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Comparison of fish prey contribution in the diet of European hake by visual assessment of stomach contents and DNA metabarcoding

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Abstract Diet studies are critical for ecosystem-level understanding of fish populations and a move toward ecosystem-based management of marine resources. European hake, *Merluccius merluccius*, a demersal mesopredator species, is one of the most important commercial fish in the Mediterranean, and as a predatory species it controls the food web. Here, we characterize the diet of *M. merluccius* with two different analytical methods, visual assessment of stomach contents and DNA metabarcoding. Seasonal and size-based differences in fish prey composition were investigated. Both methods produced complementary results with regard to fish prey contribution of European hake diet. The visual assessment approach showed significant differences between adult and juvenile hakes, while the metabarcoding approach indicated high seasonality in fish prey contribution and richness in its diet. An increase in fish prey diversity was found for both adult and juvenile hakes during the summer. *Trachurus* sp. was detected as the dominant fish prey in both methods. A broader range of fish prey species was detected by metabarcoding due to its ability to identify highly digested items from the stomach. Combining visual assessment with a novel metabarcoding approach has the potential to improve our understanding of trophic ecology, which can be useful for ecosystem-based management.

Keywords *Merluccius merluccius* · Fish prey · Seasonal shift · Next generation sequencing · Sea of Marmara

Introduction

Stomach content studies of fish allow knowledge to be gained on feeding behavior, local biodiversity, species interactions, and trophic positioning of predators and prey (Gerking 1994; Buckland et al. 2017). It is difficult to identify highly digested food items through visual assessment of stomach contents (VASC) if there are no hard pieces remaining (for example, otoliths, claws, and shells) (Hyslop 1980). Even considering these difficulties, VASC is still the most widely used and accepted method (Carreon-Martinez et al. 2011; Amundsen and Sánchez-Hernández 2019).

A relatively new topic of research is dietary characterization based on DNA and other trophic interactions (Symondson and Harwood 2014). The diet of a predator can be determined using polymerase chain reaction (PCR)-based molecular examination of stomach

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contents, which can identify species-specific DNA fragments after digestion (Symondson 2002). Using taxa-specific primers targeting short non-conserved DNA fragments and next-generation sequencing platforms, researchers can now examine the prey diversity of any predator using DNA extracted from semi-digested stomach contents (Leray et al. 2013). In this manner, the feeding behavior of fish based on prey choice enhances our knowledge of trophic interactions and community dynamics within aquatic ecosystems (Amundsen and Sánchez-Hernández 2019). Until recently, marine trophic interactions have been studied using high-throughput sequencing (HTS) to a limited extent (Leray et al. 2013). As the metabarcoding approach has become more affordable, there has been a great leap forward in DNA sequence generation using next-generation sequencing platforms that generate millions of short DNA amplicon sequences from heterogeneous mixtures, such as environmental samples (Yu et al. 2012) and semi-digested stomach contents. In addition to the fact that DNA sequence analysis may identify species that do not have recognizable physical traits, it is a good tool for automated laboratory and bioinformatics processing. In stomach content studies (Aritomi et al. 2017), morphological investigation can offer numerical indices, such as the percentage of each recognizable prey. The prey must first be evaluated quantitatively to fully elucidate the energy assimilation (Newman et al. 2010). In recent decades, there has been an increase in the use of DNA metabarcoding to study fish diet composition using HTS (Berry et al. 2015), which provides greater diversity and taxonomic resolution, as well as an increased chance of uncovering rare species (Riccioni et al. 2018; Casey et al. 2019; Bachiller et al. 2020; 2021).

The European hake *Merluccius merluccius* plays an important role in the food web; with its high trophic level (4.4), it is considered an important predator in the demersal zone (Sinopoli et al. 2012; Saygu et al. 2023). It is distributed in the northeastern Atlantic and Mediterranean Seas (Froese and Pauly 2022). Several studies have been conducted on the biology and ecology of the western and central Mediterranean (Murua 2010; Arancibia et al. 2015; Martínez-Baños et al. 2018), spatial distribution patterns, habitat preferences, recruitment (Abella et al. 1997; Caddy 2015; Khoukh and Maynou 2018), and stock assessment and management (Aldebert and Recasens 1996; Rätz et al. 2013; Druon et al. 2015; Piroddi et al. 2020). However, limited knowledge is available on hake biology, ecology, and stocks in the Eastern Mediterranean Sea (Gucu and Bingel 2011; Tserpes et al. 2016; Demirel et al. 2020).

