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ORIGINAL PAPER

Diet of two mediterranean shearwaters revealed by DNA metabarcoding

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Abstract

Information on seabird diet is key to understanding their ecological role in the marine food web. The Mediterranean Sea is a biodiversity hotspot that is experiencing a series of growing threats, including overfishing and climate change. The Scopoli's (*Calonectris diomedea*) and Yelkouan shearwaters (*Puffinus yelkouan*), two marine predators in the region, are expected to have a piscivorous diet and exploit fishery discards, but their exact reliance on different resources is still unclear. We sampled four populations in the central Mediterranean Sea and used a combination of DNA metabarcoding and stable isotopes to compare their diets and assess trophic niches. We found prey items from 38 families belonging to 21 orders. Clupeiformes and Perciformes were the main prey groups identified in both shearwater species. In fact, diet composition largely overlapped and differed by only 3% variation in the diet consumed at order level and 16% at genus level, despite sampling different populations. The results suggest high overlap of dietary and isotopic niches, while Yelkouan shearwaters occupied a wider niche space overall. Certain taxa were potentially derived from discards but are also available naturally as juvenile fish or in foraging associations with marine megafauna such as predatory fish and turtles. These findings highlight the strong dietary overlap and ecological similarities between Scopoli's and Yelkouan shearwaters, emphasising the importance of understanding their foraging dynamics in the context of resource competition and the increasing pressures on Mediterranean marine ecosystems.

Keywords Stable isotopes · Trophic level · Niche · Foraging ecology · *Puffinus yelkouan* · *Calonectris diomedea*

Introduction

Seabirds hold a significant function at the top of the marine food chain and are widely recognised as sensitive indicators of the marine ecosystem (Furness and Camphuysen 1997). The study of seabird diets provides fundamental context for their ecology and the use of seabirds as indicators within ecosystem management (Lewison et al. 2012; Carreiro et al. 2023). The Mediterranean Sea is a hotspot of marine biodiversity, which is threatened by intense anthropogenic pressures (Colloca et al. 2017; Piroddi et al. 2020; Clark et al. 2023). Its unique oligotrophic and heterogeneous oceanographic features include a summer thermocline that affects productivity (Zotier et al. 1999). Therefore, it is especially interesting to reveal the diet and trophic niches of marine predators in the region.

The Scopoli's shearwater (*Calonectris diomedea*) is a common and widely distributed species breeding in the Mediterranean Sea, while the smaller Yelkouan shearwater

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(*Puffinus yelkouan*) is distributed mainly in the central and eastern areas of the basin. The two species belong to the family *Procellariidae* and while Scopoli's shearwaters generally forage at or close to the surface (maximum dive depth 5.4 m) (Grémillet et al. 2014; Cianchetti-Benedetti et al. 2017), Yelkouan shearwaters regularly dive deeper (maximum dive depth 30.2 m) (Péron et al. 2013). The two species display high trophic plasticity, with a diet ranging from zooplankton to a variety of small to medium sized pelagic fish, which is often their main prey, but also crustaceans and squids (Péron et al. 2013; Afán et al. 2014; Grémillet et al. 2014; Thabet et al. 2019). Moreover, foraging shearwaters are often associated with other marine megafauna which either drive (predatory fish) or attract (turtles) fish to the surface (Michel et al. 2022). In fact, the decline of Atlantic bluefin tuna (*Thunnus thynnus*) has been suggested as a reason for Yelkouan shearwaters from Sardinia to repeatedly travel to distant foraging areas, rather than foraging in waters closer to their colonies (Pezzo et al. 2021).

In addition to natural foraging, shearwaters in the Mediterranean interact with fishing vessels, feeding on bait and discards (Arcos and Oro 2002; Karris et al. 2018; Michel et al. 2022). In Scopoli's shearwaters, Cianchetti-Benedetti et al. (2018) reported that 40% of tracked individuals interacted with fisheries, while Michel et al. (2022) observed such interactions on 16% of trips. The level of interaction between Yelkouan shearwaters and fisheries has not been quantified yet (Bicknell et al. 2013). However, interaction is expected due to documented accidental bycatch (e.g. Cortés et al. 2018). While fisheries do to some extent offer an alternative food source through the supply of discards (Arcos and Oro 2002; Church et al. 2019), overall intensive fishery activities are depleting resources available to seabirds (Grémillet et al. 2018). The reduction in prey is further aggravated by climate change, which impacts prey distribution, seasonality (mismatch in timing of prey availability and seabird breeding) and size (Baudron et al. 2014; Pistorius et al. 2022). Due to the impact of variation in food resources on seabird demography (Louzao et al. 2006), identification of diet and comparison within predatory guilds is an imperative but challenging aspect to seabird conservation (Barrett et al. 2007; Deagle et al. 2010; Carreiro et al. 2023).

