as "survival food" to a "regular" prey item for two demersal fish species (*N. rossii* and *N. coriiceps*) of the Southern Ocean through a multimarker metabarcoding approach. In view of the existing evidence outlined above we test the following two hypotheses:

1. A variety of GZP taxa commonly occur in the diets of both nothotheniid species as regular prey items.
2. Since *N. rossii* has semipelagic lifestyle using also the water column to feed, we expect that its prey, including GZP, are more diverse than those found in *N. coriiceps*' diet.

To test these hypotheses, we studied populations from a coastal Antarctic marine ecosystem in Potter Cove and compared results with those obtained from conventional trophic ecology studies. In addition, we tested for evidence of other factors explaining differences diet dissimilarities of these sympatric nototheniid species.

2 | MATERIALS AND METHODS

2.1 | Sampling and initial measurements

Nototheniidae were collected in South Shetland Islands waters, Potter Cove at King George Island/Isla 25 de Mayo. They were sampled in coastal waters near the Argentine scientific station "Carlini" at a site called Peñón de Pesca (62°14′ S and 58°40′ W; Figure S1). The abiotic features and biotic components of this area are described in Barrera-Oro et al. (2019). A total of 62 specimens of *N. rossii* and 64 *N. coriiceps* were collected during the austral summer of 2022 (January–March). For sampling, trammel nets (15 m long, 1.5 m deep, 2.5 cm inner mesh, 12 cm outer mesh) were deployed on a rocky bottom with red and brown macroalgal beds. For each fish specimen, we recorded the total and standard length to the nearest 0.1 cm, their weight in g and their sex. The macroscopic gonadal stage was determined according to the scale in Kock and Kellermann (1991). The stomachs were dissected, weighted with 0.01 g precision and stored at −20°C until further processing.

2.2 | Sample treatment and DNA extraction

In the laboratory, a blender and grinding tools were used to homogenize the stomach samples; countertops, dissection tools and all instruments used for DNA extraction were cleaned with 10% bleach, water and 70% ethanol. Stomachs were thawed and carefully opened in order to extract contents while avoiding rubbing the stomach walls; parasites were visually identified in two samples of *N. corriceps*, those stomachs were excluded from the analysis (46 specimens of each species were analyzed). After homogenization, DNA extraction was performed in triplicates using the Qiagen Blood and Tissue extraction kit following the manufacturer protocol, with approximately 25 mg of tissue, final elution volume was 100 uL. In every round of 24 extractions, a negative extraction control was performed, treating the empty tube as the rest of the samples. DNA quantity and quality were assessed using a Nanodrop ND-1000 (Thermo Fisher Scientific) spectrophotometer, only extracts with a 260/280 ratio of >1.5 and a concentration >10 ng/uL were used for the next steps. Triplicates were pooled prior to library preparation.

2.3 | Library preparation and sequencing

Here, we implemented a multimarker metabarcoding approach using COI and 18S. The mitochondrial COI gene is the most commonly used marker in metabarcoding studies given that it can discriminate between metazoan species with high resolution (e.g., Siegenthaler et al., 2019). The nuclear 18S v1v2 region is frequently used to target a broad spectrum of metazoans in metabarcoding studies (e.g., Wangensteen et al., 2018), it is a suitable size for the Novaseq sequencer methodology, and it allows to amplify taxa that are known to be less easily detected with COI, such as salps and some species of ctenophores (Brandt, Pradillon, & Trouche, 2021; Brandt, Trouche, et al., 2021; Günther et al., 2018, 2021).

DNA metabarcoding library preparation and sequencing were carried out by AllGenetics & Biology SL (www.allgenetics.eu). DNA concentration was quantified using the Qubit dsDNA HS Assay (Thermo Fisher Scientific). For library preparation, the "Leray XT"-fragment of the COI mitochondrial gene of 313 bp was amplified (Geller et al., 2013; Wangensteen et al., 2018). Illumina sequencing primer sequences were attached to these primers at their 5′ ends. In the first amplification step, PCRs were carried out in a final volume of 12.5 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 6.25 µL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 12.5 µL. PCR conditions consisted in an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 54.7°C for 45 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. Additionally, a fragment of the 18S (V1-V2 region) gene of ~450 bp was amplified (Blaxter et al., 1998; Sinniger et al., 2016). Illumina sequencing primer sequences were attached to these primers at their 5′ ends. In the first amplification step, PCRs were carried out in a final volume of 12.5 µL, containing 1 µL of template DNA, 0.5 µM of the primers, 3.13 µL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 12.5 µL. PCR conditions consisted in an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 49.7°C for 45 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. No PCR replicates were carried out. The oligonucleotide indices were attached in a second amplification step (PCR1 product was directly used without purification for PCR2), using 2.5 uL of PCR1 product, with identical PCR conditions but only 5 cycles and using 60°C as annealing temperature (PCR products were not purified between PCR1 and PCR2). A negative control that contained no DNA was included in

every PCR round to check for contamination during library preparation. A list of primers and sequences added during PCR1 and PCR2 can be found in the Tables S1–S3. Library size was verified using 2% agarose gels stained with GreenSafe (NZYTech) and imaging them under UV light. Subsequently, libraries were purified using the MagBind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Finished libraries (COI and 18S) were pooled in equimolar amounts according to the results of a Qubit dsDNA HS Assay (Thermo Fisher Scientific) quantification. Finally, the pool was sequenced in a fraction of an Illumina NovaSeq (2×250 bp paired-ends), adding 5% PhiX to the sequencing run, aiming for a total output of 24 Gbp.

2.4 | Bioinformatic analysis

Bioinformatic analysis of the COI sequencing data was performed following the MJOLNIR pipeline (Metabarcoding Joining Obitools and Linkage Networks In R; https://github.com/uit-metabarcoding/MJOLNIR), using the R package mjolnir v1.2 (Wangensteen, 2020) in R v4.0.4. The pipeline default settings, which were already adjusted for the COI marker gene by the provider, were retained. Taxonomy was assigned against the DUFA_COLR reference set (https://github.com/uit-metabarcoding/DUFA) with the timestamp "20210723". BOLDigger (Buchner & Leese, 2020) was run in order to double check taxonomic annotations and define thresholds. Assignments with less than 85% identity were excluded, and a >97% threshold was used to define species level, >95% to genus, >90% to family, >85% to order, class and phylum level (Macher et al., 2018; Wangensteen & Turón, 2017). Bioinformatic analysis of the 18S sequencing data was performed by applying Cudadapt v.2.8 (Martin, 2011) to remove all primers and leftover adapters and by applying functions of the R package "dada2" (version 1.18.0; Callahan et al., 2016) to conduct sequence trimming and filtering, sequence denoising according to the Divisive Amplicon Denoising Algorithm (DADA), paired-end merging, chimera detection-removal and ASV annotation against the PR2 reference set v.4.14.0 (https://doi.org/10.1093/nar/gks1160). Processed ASVs were clustered into operational taxonomic units (OTUs) using the program swarm2 (Mahé et al., 2015) with an iterative local threshold $d=4$. Clustering implementing $d=2$ and $d=1$ (the latter with fastidious option) were run (Tables S3_d=4, S4_d=2, S5_d=1), both produced more OTUs than $d=4$, therefore we used the latest value as recommended by Brandt et al. (Brandt et al., 2020; Brandt, Pradillon, & Trouche, 2021) and Günther et al. (2021). Swarm OTUs were annotated against the PR2 reference set v.4.14.0 as well. Finally, a manual correction of taxonomic annotations using the World Register of Marine Species (WoRMS; https://www.marinespecies.org) and BLASTn using NCBI database was performed.