In the Mediterranean Sea, European hake is targeted with shrimp fisheries and multispecies fisheries through bottom trawling and is also an important fish for small-scale fisheries (SSF) that use gillnets and longlines (Bensch et al. 2009). Both species share similar benthic habitats (Zengin et al. 2004). Numerous stomach content studies on *M. merluccius* with regard to its economic and ecological importance in the Mediterranean have demonstrated dietary shifts on both spatial and temporal scales (Ferraton et al. 2007; Rueda et al. 2019; Lloret-Lloret et al. 2020), with size (Bozzano et al. 1997; Mellon-Duval et al. 2017; Carrozzi et al. 2019; Lloret-Lloret et al. 2020), and evaluated predator–prey relationships (Modica et al. 2011). In the past two decades, in addition to visual stomach content analyses, metabarcoding (Riccioni et al. 2018) approaches have been used to better understand the trophic ecology of this species. *M. merluccius* is considered an opportunistic predator (Mellon-Duval et al. 2017) and ontogenetic diet shifts are common in *Merluccius* species. Ontogenetic shifts mainly occur at a range of 20 cm in total length (TL), depending on the population (Carrozzi et al. 2019). Juvenile individuals feed on small crustaceans, especially mysids, and adults feed mainly on planktivorous fish (Carpentieri et al. 2005; Fanelli et al. 2018; Carrozzi et al. 2019). Anchovies and sardines are reported to be the main fish prey in the Mediterranean Sea, and there is high variability in the abundance of these small pelagic fish across subregions (Ferraton et al. 2007; Stagioni et al. 2011; Sinopoli et al. 2012; Mellon-Duval et al. 2017; Carrozzi et al. 2019).

Here, we investigated the contribution of fish prey to the diet of *M. merluccius* using two complementary dietary examination methods: VASC and metabarcoding analysis (MA). We also compared the efficiencies and results of both methods by quantifying the seasonal and size-based differences in fish prey composition and richness. Our study demonstrates that the DNA-based dietary method provides taxonomically precise prey classification.

Materials and methods

Study site and sampling procedure

This study was conducted in the Sea of Marmara (SoM), in the northeastern part of the Mediterranean Sea (Fig. 1). The sampling locations are target areas for small-scale shrimp fisheries and industrial purse-seiners, also containing a mussel farming facility, and high tourism activities during the summer season.

Sampling operations were performed in three seasons (December 2018, April 2019, August 2019, and August 2020) using beam trawl for 30 min at 2.5 knots between the depths of 25 and 151 m using a commercial shrimp fishery vessel. A total of 322 M. *merluccius* individuals were captured as by-catch during the sampling campaign. For VASC, 228 stomach were immediately extracted and preserved in 4% buffered formaldehyde on board. For MA, 47 stomachs were dissected and preserved in 95% ethanol at – 20 °C. TL of all captured individuals was measured on board to the nearest 0.1 cm. Average TL was 27.1 cm, ranging between 12.4 and 47.0 cm.

Visual assessment of stomach contents (VASC)

Each prey item found in the stomachs was sorted, weighed, and identified to the genus or family level. *M. merluccius* diet was quantified using numerical percentage (%N) and wet weight percentage (%W) of each prey contribution, as well as frequency of occurrence (%F). Two functional groups were used to describe the main taxonomic groups (Teleostei, Invertebrata), which also allows for the inclusion of unidentifiable prey items in the estimation of trophic metrics. Index of relative importance for each prey item ($IRI = \%F \times (\%W + \%N)$) (Hyslop 1980) was standardized to allow comparisons between prey groups and factors. The IRI (%IRI) equation used was $\%IRI = (IRI/\sum IRI) \times 100$ (Cortés 1997). Results of number of non-empty stomachs were used in all calculations. Vacuity index (VI) was used to calculate the percentage of empty stomachs following the formula $VI\ (\%) = A*100/B$, where A is the total number of empty stomachs and B is the total number of examined stomachs (Hyslop 1980).

Metabarcoding (MA)

DNA was extracted from 47 stomach samples that were preserved in ethanol. Stomach samples were frozen in liquid nitrogen and homogenized using metal beads in a bead ruptor. To avoid swamping the sample with a single taxon, we attempted to subsample the homogenate as representatively as feasible. Following the manufacturer's recommendations, DNA was extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, Germany). Forensic DNA level precautions (Kemp and Smith 2005) were modified according to literature (Sepulveda et al. 2020) and applied in order to avoid contamination. Sampling equipment was sterilized by exposure to 20% commercial bleach (sodium hypoclorite 6%, hydroxide of sodium 0.3%)

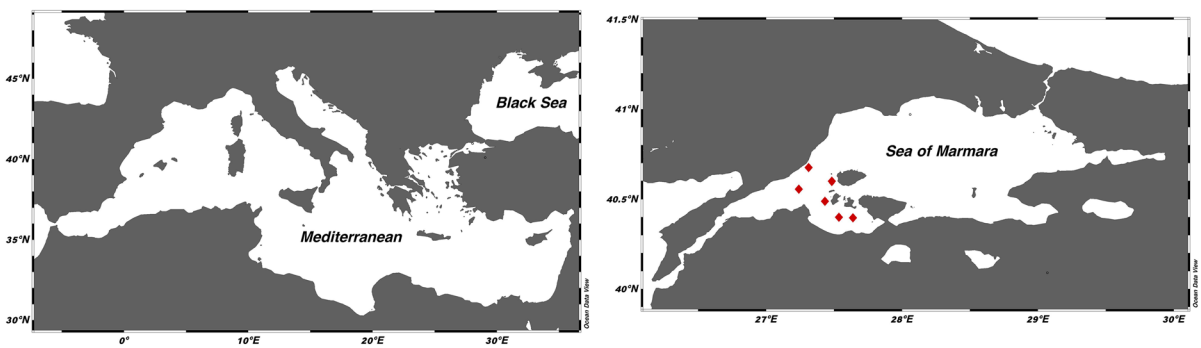

Fig. 1 Location of the Sea of Marmara in the Mediterranean basin (left panel) and sampling locations of European hake (right panel)

for 24 h. Equipment was washed with autoclaved and UVC-treated ultra-pure H2O. All equipment and cabins were irradiated with UV for 40 min before and after each round of sample processing.