The pressure on Mediterranean marine food webs is particularly marked, both through overfishing (Colloca et al. 2017) and climate change (Schickele et al. 2021). When competitors with shared limited resources coexist in a community, one or more dimensions within their niche is often adjusted resulting in niche segregation (Hutchinson 1957, 1959). Petalas et al. (2024) review niche segregation in sympatric seabirds and present three niche spaces, each with multiple dimensions: dietary, isotopic and spatial, urging research to include measurements within more than one niche space. There is potential competition for resources between Scopoli's and Yelkouan shearwaters because their breeding seasons and foraging areas overlap (Cecere et al. 2013; Cianchetti-Benedetti et al. 2018; Gatt et al. 2019). Based on studies on their foraging behaviour (Péron et al. 2013; Grémillet et al. 2014; Cianchetti-Benedetti et al. 2017), there might be a segregation due to dive capacity.

In addition to stable isotope analysis (SIA), genetic analysis of faecal samples is a prominent tool that has emerged during the last two decades and allows for improved identification of seabird diet (Barrett et al. 2007; Deagle et al. 2007). Primers specific to prey groups are applied within PCR assays to amplify prey DNA from dietary samples. The resulting amplicons are sequenced and matched with genetic reference libraries to provide a relatively detailed taxonomic and semi-quantitative assessment of prey composition. DNA analysis of faecal samples can give a detailed picture of the prey taxa consumed within the previous days (Deagle et al. 2010). On the other hand, SIA can reflect a longer period and a broader picture on trophic status and preferred foraging areas (inshore vs. offshore) (Hobson et al. 1994).

In this study, we firstly aimed to describe the diet of Scopoli's and Yelkouan shearwaters in the Central Mediterranean through DNA metabarcoding of faecal and regurgitate samples. We aimed to discuss these results in relation to previous studies on the shearwater species' diet and methodological limitations. By using a fish-specific primer, we aimed to increase the taxonomic precision for this prey type specifically and discuss if fishery discards could be facilitating access to certain taxa. We then aimed to investigate potential niche segregation within dietary items and blood stable isotope values of these two shearwaters. We expected overlap and a similar overall diet composition due to similar trophic level values found in previous studies applying stable isotope analysis, but variations in specific prey species due to different foraging behaviour. We also expected isotopic niche segregation in breeding stages that temporally overlap in the two species, driven by potential competition.

Methods

Sampling procedures

The breeding seasons of the two shearwater species are offset by approximately two months. On Malta, the Yelkouan shearwater generally lays its single egg at the end of February until mid-March, hatching occurs between end of April and mid-May, and fledging takes place by early July. The Scopoli's shearwater lays the egg in the second half of May, hatching occurs mid-July and fledging takes place in October. Hence, incubation of the Scopoli's shearwater is concurrent to the chick-rearing of Yelkouan shearwaters. Sampling occurred during both incubation and chick-rearing of the two shearwater species.

We sampled Scopoli's shearwaters at three colonies in the Central Mediterranean, Linosa (35.865, 12.865), Malta (35.900, 14.400) and La Maddalena (41.230, 9.410), while all Yelkouan shearwater samples were collected on Malta (Table 1; Fig. S1). We collected faecal samples with cloacal swabs and opportunistically when defecation occurred during handling. In the case of Scopoli's shearwaters, we collected samples from only adults. For Yelkouan shearwaters, we collected samples from both adults and from nestlings/fledglings. We also collected fresh faeces from burrow substrate from locations with only Yelkouan shearwaters nesting, but could not determine whether these samples were from adults or chicks. Furthermore, we opportunistically collected regurgitate samples during capture-mark-recapture sessions from adult Yelkouan shearwaters arriving at the colony during the chick-rearing period. Faecal samples were stored in 96% ethanol while regurgitate samples were stored frozen at −18 °C.

Additionally, we collected blood samples for stable isotope analysis from adults of both species (Table 1). Blood from both shearwaters was collected during their respective incubation and chick-rearing stage. The blood was drawn from the tarsal vein, using a syringe and 25-gauge needle in the case of Scopoli's -, and 27-gauge needle in the case of Yelkouan shearwaters. Later at the station, we centrifuged the blood to separate plasma from the red blood cells (RBC) and both fractions were stored at −18 °C.