A final refinement of both datasets consisted at first in the removal of non-target taxa (bacteria, fungi, terrestrial taxa) and contaminants (predator and human DNA). Second, we removed every OTU for which the abundance in the blank or negative controls was higher than 10% of the total reads of that OTU. Third, a minimum relative abundance filter of 0.002% was applied for each sample (Wangensteen & Turón, 2017). After all the refinement steps and bioinformatic treatment, we removed samples with a read depth of less than 1000 reads (Drake et al., 2022; Siegenthaler et al., 2022). Finally, for the construction of final tables (see Tables 1 and 2) and the multivariate analysis, reads assigned to parasitic taxa were excluded (Nematoda, Plathyhelmintes and Acanthocephala).

2.5 | Statistical analyses

The data obtained for both markers were analyzed in R (https://www.R-project.org/) using the vegan package (R version 4.2.3; Oksanen, 2019). Graphs and explorative analysis were performed using the TaxonTableTools software (Macher et al., 2021). We used relative read abundance (number of reads of one prey group divided by the number of total reads in all prey groups, RRA%) and frequency of occurrence (number of samples that contained a given prey item divided by the total number of samples, FOO%) as metrics for analyses and tables. Multivariate statistical analyses were also accomplished using the vegan package in R. *Notothenia rossii* and *N. coriiceps* data were split into three size groups, which were arbitrarily defined according to the size classes (total length groups: small ≥21.0 ≤ 29.9 cm; medium ≥30.0 ≤ 38.0 cm; large ≥ 38.1 cm), data regarding sex and sampling depth (1: ≤11 m; 2: ≥12 m ≤ 20 m; 3: ≥20.1 m); were used as factors for the analysis. Data sets for both markers were four-root transformed. Using the vegdist function we obtained a Bray–Curtis coefficient similarity matrix. Nonmetric multidimensional scaling (NMDS) plots for each of the above-mentioned factors were performed to visually check patterns of the fish diet in a two-dimensional plane according to their relevant diet similarity. Permutational Multivariate Analysis of Variance (PERMANOVA) was run with 9999 permutations using the adonis2 function to assess the differences in the diet between *N. rossii* and *N. coriiceps*, based on the species, total length, depth and sex. Finally, using the pairwiseAdonis function, we performed the multiple comparison post-hoc test.

3 | RESULTS

3.1 | COI and 18S metabarcoding output

The Novaseq sequencing runs produced 45,741,682 paired-end raw reads for the multiplexed library of COI and 47,106,864 paired-end raw reads for the multiplexed library of 18S. After all quality filtering steps, the final dataset consisted of 19,878,789 metabarcoding reads for COI and 18,015,199 reads for 18S. The sequencing depth per sample ranged from 199,182 to 820,570 reads for COI and 140,258 to 805,578 reads for 18S. For COI, 6959 reads (0.015% of total reads) clustered into 10 OTUs were found in negative controls (assigned to Arthropoda, Annelida, Cnidaria, Mollusca, Rhodophyta, human, and *Notothenia rossi*), while 52,499 reads (0.111% of total reads), comprising 25 OTUs, were found in the 18S negative controls (assigned

TABLE 1 Numbers and values of detected prey species for *Notothenia rossii* and *N. coriiceps* using COI sequences.

Phylum	Species	OTUs	Reads	RRA (%)	FOO (%)
Annelida	*Unassigned*	8	197,717	2.015	
	Neanthes kerguelensis	1	60,774	0.619	12.5
Arthropoda	*Unassigned*	38	2,047,637	20.869	
	Djerboa furcipes	1	692,996	7.062	63.636
	Bovallia gigantea	1	629,855	6.419	39.773
	Gondogeneia antarctica	1	531,224	5.414	47.727
	Cyllopus magellanicus	1	249,297	2.541	23.864
	Prostebbingia brevicornis	1	242,429	2.471	20.455
	Orchomenella rotundifrons	1	155,677	1.587	10.227
	Hippomedon kergueleni	1	153,650	1.566	19.318
	Oradarea sp.	2	122,669	1.25	44.318
	Eurymera monticulosa	1	89,399	0.911	15.909
	Monoculodes sp.	1	62,288	0.635	10.227
	Orchomenella infinita	1	52,072	0.531	20.455
	Thysanoessa macrura	1	50,976	0.519	4.545
	Euphausia superba	1	27,005	0.275	13.636
	Cyllopus lucasii	1	26,048	0.265	6.818
	Vibilia antarctica	1	18,254	0.186	13.636
	Munna sp.	1	7745	0.079	13.636
	Orchomenella pinguides	1	7586	0.077	1.136
	Charcotia obesa	1	6072	0.062	3.409
Bryozoa	*Antarctothoa* sp.	1	57,967	0.591	21.591
Chaetognatha	*Sagitta* sp.	1	19,754	0.201	4.545
Chordata	*Pygoscelis papua*	1	80,787	0.823	2.273
Cnidaria	*Haliclystus antarcticus*	1	107,627	1.097	11.364
	Unassigned	8	69,291	0.707	
	Edwardsia sp.	1	21,822	0.222	3.409
Mollusca	*Laevilacunaria antarctica*	1	555,316	5.659	36.364
	Clio pyramidata	1	244,502	2.492	9.091
	Nacella magellanica	1	144,223	1.47	5.682
	Pareledone charcoti	1	105,988	1.08	3.409
	Lamellariopsis turqueti	1	105,902	1.079	6.818
	Laevilitorina caliginosa	1	73,608	0.75	2.273
	Lissarca miliaris	1	10,813	0.11	9.091
	Aequiyoldia eightsii	1	9275	0.095	4.545
Nemertea	*Unassigned*	8	21,853	0.223	
Ochrophyta	*Unassigned*	15	200,196	2.039	
	Ascoseira mirabilis	1	15,491	0.158	54.545
	Laminariocolax aecidioides	1	7862	0.08	22.727
Rhodophyta	*Unassigned*	11	1,626,817	16.577	
	Myriogramme manginii	1	576,787	5.878	43.182
	Palmaria decipiens	1	251,285	2.561	36.364
	Sarcopeltis skottsbergii	1	38,387	0.391	20.455
	Wildemania amplissima	1	5471	0.056	14.773

Note: Summarized values from all samples regarding biodiversity (number of OTUs and their number of reads), semi-quantitative information, including the relative read abundance (RRA %) and presence/absence-based approaches with the frequency of occurrence (FOO %).

TABLE 2 Numbers and values of detected prey species for *Notothenia rossii* and *N. coriiceps* using 18S sequences.