One-step PCR (Taberlet et al. 2018) was used to amplify DNA templates using 12S MiFish-U (Miya et al. 2015) metabarcoding primers for fish (only fish prey was targeted as ≥ 90% of the gut contents were found to be fish). In the one-step PCR procedure, 8 nucleic acids were added to the 5′ end of the forward and reverse primer sequences in various combinations. Each 8-nucleotide molecular tag was distinct from the others by at least three nucleotides. To improve read quality, prevent index sequence degradation, and increase the number of reads per sequence cycle, at least one non-specific nucleotide (N) was added to the beginning of the tags once they were designed. The tags are added to the 5′ end of the primer sequence (NN + Tag + Primer) after they were generated, and the ambiguous nucleotides were inserted. In a total reaction volume of 25 µl, PCR amplifications were carried out with 12.5 µl of Amplitaq Gold 360 master mix, 1 µl of each primer (F, R), 2 µl of template DNA, and 8.5 µl of ultrapure water. Final concentrations were optimized according to PCR master mix protocol as 0.2 µM and 1 µl of each primer (5 pmol). The thermal cycler was set at 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 20 s at 72 °C, with a final extension of 7 min at 72 °C. In accordance with the multiple-tube technique, all PCR amplifications were carried out in triplicate (Taberlet et al. 1996; Goldberg et al. 2013). Negative control samples were also included in the process to ensure that contamination was not introduced. The PCR products were run on a 2% agarose gel to confirm successful amplification of the correct-sized target amplicon. AMPure XP beads were used to purify PCR yields.

The libraries were measured, standardized, and pooled after two rounds of PCR clean up before paired end sequencing on an Illumina MiSeq platform.

Bioinformatics

OBITOOLS (Boyer et al. 2016) software package was used for the bioinformatics workflow. The quality of the sequences in the fastq format from the MiSeq device and the main statistics about these sequences were checked with the FastQC tool (Andrews 2010). Forward and reverse reads corresponding to the same sample were aligned and combined by illuminapairedend code, which takes into account the coupling quality (phred score ≥ 30). After the forward and reverse sequences were merged, de-multiplexing of samples with different tags in the same fastq file was performed with the ngsfilter and obisplit commands and sequence data were prepared for processing separately and filtered according to count (≥ 10) and sequence length (minimum of 150 bp). We calculated the cut-off value of the read count based on the maximum read count in the negative controls, which was 9. Therefore, we used ≥ 10 reads as a read number cut-off for each sample. This approach is also common in the related literature (e.g., Yamamoto et al. 2017; Gehri et al. 2021). Prior to filtering, raw data were checked for such species exclusion related to insertion and deletions which were not detected in our study. More general filters as low as 100 base pairs were applied and checked. Finally, all sequences were submitted to NCBI GenBank as a batch megablast file to make taxonomic assignments, in which species level identifications based on similarities ≤ 98% were removed from the dataset.

Data analysis

VASC and MA results were analyzed according to season (winter, spring, and summer) and maturity level (adult > 25 cm and juvenile < 25 cm). Maturity classes for *M. merluccius* were chosen according to recent reproductive studies from SoM (Kahraman et al. 2017). Metabarcoding data were evaluated using two metrics, i) frequency of sequence occurrence, which is the percentage of total prey taxon in individual stomach (Ow%); and ii) relative abundance of operational taxonomic unit (OTU). OTUs are clusters of sequences based on similarity that are produced during bioinformatics processing (Nguyen et al. 2016). Here we used the proportion of unique OTU to the final OTU in each stomach as an associated index to occurrence data. Both metrics were informative for prey assemblage among seasons and between sizes in *M. merluccius* diet.

A one-way PERMANOVA to a matrix of fish prey items based on number (N) for VASC and abundance of OTU for MA were applied using Bray–Curtis similarity matrices with 9999 permutations. If significant results were found, pair-wise comparisons were carried out. Principal coordinate analysis (PCoA) was used to assess and visualize dissimilarity of significant variables

in diet. Fish prey were superimposed as vectors based on Pearson rank correlation (>0.5). Multivariate analyses were performed with PRIMER7+PERMANOVA software (v.7.0; PRIMER-E).

Results

Prior to data analysis, prey accumulation curves were constructed to determine whether sample size was sufficient for a representative description of the diet. Sample order was randomized across 999 permutations by non-parametric estimator (Jacknife 1) using PRIMER v7 (Clarke and Gorley 2006). Although the number of stomachs for MA was found to be sufficient to describe fish prey in the diet, the accumulation curves for VASC did not reach a plateau (Fig. 2).