Molecular diet analysis

We extracted DNA from 206 samples in total using the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Germany) and followed manufacturer protocols. For large samples, we weighed-in between 180 and 220 mg (*n*= 21), but in most cases the entire sample, including the swab, was extracted and no sample weight could be obtained (*n*= 185). Similar to Masello et al. (2023), we added 2–3 bashing beads (ZR Bashing BeadTM 2.0 mm, Zymo Research, USA) to each sample to ensure proper homogenization during mixing in a Disruptor GenieTM (Scientific Industries SITM, USA).

We mixed each regurgitate sample from 2021 in an open tray using a spatula, and took out a small proportion from the homogenous paste for DNA extraction. In between samples, the tray and tools were cleaned with chlorine bleach and 96% ethanol. For the 2019 and 2020 samples, we took out a small proportion of the homogenous paste without any mixing. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), same as for the faecal samples. We measured nucleic acid concentration with a ThermoScientific Nanodrop 2000 micro-volume UV-VIS spectrometer for all samples (Thermo Fisher Scientific, USA) and when

Table 1 Number of dietary samples sequenced and blood samples collected from Scopoli's shearwaters (*Calonectris diomedea*) and Yelkouan shearwaters (*Puffinus yelkouan*) per colony, breeding stage, year and age. For faecal & regurgitate samples, numbers of sequenced samples and number of positive samples for prey DNA as per sequencing are given

Species	Colony	Year	Breeding stage	Age	Sample type	*N*	*N* (positive for prey DNA)
Scopoli's shearwater	La Maddalena	2020	Chick-rearing	Adult	Faecal	22	15
				Adult	Red blood cells	22	–
	Linosa	2021	Chick-rearing	Adult	Red blood cells	33	–
		2020	Incubation	Adult	Faecal	15	4
				Adult	Red blood cells	31	–
			Chick-rearing	Adult	Faecal	23	7
				Adult	Red blood cells	24	–
	Malta	2020	Chick-rearing	Adult	Faecal	8	5
				Adult	Red blood cells	15	–
Yelkouan shearwater	Malta	2021	Incubation	Adult	Red blood cells	6	–
			Incubation & Chick-rearing	Adult	Faecal	23	15
			Chick-rearing	Chicks	Faecal	34	12
				Unknown	Faecal (Burrows)	11	7
				Adult	Regurgitate	10	10
				Adult	Red blood cells	30	–
		2020	Chick-rearing	Adult	Regurgitate	6	6
				Adult	Red blood cells	11	–
		2019	Chick-rearing	Adult	Regurgitate	5	5

An additional three samples from Yelkouan shearwaters were sequenced but did not give any yields and were omitted from the table, resulting in a total of 89 and 68 successfully sequenced samples for Yelkouan and Scopoli's shearwater. N positive to prey DNA shows the number of sequenced samples which yielded prey DNA. Blood (RBC, centrifuged blood cell component) was analysed for stable isotope values of δ^{13}C and δ^{15}N concentration was over 100 ng/µl, dilution by a factor of 20 was carried out prior to PCR amplification.

In order to identify prey to the family level, we applied the Metazoa primer pair (mlCOIintF/jgHCO2198; Leray et al. 2013) in PCR amplifications on all samples (Table S1). PCR amplification and sequencing preparation was carried out in a separate lab-room to DNA extractions and under a UV clean bench (Cleaver Scientific, UK). Samples with a positive PCR result were analysed further with a primer specific to fish (12 S, Xavier et al. 2018). On Yelkouan shearwater samples only, we applied a third primer specific to cephalopods (16 S, Berry et al. 2017). We carried out an index PCR following the procedure described in Masello et al. (2023) on samples with a positive PCR result. In total, we sent 68 samples from Scopoli's shearwater and 92 samples from Yelkouan shearwaters for sequencing (Table 1), together with negative controls for each primer per study species. Sequencing was carried out on a MiSeq desktop sequencer (Illumina, USA) at SEQ-IT GmbH & Co. KG, Kaiserslautern, Germany.