Phylum	Species	OTUs	Reads	RRA (%)	FOO (%)
Chordata	*Salpa thompsoni*	28	8,798,534	64.695	100
	Molgula sp.	11	272,593	2.004	23.333
	Ihlea racovitzai	6	43,372	0.319	31.111
	Salpa sp.	72	11,627	0.085	68.889
Arthropoda	*Glyptonotus antarcticus*	19	730,775	5.373	45.556
	Euterpina acutifrons	3	36,045	0.265	14.444
	Unassigned	40	19,608	0.152	
	Vargula hilgendorfii	1	6731	0.049	10
Rhodophyta	*Chondrus crispus*	2	431,643	3.174	35.556
	Pyropia sp.	3	187,572	1.379	17.778
	Sarcodia sp.	1	43,314	0.318	7.778
	Palmaria palmata	1	21,464	0.158	22.222
	Unassigned	5	7989	0.062	
Chlorophyta	Unassigned	5	588,652	4.328	
Mollusca	*Clio pyramidata*	6	208,279	1.531	13.333
	Borsonia sp.	1	104,858	0.771	12.222
	Unassigned	17	49,479	0.383	
	Gaimardia trapezina	11	38,402	0.282	18.889
	Mytilus edulis	1	34,144	0.251	2.222
Ochrophyta	*Desmarestia* sp.	1	347,800	2.557	78.889
	Ectocarpus siliculosus	1	33,349	0.245	44.444
Annelida	Unassigned	15	206,102	1.515	
	Flabelligera affinis	1	44,853	0.33	11.111
Cnidaria	*Myxidium* sp.	1	109,246	0.803	37.778
Nemertea	*Antarctonemertes valida*	5	16,988	0.125	13.333
Bryozoa	*Membranipora* sp.	1	9610	0.071	23.333

Note: Summarized values from all samples regarding biodiversity (the number of OTUs and their number of reads), presence/absence-based approaches with the frequency of occurrence (FOO %), and semi-quantitative information, including the relative read abundance (RRA %).

to Arthropoda, Mollusca, Nematoda, Platyhelminthes, Rhodophyta, Rotifera, Streptophyta, and Chordata), including DNA extraction and PCR controls. Rarefaction curves (Figure S2) suggest that sequencing depth was enough to recover all taxa in the stomachs, given that all individuals reached an asymptote. Reads were clustered into 791 eukaryotic OTUs for COI and 1758 eukaryotic OTUs for 18S (3511 ASVs were clustered into these OTUs). After the final refinement, 492 OTUs were assigned at least to phylum level for 18S, while 192 OTUs were annotated for COI. Moreover, some samples were excluded from the analysis given that they were represented by less than 1000 reads (4 and 2 samples for COI and 18S dataset, respectively).

3.2 | COI and 18S findings on the diet spectrum of both nototheniids

The prey items detected using COI and 18S metabarcoding sequencing on stomach contents of *N. rossii* and *N. coriiceps* were assigned to a broad spectrum of taxa, corresponding to metazoan phyla but also algae and other eukaryotic groups such as Rotifera or Bryozoa. The RRA% of each prey phylum revealed in the COI and 18S output are shown in Figures 1 and 2, respectively. It is noteworthy that arthropods in COI results, and salps in 18S results are important prey groups for both fish species. When counting the RRA for all samples (both predator species), for COI, 52.7% of the reads corresponded to the phylum Arthropoda, 25.5% to Rhodophyta, 12.8% to Mollusca, 2.7% to Annelida, 2.3% to Ochrophyta and 2% to Cnidaria. The rest of the prey phyla represented less than 0.8% of the RRA. When counting the RRA of prey items for 18S, we found that 67.1% of the reads corresponded to phylum Chordata (with the most abundant being assigned to salps), 5.94% to Arthropoda, 5.09% to Rhodophyta, 4.33% to Chlorophyta, 3.28% to Mollusca, 2.56% to Ochrophyta,1.85% to Annelida, and 0.87% to Cnidaria. The rest of the phyla represented less than 0.26% of the RRA.

The metazoan prey list detected using the COI and 18S sequences are summarized in Tables 1 and 2, respectively, where we present the taxa found in the stomach contents of *N. rossii* and *N. coriiceps*. The prey taxa are organized from the most abundant to less represented phylum. All OTUs that were not assigned to species level were clustered together. Frequency of occurrence was calculated for each

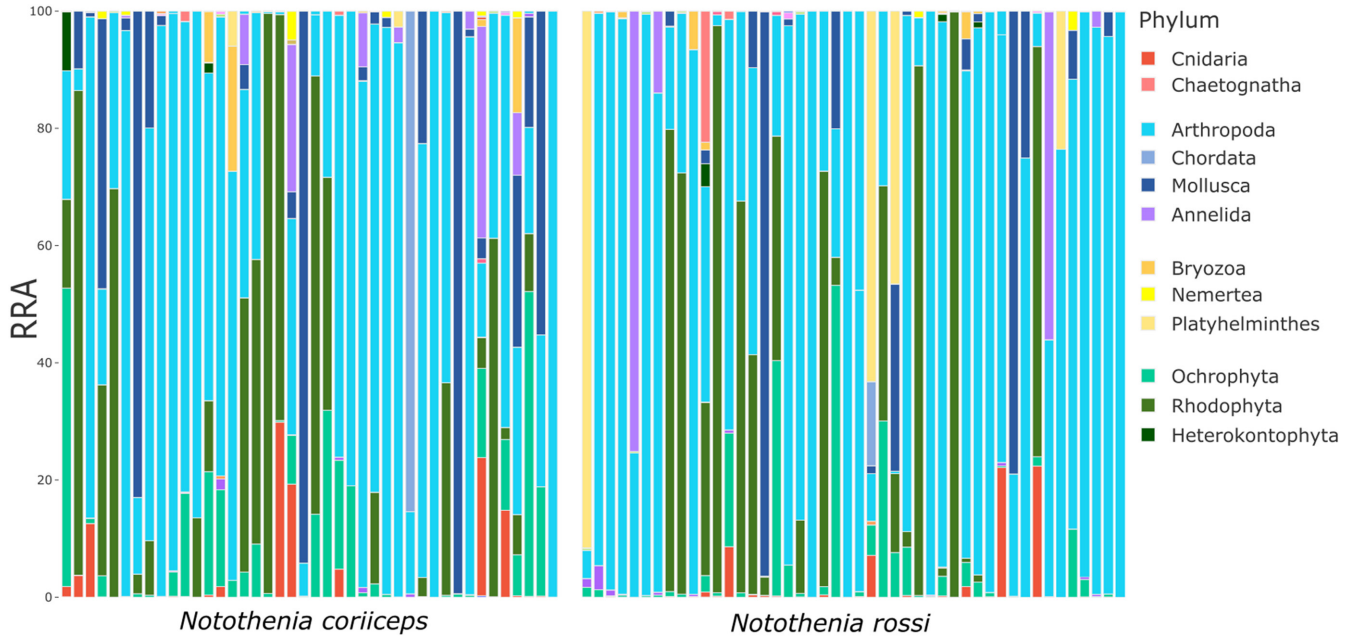

FIGURE 1 Relative read abundances for COI metabarcoding output (number of reads for each phylum, divided by the total amount of reads). Each column represents an individual. The COI reads grouped under Chordata mainly represented reads assigned to the penguin *Pygoscelis papua*. Phyla showing less than 0.02 RRA% are not displayed in the graph.