Diet composition based on VASC

A total of 228 stomachs (108 juveniles, 110 adults) were analyzed. Vacuity index (VI) was calculated as 46.9% for all samples, 52.8% for juveniles, and 40.2% for adults. Significant differences for VI% were found among seasons ($H(2)=7.71$, $p<0.01$), with the highest in winter (65.2%) and lowest in spring (34.0%). Teleosts were the dominant prey group in hake diet both for adults and juveniles in all seasons (IRI > 50%, Table 1). The horse mackerel *Trachurus* sp. was the most dominant teleost prey species for adults in winter and spring seasons (IRI 62.3% and 94.7%, respectively), while juveniles preferred other teleosts, also demonstrating cannibalism with 20% IRI in winter.

PERMANOVA results for stomach contents revealed that dietary preferences were significantly different between juveniles and adults (pseudo-$F=6.526$, $p=0.003$), among seasons (pseudo-$F=6.190$, $p=0.001$) and for season and size (pseudo-$F=2.486$, $p=0.039$). According to pair-wise tests, summer season was significantly different in prey composition than winter and spring ($t=2.041$ $p=0.014$, $t=3.296$ $p=0.001$, respectively). For season and size, only spring was significantly different in fish prey composition for juveniles and adults ($t=3.385$ $p=0.001$). The principal coordinate analysis (PCoA) plot based on the Bray–Curtis dissimilarity was computed using cumulative prey composition (N) of hake diet. PCoA reflected

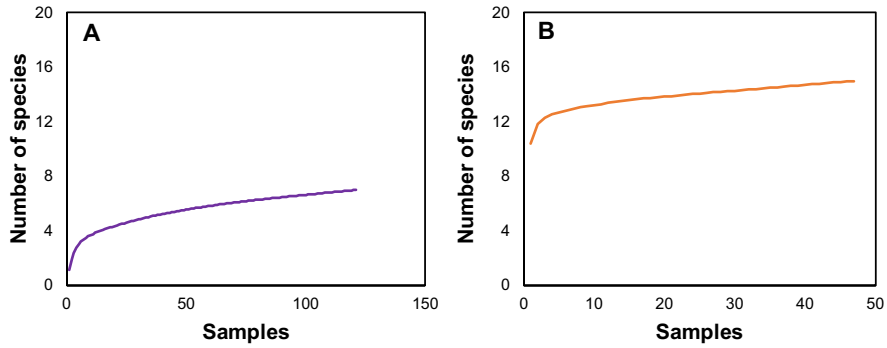

Fig. 2 Sample-based fish species richness curves for **a** the visual assessment and **b** metabarcoding data

Table 1 Results of the visual assessment of stomach contents. Main prey groups in *M. merluccius* diet (*F%*, frequency of occurrence; *N%*, number of prey items; *W%*, weight of prey items; *IRI%*, standardized relative importance)

Prey items	Juvenile (≤25 cm)				Adult (>25 cm)			
	F%	N%	W%	IRI%	F%	N%	W%	IRI%
Trachurus sp.	5.88	4.84	6.17	0.88	44.29	35.90	54.58	61.07
Engraulis encrasicolus	7.84	6.45	9.07	1.65	1.43	0.85	0.49	0.03
Merluccius merluccius	5.88	4.84	5.57	0.83				
Gaidropsaurus sp.	1.96	1.61	7.39	0.24				
Parablennius sp.					1.43	0.85	3.19	0.09
Unidentified Teleostei	62.75	53.23	56.36	93.08	38.57	27.35	29.43	33.38
Overall Invertebrata	25.48	29.03	15.44	3.33	35.71	35.03	12.31	5.43

that the first (PCo1) and the second (PCo2) components explained 88.6% of the total variance, with a clear shift being apparent for size and season. Superimposed correlation vectors (>0.5) indicated *Trachurus* sp. were responsible for dietary dissimilarities between juvenile and adult hake samples, while other fish prey and invertebrates were responsible for seasonal dissimilarities mainly in summer season between size (Fig. 3).

Metabarcoding results by fish primers

DNA taken from 47 hake (18 juveniles and 29 adults) stomachs was amplified and sequenced. Although no visible bands were detected on agarose gels for negative controls, they were tagged and sequenced with other sample pools to see if low-abundance contaminants could be detected. No sequences with tags assigned to negative control samples were found after filtering for QC, minimum count, and minimum length. A total of 19,486 reads obtained from sequences with tags assigned to negative control samples were removed from the data set after filtering for QC, minimum count, and minimum length. Samples were also checked for sequences from distinct samples in the sequencing pool that may have been recombined due to tag jumping, but none were detected.

A total of 10,741,542 sequencing reads were dereplicated into 782 distinct OTUs after quality control, filtering, and the removal of uncommon haplotypes. Only 73 (10%) of the 782 OTUs were recognized to the genus or species level. Prey species were identified by removing prey sequences identified above genus level and combining identical taxa with separate barcode sequences (Table 2).