Bioinformatics

Molecular operational taxonomic units (MOTUs) were identified using a custom workflow implemented in GALAXY (Masello et al. 2021; The Galaxy Community 2022), which consisted of the following steps: 1) sequence quality assessment with FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc; accessed on 12 Nov 2024), 2) adapter and quality trimming of the paired-end reads with TRIMMOMATIC (minimum quality score of 20 over a sliding window of 4 base pairs; Bolger et al. 2014), 3) merging of the overlapping paired-end read pairs using FLASH (Magoč and Salzberg 2011), 4) conversion of sequence files to FASTA with the FASTX-Toolkit (https://github.com/agordon/fastx_toolkit; accessed on 12 Nov 2024), 5) extraction of amplicons from the FASTA files in MOTHUR (Schloss et al. 2009), 6) removing identical replicates (dereplicate, plus strand), 7) detecting and removing chimeric sequences (de novo, minimal abundance ratio of parent vs. chimera 2, 'no' vote pseudo count 1.4, 'no' vote weight 8, minimum number of differences in segment 3, minimum divergence from closest parent 0.8, minimum score 0.28), and 8) clustering sequences into MOTUs, rejecting if identity was lower than 0.97, with VSEARCH (Rognes et al. 2016). To reduce index assignment errors and thus cross contamination from other samples in the batch, we applied a filtering step for index reads with a quality score threshold of 26 as recommended by Wright and Vetsigian (2016). Finally, using the BLASTn algorithm (Altschul et al. 1990), MOTU sequences were matched to reference sequences in the National Center for Biotechnology Information (NCBI) GenBank nucleotide database, using a cut-off of 90% minimum sequence identity and a maximum e-value of 0.00001.

For the taxonomic assignments, we used the percentage similarity of the query and the reference sequences, maintaining a BLASTn assignment match greater than 98%, and a minimum sequence length of 190 base pairs, as short fragments are less likely to contain trustworthy taxonomic information (Deagle et al. 2009; Vesterinen et al. 2013). MOTUs were assigned to the species level only in cases where the BLASTn assignment match was > 99%, and all retained hits of a MOTU, with the same quality criteria (sequence identity and length), corresponded to the same species. Otherwise, we assigned the MOTU to the lowest common taxonomic level (e.g. genus or family; Kleinschmidt et al. 2019). If two species resulted in the same match percentage, we assigned the next common taxonomic level. Consequently, in the case of the Metazoa primer, only family level assignments were possible. Of the identified MOTUs we only retained those that occur in the Mediterranean Sea at their assigned taxonomic level. The raw data set included a wide range of non-specific contaminant DNA (e.g., human, bacterial) that could be excluded as potential prey taxa. Based on previous literature fish, cephalopods, marine crustaceans and pelagic tunicates were considered potential prey (Sarà 1993; Bourgeois et al. 2011; McInnes et al. 2017a; Thabet et al. 2019).

Sequencing success and positive samples

A total of 251 and 120 MOTUs were obtained for Yelkouan and Scopoli's shearwaters respectively, with several identified as non-prey with an especially high number of sequences arising from the study species DNA (Procellariidae, Table 2). The yields of prey DNA varied with collection method in respect to non-prey DNA, with higher yields for opportunistic regurgitate and faecal samples and

Table 2 Sequencing results measured as the percentage of non-prey DNA sequences and prey DNA (fish, cephalopods, marine crustaceans and pelagic tunicates) for Scopoli's (*Calonectris diomedea*) and Yelkouan (*Puffinus yelkouan*) shearwaters. Valid sequences are those belonging to molecular operational taxonomic units with BLASTn assignment match greater than 98%, a minimum sequence length of 190 base pairs and more than twice the number of reads found in controls. Within range sequences refer to taxa occurring within the mediterranean range

	Scopoli's shearwater		Yelkouan shearwater	
	Total sequences	% of total sequences	Total sequences	% of total sequences
Non-prey DNA	505,090	97.86	569,880	73.36
of which Procellaridae	490,788	95.09	560,998	72.22
Prey DNA	11,041	2.14	206,910	26.64
of which Valid	8041	1.56	165,766	21.34
Within range	7987	1.55	139,520	17.96

the lowest yields from cloacal swabs (Fig. S2). Samples were retained for further analysis if any number of reads from prey MOTUs were detected, but excluded samples with non-Prey DNA only. Moreover, prey MOTUs were retained in any sample on the condition that the number of reads was larger than twice than any number of reads for specific MOTUs detected in controls. We then converted the number of reads to presence/absence values (1/0) for each MOTU. Prey MOTUs were identified in 31 Scopoli's shearwater samples and in 55 Yelkouan shearwater samples and we used these sample numbers to calculate % frequency of occurrence per MOTU (Barrett et al. 2007) for each shearwater species respectively.