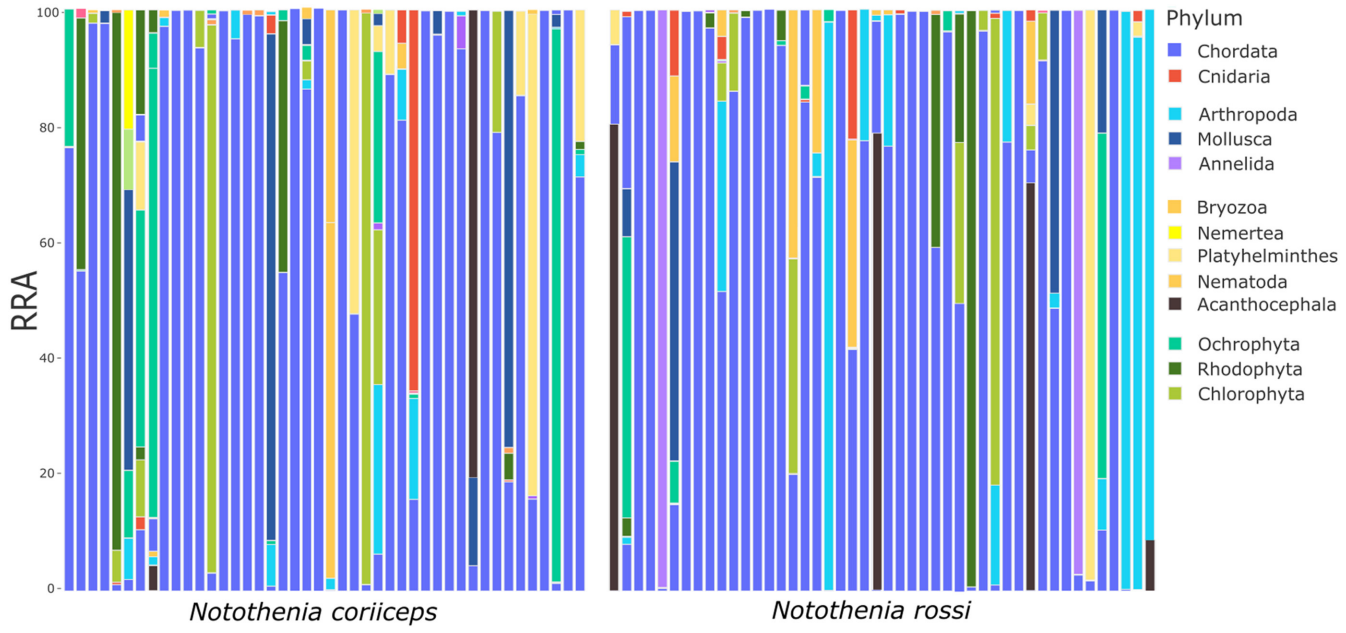

FIGURE 2 Relative read abundances for 18S metabarcoding output (number of reads for each phylum, divided by the total amount of reads). Each column represents an individual. The 18S reads grouped under Chordata mainly represented reads assigned to different species of salps. Phyla showing less than 0.09 RRA% are not displayed in the graph.

species, and only species (or clusters) that showed more than 0.05 RRA% are presented in the tables (complete tables can be found in the Supplementary material section, Tables S1 and S2). The phyla Platyhelminthes, Rotifera, and Nematoda were not included in the list. In the 18S output, we observed reads assigned to salps (*S. thompsoni*) in the gut contents of all individuals from both species, which was the only prey item that was shared between all investigated animals. The second most common food item for both species was the brown algae *Desmarestia* sp. (78.88 FOO%, 18S output), and the third most frequent prey item consumed by both species was the benthic amphipod *Djerboa furcipes* (63.636 FOO%, COI output).

All prey items listed in Tables 1 and 2 (assigned to species level) were assigned to three functional groups: benthic, pelagic, or benthopelagic according to the description in the literature of the adult

and larval stage (if applicable). Figure 3 shows the number of species (here only OTUs assigned to species level were considered) that correspond to each functional group. Both markers here showed that *N. rossii* and *N. corriceps* prey on benthic, pelagic, and benthopelagic prey in similar amounts. For both species, the proportion of benthic items was higher than the pelagic ones, specially taking in account that most of the benthopelagic prey species included here, show a benthic adult stage and pelagic larvae.

3.3 | Focus on arthropods and gelatinous taxa

The phylum Arthropoda was the most represented group in the COI output, while the phylum Chordata (including mostly reads assigned to salps) was the most frequently occurring prey item in the 18S dataset. When considering only the reads (RRA%) assigned to arthropod taxa, amphipods represented the largest number of reads (52.7%) in the COI dataset, while isopods were the most represented ones (90.7%) in the 18S output (Figure 4). For COI, the amphipod species *Djerboa furcipes*, *Bovallia gigantea*, *Gondogeneia antarctica*, *Cyllopus magellanicus*, *and Prostebbingia brevicornis* presented the highest values in RRA% and FOO%, whereas the isopod *Glyptonotus antarcticus* was the most represented in terms of RRA% and FOO% among 18S sequences. Reads assigned to Antarctic krill (*Euphausia superba*) were poorly represented in both datasets.

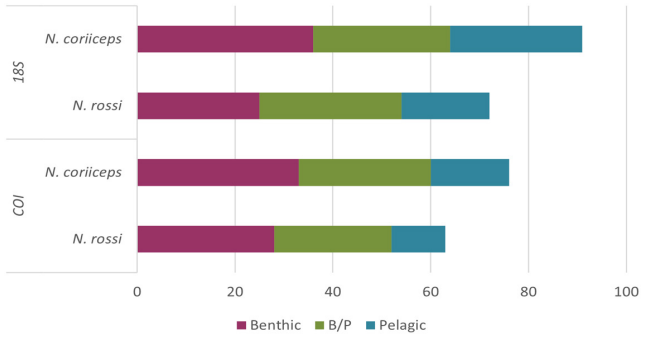

FIGURE 3 Number of benthic, pelagic, or benthopelagic (B/P) prey species for *Notothenia coriiceps* and *N. rossii*.

For both fish species, we found reads that could be assigned to gelatinous invertebrates. In Figure 5, we present the RRA% assigned to gelatinous taxa (including Appendicularia, Thaliacea, Cnidaria, Chaetognatha, and Ctenophora) at class level. Staurozoa and Hydrozoa were the most represented classes within COI sequences, with the benthic species *Haliclystus antarcticus* showing the highest number of reads, consumed by both species. The pattern changes substantially for the 18S dataset, in which the class Thaliacea (including salps, pyrosomes and doliolids) is by far the most represented with 98.7 RRA%. Here, the species *Salpa thompsoni*, *Ihlea racovitzai*, and *Salpa maxima* were represented with the highest values of RRA% and FOO%. For COI, we found in total 15 OTUs, of which only 3 were assigned to species level, corresponding to cnidarians and chaetognaths; while 18S presented 170 OTUs of gelatinous taxa, of which 13 were assigned to species level, corresponding to Chordata (appendicularians and salps), Cnidaria, Chaetognatha, and Ctenophora. Based on the total of OTUs assigned to species level, we found for *N. coriiceps*: 1 species of appendicularian, 3 species of salps, 1 species of ctenophora, 11 species of cnidarians, and 3 species of chaetognaths. For *N. rossii*, we found OTUs assigned to