The most frequently (total 100% Ow, Fig. 4a) detected prey species in *M. merluccius* diet were *E. encrasicolus*, *Sardina pilchardus*, *Atherina hepsetus*, *Trachurus trachurus*, and *Symphodus cinereus*, respectively. Samples collected in winter contained high occurrence of *Coris julis* and *Gobius pagenellus* ($N=11$, 100%, and 91% O_w, respectively, Fig. 4a). *Scomber scombrus* was only detected in winter samples. Contrarily, two species, *Pomatoschistus minutus* and *Lesueurigobius friesii*, were absent in winter samples, but detected in all samples collected in spring and summer ($N=36$, 100% Ow, Fig. 4a). While there was no clear difference in the occurrences of identified prey species

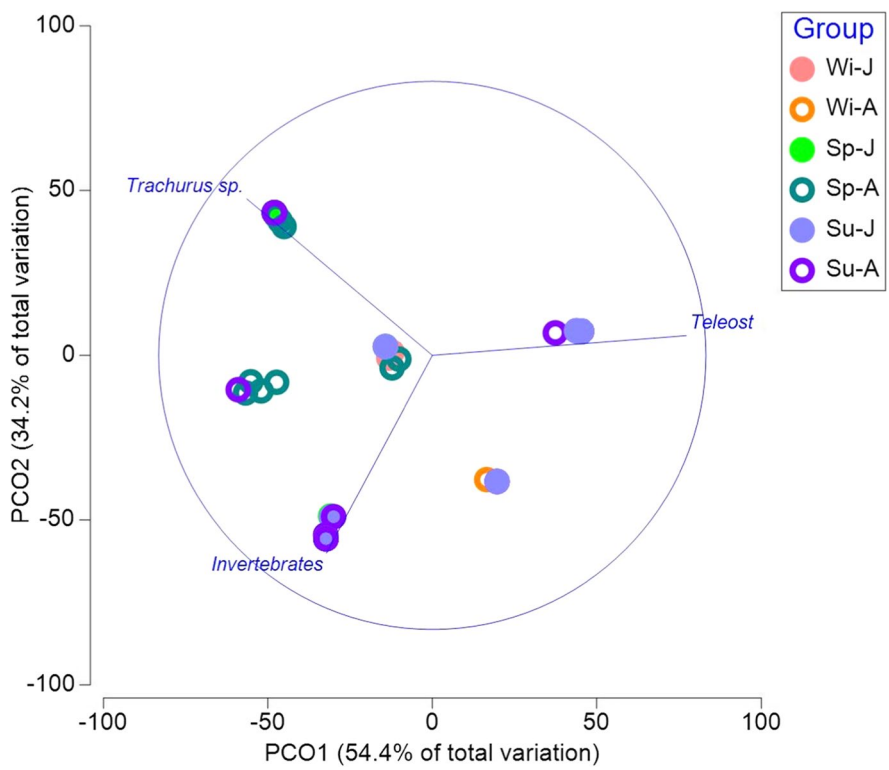

Fig. 3 Results of principal coordinate analysis (PCoA) derived from visual assessment of stomach content composition based on number of prey species (N) in *M. merluccius* according to maturity (juveniles, dots; adults, circle) and season (winter, orange; spring, green; summer: purple) in the Sea of Marmara. Superimposed vectors of prey groups (correlation > 0.5) are indicated by blue lines

Table 2 Presence–absence of fish prey taxa identified in *M. merluccius* stomach contents via metabarcoding (MA) and morphological approaches (VASC), according to season

Species	Overall		Winter		Spring		Summer	
	VASC	MA	VASC	MA	VASC	MA	VASC	MA
Atherina hepsetus	+	+	−	+	−	+	−	+
Balistes capriscus	−	+	−	−	−	+	−	+
Chelon labrosus	−	+	−	−	−	+	−	+
Coris julis	−	+	−	+	−	+	−	+
Engraulis encrasicolus	+	+	+	+	+	+	+	+
Gaidropsaurus sp.	+	−	+	−	−	−	−	−
Gobius paganellus	−	+	−	−	−	+	−	+
Parablennius sp.	+	−	−	−	−	−	+	−
Lesueurigobius friesii	−	+	−	−	−	+	−	+
Macroramphosus scolopax	−	+	−	+	−	+	−	+
Merluccius merluccius	+	−	+	−	+	−	+	−
Pomatoschistus minutus	−	+	−	−	−	+	−	+
Sardina pilchardus	−	+	−	+	−	+	−	+
Scomber scombrus	−	+	−	+	−	−	−	−
Scyliorhinus canicula	−	+	−	−	−	+	−	−
Solea solea	−	+	−	−	−	−	−	+
Symphodus cinereus	−	+	−	+	−	+	−	+
Trachurus trachurus	+	+	+	+	+	+	+	+

between individuals (Fig. 4a), a large variation in the relative abundance of OTU existed between samples. Some fish prey were represented by very low or very high relative abundance. Particularly the species with very high values were *E. encrasicolus* (0.05–99.9%), *S. pilchardus* (0.001–99.9%), *A. hepsetus* (0.001–49.9%), and *T. trachurus* (0–99.9%, Fig. 4b). In 47 samples, 11 mainly comprised *E. encrasicolus* and *T. trachurus* (50.1–99.1%), two were dominated by *S. pilchardus* (99.4–99.9%), and the remaining samples mainly comprised *A. hepsetus*, *P. minutus*, and *L. friesii* (max. 49.9%, Fig. 4b). There was an obvious difference in hake diet among seasons, and winter and spring seasons were dominated by *E. encrasicolus* and *T. trachurus*, while prey diversity was highest in summer having no clear dominant species (<50%, Fig. 4b).