Stable isotope analyses and baseline correction

The stable isotope (SI) values of carbon and nitrogen were measured on ~ 0.3 mg aliquots of dried red blood cells (RBC) weighed in tin capsules. The analysis was conducted at the LIENSs laboratory (France) using a Delta V Plus isotope ratio mass spectrometer equipped with a Conflo IV interface (Thermo Scientific, Bremen, Germany) and a Flash 2000 elemental analyser (Thermo Scientific, Milan, Italy). Calibration was performed using certified reference materials (details in Supplementary Information, Section A), with a two-point calibration method employing the working standards USGS-61 (caffeine) and USGS-63 (caffeine). Internal laboratory standards, acetanilide (Thermo Scientific) and peptone (Sigma-Aldrich) were analysed together with the samples, yielding an analytical precision of < 0.15‰ for δ^{15}N and < 0.10‰ for δ^{13}C. Results are reported in δ units, representing deviations from standards (Vienna Pee Dee Belemnite for δ^{13}C and atmospheric N_2 for δ^{15}N), using the formula:

$$\delta\,^{15}\mathrm{N}\ \mathrm{or}\ \delta\,^{13}\mathrm{C} = [(R_{sample}/R_{standard}) - 1] \times 10^3$$

where R is ^{15}N/^{14}N or ^{13}C/^{12}C, respectively. Fluctuation of δ^{13}C and δ^{15}N SI values in marine environments occur due to differences in baseline resources along a latitudinal gradient (Rumolo et al. 2016; Campioni et al. 2022). To account for this latitudinal gradient, we calculated the difference between the two reported mesozooplankton means from the Tyrrhenian Sea and the Sicily Channel, the primary foraging areas of the populations in our study, applying this adjustment to our SI values as previously described in Michel et al. (2025).

Statistical analysis

We tested for differences in community composition using permutation tests in the R package vegan (Oksanen et al. 2022). To visualise differences in diet composition between shearwater species, colonies and sample types, we employed non-metric multidimensional scaling (NMDS; Kruskal 1964) on presence/absence data with the 'metaMDS' function in the same package. NMDS ordinates the data and reduces information down to two dimensions for easier visualisation and interpretation. The 'metaMDS' function allowed us to assess how well the two-dimensional configuration matched the original data using a stress parameter, where values < 0.05 indicate excellent agreement, < 0.1 very good, and < 0.2 a good representation. In our tests, the stress values were below 0.2, indicating a good fit. To improve visualisation in NMDS plots we also used the function 'ordiellipse' to obtain the centroid per shearwater species and 'ordihull' to create polygons around the points per species. We further used permutational multivariate analysis of variance (PERMANOVA) in the function 'adonis' to assess the variation in diet between shearwater species, colonies and sample types and present the test statistics. Finally, in order to assess how well our sample sizes captured the diversity of prey, we plotted rarefaction curves for MOTUs for each shearwater species using the function 'rarecurve' in package vegan (Oksanen et al. 2022).

To calculate the trophic level, we followed the framework by Weiss et al. (2009) and the same base values as those used by Péron et al. (2013) and Thabet et al. (2019) in studies from the central Mediterranean:

$$\mathrm{TL} = \mathrm{TL}_{\mathrm{copepods}} + 1 + (\delta^{15}\mathrm{N}_{\mathrm{S}} - \delta^{15}\mathrm{N}_{\mathrm{copepods}} - \Delta d_{\mathrm{RBC}})/3.4$$

where $\mathrm{TL}_{\mathrm{copepods}}$ is the estimated TL of copepods (2.0), $\delta^{15}\mathrm{N}_{\mathrm{S}}$ is the value in respective shearwaters; $\delta^{15}\mathrm{N}_{\mathrm{copepods}}$ is the mean nitrogen isotope value of copepods in the western Mediterranean Sea (2.8‰, Cardona et al. 2012); and Δd_{RBC} is the discrimination factor for blood (2.6‰, mean from seabirds summarised in Thabet et al. (2019) and 3.4‰ is an assumed constant trophic enrichment factor (Post 2002). While different diet-tissue discrimination factors are found between for example blood and feathers, whole blood and the blood cell component of blood have close to identical isotopic signatures (Cherel et al. 2014). To test whether trophic level differed between years, species, site and breeding stages we used separate Generalised Liner models (GLM) for each categorical predictor variable. To test breeding stage and site by species we constructed composite categorical variables (e.g. "SCSH_Incubation").