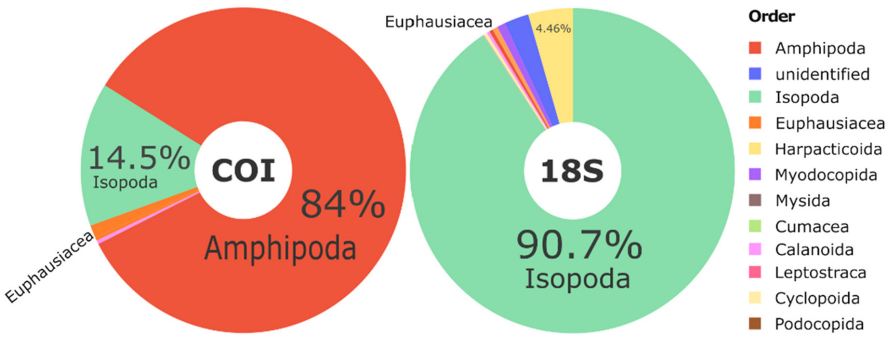

FIGURE 4 Arthropod reads in RRA% recovered for both fish species in the COI (left) and 18S dataset (right).

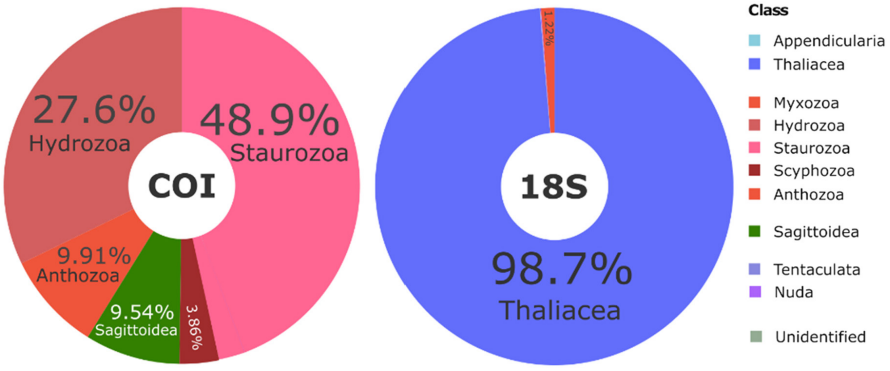

FIGURE 5 Reads assigned to gelatinous taxa (RRA%) recovered for both fish species in the COI (left) and 18S (right) dataset.

1 species of appendicularian, 4 species of salps, 1 species of ctenophora, 9 species of cnidarians, and 3 species of chaetognaths.

3.4 | Multimarker approach

Metabarcoding of *N. rossii* and *N. coriiceps* stomach contents, using COI and 18S markers, recovered 192 versus 508 OTUs, respectively, and 69 versus Totally 83 species were assigned to COI and 18S sequences, respectively (these numbers include parasites). Nevertheless, the species-level assignments were almost all incongruent among markers, which is shown in Tables 1 and 2, where prey species lists of *N. rossii* and *N. coriiceps* were pooled together. From the complete list of species, only 8 were identified with both markers: the chlorophyte *Bathycoccus prasinos*, the mollusks *Nacella magellanica*, *Cyamiomactra laminifera* and *Clio pyramidata*, the nemerteans *Parborlasia corrugatus* and *Antarctonemertes valida*, the krill species *Euphausia superba* and *Thysanoessa macrura* (Figure S4). When focusing on gelatinous taxa, none of the species found with COI were shared with 18S metabarcoding results, only at the genus level, the staurozoan (Hydrozoa) *Haliclystus* and the chaetognath *Pseudosagitta* were present in both datasets. At family level, only Campanulariidae was annotated with both markers, at class level these were the Anthozoa, Scyphozoa, Staurozoa, Hydrozoa, and Tentaculata; and at phylum level Ctenophora, Cnidaria, and Chaetognatha were sequenced with both markers.

3.5 | Multivariate analysis

Visual inspections of each NMDS plot did not reveal a clear pattern among diets for both markers (COI and 18S, see Figure S3). PERMANOVA outputs showed that there are no differences among the composition of diets considering all the factors and the interactions between them for the COI marker. Although the pairwise comparisons of this marker did not show significant differences between the diet of the specimens, a clear trend was identified: both nototheniids consumed higher proportions of rhodophytes and amphipods from the family Pontogeneiidae (i.e., *Gondogeneia antarctica*, *Bovallia gigantea*, *Prostebbingia brevicornis*, *Djerboa furcipes* and *Eurymera monticulosa*) at the 12–20 m depth strata in comparison to other sampling depths. Regarding the 18S marker, adonis analyses showed a significant effect of the fish size regardless of the species ($r^2 = 0.04$, $p < 0.05$). Pairwise PERMANOVA showed a significant difference between the "small" specimens (total length ≥ 21.0 ≤ 29.9 cm) and the "medium size" specimens (total length ≥ 21.0 ≤ 29.9 cm; $r^2 = 0.04$, $p < 0.05$). Even though *Salpa thompsoni* was detected as a main prey for all fish size classes, the "small" specimens consumed a minor proportion of this item; this fish group's stomachs contained a higher proportion of *Desmarestia* and *Ectocarpus* (Ochrophyta) and the cnidarians (*Myxidium*) than the other groups. Besides, *Glyptonotus antarcticus* (isopod) was mainly preyed upon by fishes from the "medium" size group.

4 | DISCUSSION

We provide here the first high-resolution taxonomic approach to the diet of two key demersal fish species, *Notothenia rossii* and *N. coriiceps*, in Potter Cove, South Shetland Islands, based on multimarker DNA metabarcoding, with special attention to the role of easily digestible prey that may have been overlooked in previous studies. In this context, we were able to test our hypotheses, confirming that gelatinous zooplankton (GZP) taxa commonly occur in the diets of both species. Although we expected *N. rossii* to have a greater diversity of GZP prey taxa than *N. coriiceps*, given its migratory habits within the water column, both species consumed salps, appendicularians, cnidarians, chaetognaths and ctenophores in similar read proportions and frequency of predation, with salps being the most abundant prey group.

4.1 | *Notothenia rossii* and *Notothenia coriiceps* are omnivorous

Both species showed a wide spectrum of prey groups in their diet, including a diverse set of metazoans and algae. The most represented groups were Arthropoda, Chordata (with salps being the most abundant), Rhodophyta, Mollusca, Chlorophyta, Annelida, and Ochrophyta. The importance of algae in the diet of *N. rossii* and *N. coriiceps*, which inhabit Antarctic coastal areas is broadly acknowledged (Moreira et al., 2020 and references herein), not only in Potter Cove but also in other areas of the western Antarctic Peninsula (compiled in Barrera-Oro, 2002). Our findings, using COI and 18S, also revealed the importance of primary producers like *Desmarestia* sp. in both notothenioids' diets. Our results showed a similar pattern to that found in previous studies based on traditional methods, where, in addition to krill, notothenioids also consumed a wide array of other taxa including amphipods, copepods, squid and other fish (Barrera-Oro, 2003; Barrera-Oro et al., 2019, Hollyman et al., 2021; Moreira et al., 2020, 2023; Stefanov, 2022). Noteworthy, the results obtained from the DNA metabarcoding approach revealed the important role gelatinous taxa, mainly salps (in terms of frequency of ingestion and read dominance), in the diet of these notothenioids.