PERMANOVA results and pair-wise comparisons for metabarcoding results showed that diet preferences were significantly different between seasons (pseudo-$F = 12.301$, $p = 0.001$), and for season and size (pseudo-$F = 2.699$, $p = 0.018$). In pair-wise tests, prey compositions in all seasons were significantly different ($p < 0.05$). The differences were mainly found in winter in which prey diversity was very low and dominated by *Trachurus* sp. and *E. encrasicolus*, while a gradual increase was observed from spring to summer. In addition, for season and size pairwise tests, only summer was marginally insignificant in fish prey composition between juveniles and adults ($t = 1.511$, $p = 0.06$). The principal coordinate analysis (PCoA) plot based on the Bray–Curtis dissimilarity was computed on the OTU of hake diet. PCoA reflected that the first (PCo1) and the second (PCo2) components explained 70.3% of the total variance with a clear shift among seasons. Superimposed correlation vectors (> 0.5) indicated *T. trachurus* and *E. encrasicolus* were responsible for dietary dissimilarities between juvenile and adults in winter and spring samples, while *S. cinereus*, *P. minutus*, *L. friesii*, and *A. hepsetus* were responsible for dissimilarities in summer season (Fig. 5).

Discussion

Two dietary examination approaches were used to investigate the composition of fish prey in the stomachs of *M. merluccius*. Both methods led to complementary knowledge regarding diet. Based on the VASC results, our study confirms that *M. merluccius* is a piscivorous predator that feeds on various pelagic, demersal, and benthic prey taxa. Contrary to

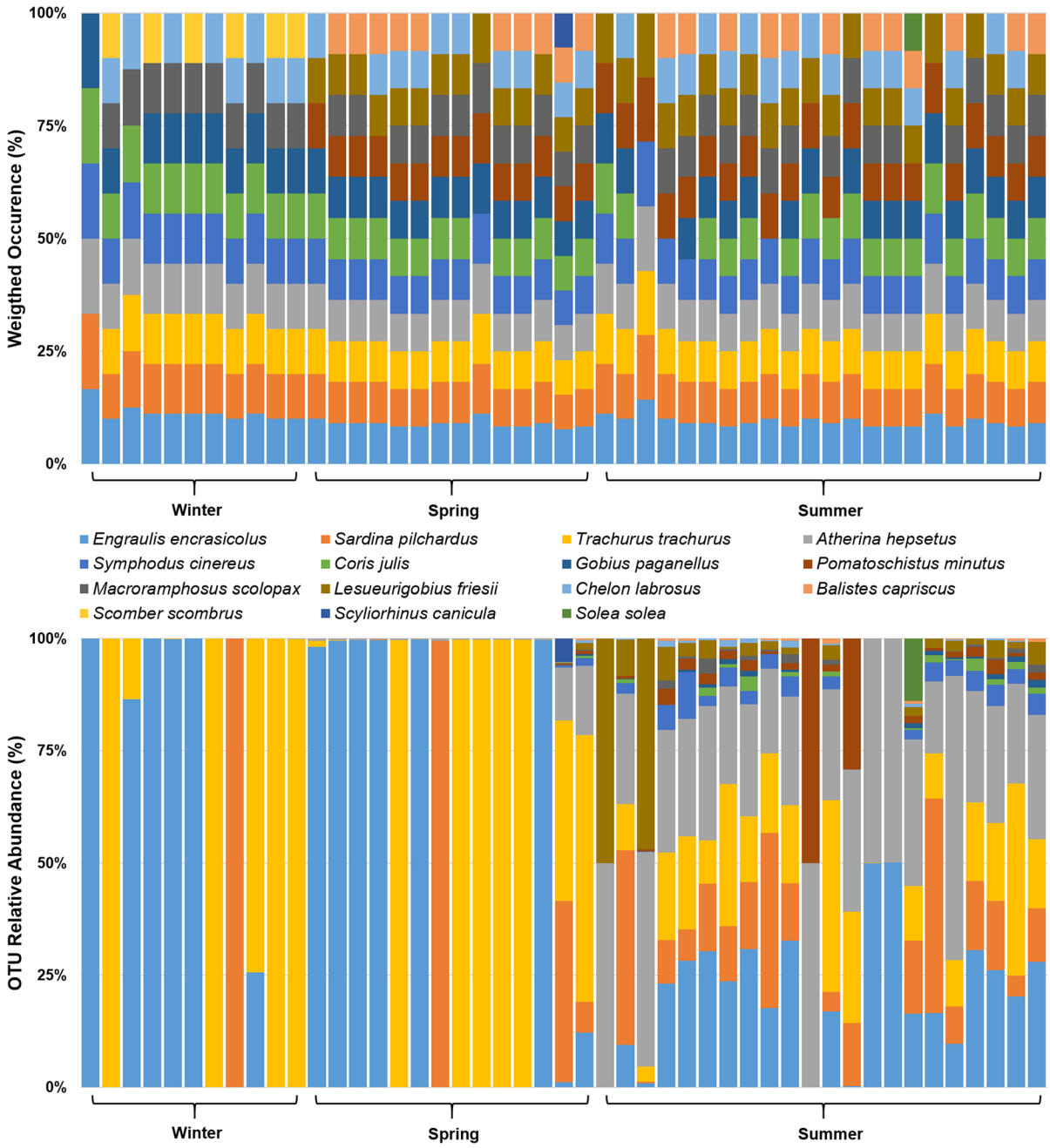

Fig. 4 Weighted occurrence (Ow%) and relative abundance of OTU of fish prey in the stomach of *M. merluccius* according to three seasons detected using the 12S rRNA. Bars represent each hake stomach ($n = 47$), colors represent 15 different fish prey