Diet dissimilarity analyses showed that, within the four factors considered (species, total length, sampling depth and sex), only for the 18S marker the fish size influenced the diet composition of both notothenioids. The diet overlap between *N. rossii* and *N. coriiceps* throughout their ontogeny has already been evaluated at Potter Cove. Early juveniles, juveniles and adult stages showed between 45% and 55% of diet overlap (Barrera-Oro, 2003; Moreira et al., 2014). Our results align with these findings, as according to COI, *N. rossii* and *N. coriiceps* share 41 prey items at species level, and 23 at class level, and according to 18S, 39 prey items at species level and 31 at class level were present in the diet of both species, see Figure 6. Although the food overlap between species seems to be high and may reflect competition under conditions of limited resource availability, it was addressed in previous studies that there is

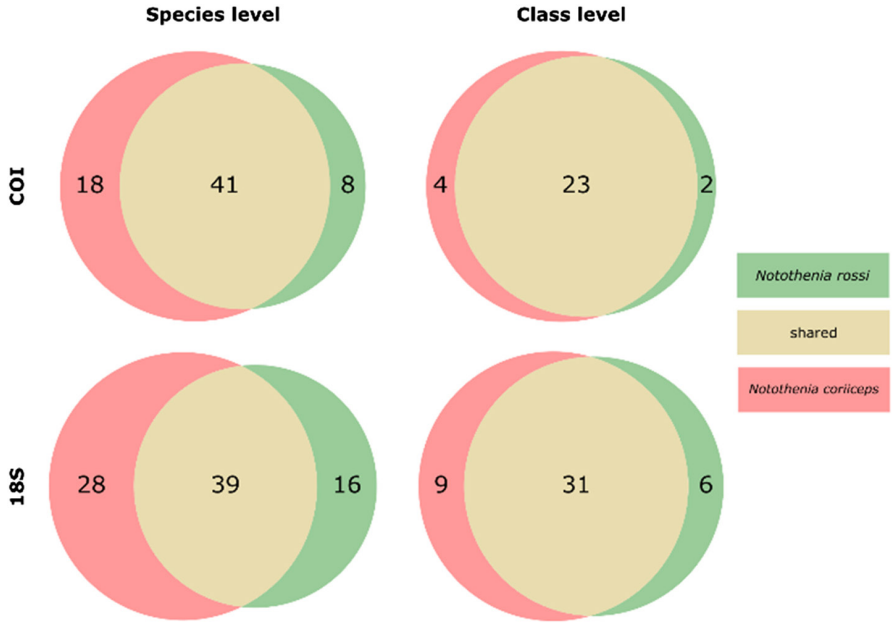

FIGURE 6 Venn diagrams, comparing the number of prey items at species and class level for *Notothenia rossi* (in green) and *N. coriiceps* (in red), shared items are shown in yellow.

no substantial competition but utilization of different feeding strategies and a specific selection of the prey items (Barrera-Oro, 2003; Gröhsler, 1994; Moreira et al., 2014).

In the literature, it was described that *N. rossii* feeds on benthic and pelagic prey while *N. coriiceps* feeds on a wide range of benthic organisms (Barrera-Oro et al., 2019). On the contrary, based on the DNA reads that could be assigned to species level, we found slightly higher number of benthic prey taxa for *N. coriiceps* compared to *N. rossii* (COI: 33, 18S: 36 vs. COI: 28, 18S: 25), for pelagic prey taxa, *N. coriiceps* also fed on a higher number of species than *N. rossii* (COI: 16, 18S: 27 vs. COI: 11, 18S: 18); while the number of bentho-pelagic prey or species switching between both realms throughout their ontogeny showed no clear differences among predators (COI: 27, 18S: 28 vs. COI: 24, 18S: 29). Thus, based on the presence data obtained in this study we can assume that both fish species prey on benthic and pelagic taxa (Figure 3).

As mentioned before, we detected a wide trophic spectrum of different taxa belonging to 17 phyla, from primary producers like *Desmarestia* sp. to highly abundant invertebrates such as the amphipod *Djerboa furcipes* or the isopod *Glyptonotus antarcticus*. All these taxa were already described to be very abundant in Potter Cove, where the characterization of community composition and their changes over the last decades has been addressed implementing a broad spectrum of techniques from molecular tools to models (Abele et al., 2017; Marina et al., 2018; Sahade et al., 2015; Wiencke et al., 2008). This wide breath of existing knowledge on this particular inshore community constitutes a unique opportunity to pursue ecosystem monitoring in order to detect and predict probable future changes that might impact the structure and dynamics of its local food web. Focusing on the study of keystone species within the food web will certainly help to address these challenges. Therefore, coupling previous information and the results obtained in this study, we propose that *N. rossii* and *N. coriiceps* could act as "natural samplers" of Potter Cove ecosystem and this could also apply for other coastal assemblages of the Northern Antarctic Peninsula where the species are distributed.

4.2 | The importance of a multimarker approach

Metabarcoding of COI and 18S fragments recovered 192 versus 508 OTUs, respectively, and 69 versus 83 species were assigned to COI and 18S OTUs, respectively. Nevertheless, the species-level assignments were almost all incongruent among markers (see Tables 1 and 2, and Figure 6). Similar results were obtained in other multimarker metabarcoding studies (Pappalardo et al., 2021; van der Reis et al., 2018, Wangensteen et al., 2018), where the output provided by different markers were complementary. These incongruences can be explained by two main factors: differential amplification and sequencing success of each marker for specific groups, and taxonomic coverage of their associated reference databases.

Even though the mitochondrial COI gene is the most commonly used marker in metazoan (meta)barcoding studies, providing high resolution taxon discrimination, COI was often less successfully amplified than other markers (e. g. Pappalardo et al., 2021; van der Reis et al., 2018). This is related to the fact that the high degeneracy of the DNA regions in the protein-coding COI gene limit universal amplification in eukaryotes (Deagle et al., 2014); and this is particularly true for tunicates, where the high mutation rate in the primer binding sites can affect the amplification success (Goodall-Copestake, 2014, 2017; Ruiz et al., 2020). On the other hand, the nuclear 18S marker is frequently used to target a broad spectrum of eukaryotic phyla because it has highly conserved regions (so-called stem regions of the ribosomal RNA gene), yet it suffers often from a lower discriminatory power compared to mitochondrial markers (Clarke et al., 2017; Wangensteen et al., 2018). In our results, the resolution of 18S v1-2 output was comparable to that of COI, but different taxa were detected with the two datasets, with salps being

the most evident difference among our results, as they were only annotated with 18S. Previous studies have shown a good relationship between the 18S read abundance of ctenophores and cnidarians, and morphological stomach inventories (Günther et al., 2018, 2021). Moreover, compared to COI, gelatinous taxa were better identified in diet studies when implementing 16S (Ayala et al., 2018), or 18S (Ayala et al., 2018; Günther et al., 2021; McInnes et al., 2017; van der Reis et al., 2018), with salps (Thaliacea) and ctenophores barcoded with a significantly higher success using 18S v1-2 than COI (Günther et al., 2018; Pappalardo et al., 2021).

The taxonomic coverage of reference databases is a clear issue that explains, in part, the different results among COI and 18S, but also it is evident when looking at the percentage of unassigned OTUs at least at phylum level (72% for 18S vs. 37% for COI), an issue already addressed by other authors (Leray & Knowlton, 2016; Wangensteen et al., 2018). Database gaps are not equally distributed among metazoan groups, e.g., when checking salp sequences on DUFA and BOLD databases (used in this study for COI), only sequences of *Salpa thompsonii* were present there; the remaining salp species detected with 18S (*Ihlea racovitzai*, *Salpa maxima*, *Iasis cylindrica*) do not have COI sequences in those repositories yet. Nevertheless, BOLD and PR2 are more reliable databases than NCBI, given that there is a growing effort to curate them (Hebert et al., 2003; Porter & Hajibabaei, 2018; Radulovici et al., 2021). The expansion and curation of the DNA reference databases (and making them public) is necessary in order to optimize the use of DNA metabarcoding outputs in general (Cristescu, 2014; Radulovici et al., 2021).

Finally, parasites, including nematodes, platyhelminthes and acanthocephala, were amplified successfully with the 18S v1-v2 fragment, however grossly overlooked with the COI sequencing. These reads were not included into the diet analysis given that parasites are not fish prey items, but can add valuable information given that the parasitic intestinal fauna is poorly studied for these notothenioids species (Muñoz & Rebolledo, 2019).

4.3 | Intraspecific prey diversity identified with 18S v1–v2 sequences

With the 18S metabarcoding data, we obtained multiple OTUs for the following species: *Salpa thompsoni* (28 OTUs), *Ihlea racovitzai* (6), *Salpa maxima* (36), *Glyptonotus antarcticus* (19), *Gaimardia trapezina* (11), *Nacella magellanica* (14), *Pseudosagitta lyra* (8), and 20 further (see Tables S1 and S2). This can be explained in two different ways: the different OTUs represent cryptic species or at least genetically divergent subpopulations, or those OTUs were clustered into different units to bioinformatic artifacts. The cryptic species hypothesis is supported by the fact that many planktonic species that were thought to be widely distributed exhibit significant genetic structure and possibly represent cryptic species complexes (Dawson & Jacobs, 2001 for Scyphozoa; Govindarajan et al., 2005 for Hydrozoa). In the Southern Ocean, numerous cryptic species have been described over the last decades, for example, cryptic species were discovered for *Glyptonotus antarcticus*, which presents four divergent groups distributed around Antarctica (Held & Wägele, 2005).

Considering the possibility that the multiple OTUs same species are a bioinformatic artifact, we have to consider the following. As it was stated above, our processed ASVs were clustered into OTUs using swarm2 with $d=4$. This means that after denoising (detecting erroneous sequences and merging them into a mother sequence), we combined the ASVs into meaningful biological entities aiming to approach species level (OTUs) (for more discussion about why to denoise and cluster see Antich et al., 2021 and Alberdi et al., 2018). The d value is the clustering distance threshold, or maximum number of differences allowed between two OTUs (Mahé et al., 2014), thus it should take into consideration the variability of the fragment. The 18S v1-v2 region is highly variable within 18S for metazoans (Hadziavdic et al., 2014). Here we used $d=4$, as in Günther et al. (2021) who analyzed stomach contents of tuna fish using the 18S v1-v2 region. Nevertheless, in previous studies $d=1$ value was tested in ribosomal DNA and has been implemented in dietary studies when amplifying 18S v7 (e.g., Guardiola et al., 2015). Similarly, van der Reis et al. (2018) sequenced the 18S v4 fragment and used $d=2$ for clustering. These approaches yielded multipleOTU per species, from which a representative OTU sequence was chosen for the analyses. In this study, we used all the different OTUs obtained for the same species when running the diversity and multivariate analysis, expecting to address the highest variability possible.

Finally, the taxonomic assignment was performed here through the Naive Bayesian Classifier algorithm using the PR2 database, with a threshold of 97% similarity. This is widely used algorithm in literature and it is supported by the fact that 18S fragments v2, v4 and v9 yielded the highest taxonomic resolution at cut-off values ranging from 95–100% sequence identity (Hadziavdic et al., 2014). Nevertheless, it is evident that the reference database has to be optimized for the southern hemisphere diversity and particularly for the Southern Ocean. For example, in our results, 14 OTUs were assigned to *Nacella magellanica*, but it is known that this is not a very abundant species in Antarctica, and particularly in Potter Cove, where *Nacella concninna* is the most abundant species of the genus (de Aranzamendi et al., 2011), thus it is likely that the assignment was erroneous. We propose that a better adjustment of d clustering value and an increased coverage of the regional database for the Southern Ocean has to be developed in order to be confident with the results obtained with 18S DNA metabarcoding, in terms of number of species and taxonomic assignments.

4.4 | Making visible the invisible: Detection of salps as a major prey

The class Thaliacea (comprising mainly salps) was the most represented prey in the diet of both fish species, reaching 98.7 RRA% for 18S. *Salpa thompsoni*, *Ihlea racovitzai*, and *Salpa maxima* were the prey items that showed the highest values (e.g., *S. thompsoni* represented for *N. coriiceps*: 73.55 RRA% and 100 FOO%, and for

N. rossii: 62.76 RRA% and 100 FOO%). In Potter Cove, the ingestion of salps was registered by morphological analysis for *N. coriiceps* (2.8 weight as percent of the total prey items), but not for *N. rossii* (data collected from the end of winter 1985 to autumn 1986, $n = 992$; Barrera-Oro, 2003; Casaux et al., 1990). More recently, at the same site and also with the same approach, an increase in the ingestion of salps as occasional and secondary prey in both notothenioid species was registered (0.02–7.2% for *N. rossii*, 0–16.54% for *N. coriiceps*; Barrera-Oro et al., 2019). Likewise, in the neighbor locality of Admiralty Bay, King George Island/Isla 25 de Mayo, salps constituted 35.8% and 43.3% of the total prey weight of *N. coriiceps* and *N. rossii*, respectively (data obtained during winter 1977 and summer 1979/1980, $n = 683$; Barrera-Oro, 2003; Linkowski et al., 1984). Even though our results comprise samples collected only in one summer (2022), it is noteworthy that by implementing DNA metabarcoding we could show that the importance of salps in the diet of *N. coriiceps* and *N. rossii* is higher than previous visual inspections of the diet suggested. This striking new finding demonstrates that salps comprise an important link in the trophic web of Potter Cove, given that notothenioids are key nodes in its food web (Marina et al., 2018).

Salpa thompsoni is the most abundant salp in Antarctic waters, and can be an important component for the pelagic realm during spring and summer through the formation of extensive and dense blooms, comprising up to 90% of the total zooplankton biomass by fresh mass north of 62° S during the austral summer (Casareto & Nemoto, 1986; Perissinotto & Pakhomov, 1998). This species has been shown to perform vertical migrations in offshore waters, reaching between 0 and 75 m depth during the night, and between 200 and 300 m at daytime (Casareto & Nemoto, 1986). Thus, it is not surprising that notothenioids prey on very abundant species like *S. thompsoni*, that can also be found in deep or benthic environments. Moreover, as it was mentioned in section 3.3, amphipods also constituted an important diet component for both fish species (84 RRA% for COI). Hyperiid amphipods have been described as symbionts of GZP and this association is often specific, involving salps, siphonophores, scyphozoans, and ctenophores (Madin & Harbison, 1977; Ohtsuka et al., 2009). This is the case of the *Vibilia* species that were registered in this study in the diet of both notothenioids (see Table 1), which are exclusively associated with salps, and a single salp can be colonized by many adult amphipods (Havermans et al., 2017). Therefore, GZP and particularly salps, may represent prey aggregating systems for predators targeting their more lipid-rich crustacean symbionts, which may be one of the reasons that explains the presence of salps in the diet of predators. Moreover, salps show the highest nutritional values among all gelatinous taxa (Dubischar et al., 2006, 2012; Henschke et al., 2016; Thiebot & McInnes, 2020).