other studies, the vacuity index was higher in juveniles than in adults (Carrozzi et al. 2019; Zorica et al. 2021). This may be due to the prey capture capacity in adult piscivore (D'lglio et al. 2022). On the contrary, the vacuity index in feeding studies may also be indication that different size groups distribute at diverse depths (Morato et al. 2003; Gül and Demirel 2022). Ontogenetic dietary changes by size were observed through VASC, and benthic invertebrates were consumed more by juveniles, whereas

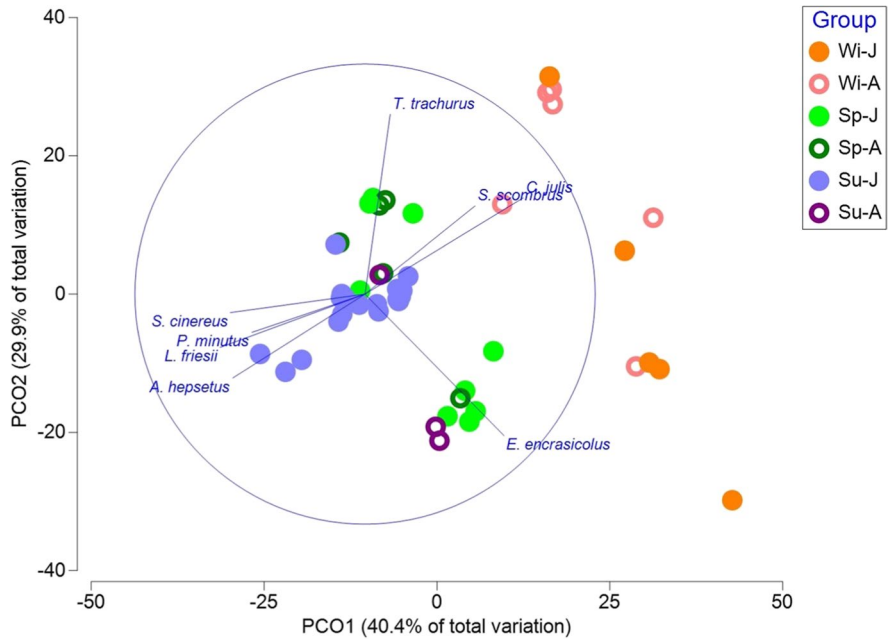

Fig. 5 Results of principal coordinate analysis (PCoA) derived from metabarcoding analysis based on OTU in *M. merluccius* by maturity (juveniles, dots; adults, circle) and season (winter, orange; spring, green; summer, purple). Superimposed vectors of prey species (correlation > 0.5) are indicated by blue lines

Trachurus sp. was the main prey of adults. Seasonal differences in prey composition were observed in adults and juveniles.

In this study, we only used fish primers in the metabarcoding approach; thus, MA results revealed seasonal changes in prey composition, with higher diversity of fish prey in the summer. However, no clear size-based variation was found, which we interpreted as owing to the lack of invertebrate contribution in our MA data, as juvenile diets contain important invertebrate contributions. With the possibility of greater taxonomic precision when more sequences and longer reads become available, metabarcoding may be able to identify food items at taxonomic levels similar to morphological dietary analyses. Furthermore, metabarcoding discovered predation on taxa such as larval fish that is unlikely to be accounted for by morphological dietary analyses, demonstrating that predator diets are more diversified than previously believed (Ley et al. 2014).

Specifically, compared to VASC, we identified six different fish prey, of which two were at the species level, three at the genus level, and one belonged to the functional group of Teleostei containing unidentified highly digested fish prey, whereas MA was able to identify 15 fish prey to the species level. Our results are consistent with those of previous studies, which reported that clupeids, carangids, and gobies were the main fish prey taxa (Mellon-Duval et al. 2017; Riccioni et al. 2018; Carrozzi et al. 2019). In addition, *M. merluccius* is known for its cannibalistic behavior, and we also observed *M. merluccius* as prey only in juvenile stomachs with high IRI% in winter. However, owing to the nature of MA, the DNA of the studied species itself was ignored and discarded from the identified prey list. Our strategy to limit self-contamination while extracting stomach contents was to avoid including the tissues of the predator species itself to the greatest extent possible. As a result, DNA for PCR may be recovered from portions of prey tissues that have been isolated, simultaneously resulting in possible loss of data owing to cannibalistic behavior, either through extracted DNA or the material being bioinformatically below the copy number/identification rate thresholds.

Surprisingly, the MA results for European hake fish prey composition revealed the presence of the previously non-resident fish species *Macrorhamphosus scolopax* in the SoM. According to the most recent and comprehensive checklists for fish found in Turkish and Aegean waters (Bilecenoğlu et al. 2014; Gerovasileiou et al. 2017), this species was present in the Aegean Sea and in the greater Mediterranean Sea but has not been previously recorded in the SoM (Froese and Pauly 2022). As a transition zone between the Mediterranean and Black Seas, the

benthic environment of the SoM reflects the characteristics of the Mediterranean Sea (Cinar et al. 2021). Therefore, it is unclear whether *M. scolopax* may be resident in the SoM, but has not been recorded yet, or whether its occurrence in the diet of *M. merluccius* throughout the sampling period may be due to hake migration between the SoM and Aegean Sea, as DNA traces from fish prey eaten would be present several days after consumption (Carreon-Martinez et al. 2011; Lazic et al. 2021) in another sea. The trophic linkages within complex marine ecosystems can be better understood when diets are sampled at diverse times of prey availability and a more representative dietary analysis is performed by combining VASC with metabarcoding.

There are advantages to the DNA metabarcoding procedure used here because this method can be applied to larger-scale research as it permits simultaneous assessment of the diet composition of many individuals. As a result, the quality and breadth of the reference databases are critical to DNA-based dietary analysis, which is still in its infancy and hindered by a lack of completeness in these databases (Lazic et al. 2021). Such efforts should be performed simultaneously with conventional VASC to develop DNA databases for unrecognized groups (Hajibabaei et al. 2007). Diet metabarcoding is generally associated with inadequate DNA reference databases (Burgar et al. 2014), but morphological analysis also faces issues where knowledge is lacking, digestion eliminates crucial diagnostic features, or vouchers are unavailable (Alonso et al. 2014). When databases have been incomplete, researchers have adopted the MOTU strategy (Symondson and Harwood 2014). This incompleteness of databases may occur at the family level or lower, which supports the need to create reference databases for DNA barcodes with high resolving power (Deagle et al. 2014), particularly those that are appropriate for metabarcoding semi-digested prey items. Specific MOTUs and MOTU diversity can be monitored to offer metrics for ecosystem change, even in the absence of reference sequences.

Other disadvantages to metabarcoding include amplification bias, which can affect the results of fish diets (Deagle et al. 2014). There are inherent biases involved in primer selection when interpreting metabarcoding data, and the amplification output does not perfectly match the original DNA (Casey et al. 2019). Sequence clustering, where the number of OTUs or species detected is dictated by the sequence similarity cut-off used, and taxonomic assignment, which uses a sequence identity threshold of 98%, are two examples of bioinformatics analysis phases that can introduce bias. This threshold was determined (Miya et al. 2015) to recover the species composition of stomach material to the fullest extent possible, while avoiding incorrect taxonomic classification. However, the most challenging aspect is that a species must be pre-recorded in the DNA reference library for matching. According to our evaluation, the percentage of OTU findings using relative abundance as a proxy for relative prey volume was comparable to weighted percentage of occurrence or relative abundance of OTU, indicating that they can differentiate between seasonal changes. Research using DNA may be skewed by secondary consumption, which reveals the prey consumed by the main prey; this is affected by digesting speeds, which can lead to inaccuracies. DNA from secondary prey is expected to comprise a smaller percentage of total OTU reads when compared to primary prey (Jakubavičiūtė et al. 2017; Taberlet et al. 2018), but the actual contributions will vary by instance; therefore, standard error indices cannot be used. Our findings are consistent with recent research (Riccioni et al. 2018), which suggests that the relative abundance of OTU can be utilized as an auxiliary dataset because of its high performance in representing the contribution of prey to the ecosystem. In addition to examining the implications of varied resolutions among various ecosystem components, future studies should assess the effects of higher-resolution food webs on the operation of ecosystem models. The sequencing work needed to generate these high-resolution data is easily manageable by conventional dietary investigations. To maximize the utility of HTS, morphological and DNA techniques must be combined. Understanding the underlying taxonomy of significant food groups will be a major benefit for morphology, as it would make more reference DNA sequences available to more precisely reflect dietary composition in future analyses.

Dietary analyses are crucial for understanding the ecological roles, trophic positions, and linkages of marine organisms. New high-throughput technologies should be incorporated into dietary research to inform fishery management and to contribute to marine conservation. Owing to the limitations of the spatial and temporal scopes of novel approaches, combining the

isotope analysis and metabarcoding approach with long-term dietary studies using conventional methods will enhance our knowledge on the ecological roles and functions of species.

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Author contribution GG, EK, and ND: data curation, visualization, formal analysis, investigation, writing—original draft preparation, and writing—reviewing and editing. ND: supervision, funding acquisition, and conceptualization.

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Data availability The NCBI BioProject database was used to store all of the DNA metabarcoding reads of prey species collected from the stomach contents, with the BioProject ID PRJNA807866.

Declarations

Ethics approval Fish samples in this study were collected through commercial shrimp fisheries in the Sea of Marmara. All fishes were retained and used as per international guidelines for the care and use of animals.

Conflict of interest The authors declare no competing interests.

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