Even though the food web in Potter Cove is very well studied, and salps have been included in local food web models (Marina et al., 2018), their importance has been substantially underestimated. Salps have historically been ignored because they are difficult to sample and assumed to be unimportant in food webs and biogeochemical cycles based on their gelatinous body structure. However, recently, it was demonstrated that they play a major role in carbon sequestration (especially in the Southern Ocean, Décima et al., 2023; Phillips et al., 2009) and are key components of marine food webs as a food source for at least 202 species including fish, turtles, and crustaceans (Henschke et al., 2016). In this way, our results are validating the paradigm shift from GZP, and especially salps, being considered as "survival food" to being considered a "regular" prey item for two demersal fish key stone species, and thus playing an important role in the Antarctic food webs.

Finally, it is worth mentioning that salps show a high nrDNA copy number (Jue et al., 2016), particularly *S. thompsoni* shows 3 times more copy 18S:16S ratio than the congeneric *S. fusiformis* (9:1 vs. 3:1; Goodall-Copestake, 2018); thus, the high proportion of reads related to salps found in this study can also be influenced by the potential inter-specific differences in the target gene copy number. Further investigation is crucial to understand and correct for these biases, ensuring more accurate ecological assessments of the importance of salps in environmental samples analyzed using DNA metabarcoding.

4.5 | Salps on the menu: Perspectives in the context of climate change-driven species shifts

In recent years, an increase of salps occurrence in Potter Cove, as well as in their morphological identification in the diet of the notothenioid species inhabiting the cove, was registered (Barrera-Oro et al., 2019). Simultaneously, a decrease in krill consumption by the same fishes was registered based on the traditional identification method (Casaux et al., 1990; Moreira et al., 2014, 2023). Likewise, our results, based on multimarker metabarcoding analyses, indicate that krill did not represent an important prey item for *N. rossii* and *N. coriiceps* during the summer season in 2022, while salps comprised the most represented prey item in the 18S output. At this stage, it is not possible to disentangle whether these differences are related to method-related biases or climate-change induced changes in feeding patterns, since this is the first metabarcoding study on notothenioid diets in Potter Cove. Indeed, researchers have noted that the lack of gelatinous zooplankton (GZP) in specimens is likely attributable to the preservation method employed, gelatinous prey tend to disintegrate during the freeze/thaw process, especially impacting ctenophores, which were frequently observed in fresh stomach contents (Hollyman et al., 2021). Further metabarcoding studies on historical and newly collected notothenioid samples, coupled with monitoring of zooplankton biomass in the region, would be needed to provide evidence for such a climate-change-driven shift in prey. Predation on GZP has been registered in many marine species, being more common in the Arctic and the Antarctic than in lower latitudes (Thiebot & McInnes, 2020). It has been previously reported in *N. rossii* diets at South Georgia (Davenport, 1998; Hoshiai, 1979; Tarverdiyeva, 1972) and Potter Cove (Barrera-Oro, 2002, 2003; Barrera-Oro et al., 2019). Of all gelatinous prey items, salps are the most frequently recorded in the diet of invertebrate predators, and this may be related to their

high abundance and their high carbon and protein content in comparison to other GZP (Carroll et al., 2019; Dubischar et al., 2012; Henschke et al., 2016; Thiebot & McInnes, 2020). Alternating sexual and asexual reproduction, *Salpa thompsoni* can reach high population densities under favorable environmental conditions such as poor sea-ice cover in winter (Daponte et al., 2001). This pattern contrasts sharply with that of krill, where the decline in krill populations is related to the reduction of ice during the winter (Atkinson et al., 2017, 2019; Loeb et al., 1997). Thus, in face of a global warming trend (IPCC, Shukla et al., 2019), with presumably higher frequency of warm winters and less sea-ice coverage, we can expect more salps blooms and a poorer development of krill populations, This scenario presents salps as a potential alternative prey to Antarctic krill in the Southern Ocean food web (McCormack et al., 2021; Queirós et al., 2024). Hence, it is important to invest effort in detecting possible changes in predator–prey dynamics of these competing species in the next decades.

5 | CONCLUSIONS

In the face of the accelerated climate change that the Southern Ocean and, in particular, the Antarctic Peninsula and surrounding islands are experiencing, understanding trophic links and energy flow, and their associated changes, has become a research priority. This multimarker metabarcoding approach provides new insights into the trophic ecology of these two notothenid species, which are also of commercial interest. Here, we confirm the relevant consumption of salps and reveal the important, but so far overlooked, role that gelatinous zooplankton has as a prey in the diet of both fish species. This study provides a temporal and spatial snapshot of the dietary patterns of *N. rossi* and *N. coriiceps*. We recommend establishing a multi-annual survey of notothenid diets based on a combination of COI and 18S metabarcoding, in which these fish function as natural samplers of climate-change-driven shifts in diversity, distributions and abundances that the Southern Ocean species. Such an effort may shed further light on the shift from a krill-dominated to a salp-dominated local food web through the replacement of krill by salps as major prey and the impact this may have on energy transfer to higher trophic levels.

AUTHOR CONTRIBUTIONS

The conception or design of the study: MBR, CH, FL. The acquisition, analysis, or interpretation of the data: MBR, CH, EM, MN, SN. Writing of the manuscript: MBR, CH, EM, MN, FL.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) in the framework of the priority program SPP 1158 "Antarctic Research with comparative investigations in Arctic ice areas" by the following grant HA 7627/3-1, LE 2323/11-1. CH is supported by the Helmholtz Young Investigator Group "ARJEL – Arctic Jellies" with the project number VH-NG-1400, funded by the Helmholtz Society and the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research. The authors want to particularly thank the members of the Carlini (former Jubany)—Dallmann staff. Logistic and financial support for the sample collection and processing was also provided by the Dirección Nacional del Antártico, Instituto Antártico Argentino (PICTA 0100), and Fondo para la Investigación Científica y Tecnológica [PICT 2018-03310 Res.401/19]. Finally, we would like to thank the reviewers, whose efforts have greatly improved the manuscript. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All raw sequences have been stored in the Sequence Read Archive (SRA) under accession numbers BioProject ID PRJNA1047314, and BioSamples ID SAMN38529455-SAMN38529651.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ruiz, M. B., Moreira, E., Novillo, M., Neuhaus, S., Leese, F., & Havermans, C. (2024). Detecting the invisible through DNA metabarcoding: The role of gelatinous taxa in the diet of two demersal Antarctic key stone fish species (Notothenioidei). *Environmental DNA*, *6*, e561. https://doi.org/10.1002/edn3.561