To measure isotopic niche segregation, we used the package SIBER (Jackson and Parnell 2023) to calculate niche width, as Standard Ellipse Areas, of each shearwater species based on the adjusted isotope values of δ^{13}C and δ^{15}N. Since the breeding stages of the two shearwaters are temporally staggered, we specifically compared the Scopoli's shearwater incubation period with the Yelkouan shearwater chick-rearing period (temporally overlapping) and the chick-rearing periods of both species (temporally distinct). We estimated the Standard Bayesian Ellipse Area (SEA_B) (20,000 iterations, burn-in 1000, thin 10) in addition to Standard Ellipse Area corrected for small sample sizes (SEA_C) (Jackson et al. 2011). With 4000 posteriors, we expressed overlap between the ellipses as the proportion of non-overlapping area of the two ellipses. We carried out all statistical analyses within the R environment (v. 4.2.3–4.4.1) (R Core Team 2024).

Results

Prey composition of Scopoli's and Yelkouan shearwaters

Both shearwater species fed on fish, crustaceans and cephalopods, while in one Yelkouan shearwater sample we also found a pelagic tunicate (Table 3). In total, we found prey items from 37 families which belong to 21 orders (Table 3, Table S2).

We found low variation between the diet consumed by Scopoli's and Yelkouan shearwaters. With MOTUs at order level, shearwater species explained only 3% variation in the diet consumed (NMDS $F = 2.937$, $R^2 = 0.034$, $P = 0.016$; Fig. 1) and at genus level shearwater species explained 16% of the fish diet consumed (NMDS $F = 10.649$, $R^2 = 0.162$, $P = 0.001$; Fig. 2, Fig. S3). Diet at order level was largely overlapping across sample types (NMDS $F = 1.556$, $R^2 = 0.054$, $P = 0.113$, Fig. S4) and colonies (NMDS $F = 2.956$, $R^2 = 0.066$, $P = 0.006$; Fig. S5) for both shearwater species. In Yelkouan shearwaters diet overlapped for the two age classes sampled in that shearwater species (NMDS $F = 2.281$, $R^2 = 0.084$, $P = 0.061$, Fig. S6).

Overall, Perciformes contributed greatly to the diet of both shearwaters. The main differences between the consumed prey were that Scopoli's shearwaters consumed a higher proportion of Clupeiformes, while Syngnathiformes were detected more frequently in Yelkouan shearwaters (Table 3; Fig. 3). At genus level, depth-ranges of prey were obtained from fishbase.se (Froese and Pauly 2024) (Fig. 4). Moreover, at this taxonomic resolution, it is evident that *Trachurus* had a high FO in both species, but was more prevalent in the Yelkouan shearwater diet. Both shearwaters preyed similarly on *Engraulis*, while more Scopoli's shearwater individuals consumed *Sardinella*, *Auxis*, *Phycis* and *Thunnus*, and more individuals of the Yelkouan shearwater consumed *Macroramphosus*, *Mullus* and *Scomber* (Table 3; Fig. 4, Fig. S7). Rarefaction curves demonstrate that there might be higher diversity than what our sample sizes were able to capture for both shearwater species (Figs. S8 and S9).

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Trophic ecology

The means of adjusted $\delta^{13}C$ and $\delta^{15}N$ values for both shearwaters and years showed little variation, and high overlap between the species (Table 4; Fig. 5). The trophic level calculated for both Scopoli's and Yelkouan shearwaters were very similar (Table 4), with no significant differences detected by year, species or site using GLMs. This was also the case when calculating trophic level per breeding stage (Table S3), but the trophic level was higher for the incubation period in Scopoli's shearwaters (GLM parameter estimate $= 0.345 \pm 0.122$, $P = 0.050$).

Niche width was narrowest in Scopoli's shearwaters during the chick-rearing period (SEA_B mean $= 0.372$ 95% CI $= 0.296–0.449$) and widest in Yelkouan shearwaters during the chick-rearing period (SEA_B mean $= 0.821$ 95% CI $= 0.576–1.08$) (Table S3, Figs. S10 and S11). The overlap between Scopoli's and Yelkouan shearwater SEA_B in their respective chick-rearing periods was higher than the overlap between incubating Scopoli's shearwaters and chick-rearing Yelkouan shearwaters (Fig. S12).

Discussion

Diet in comparison to previous studies

With this study, we assessed the diet of two shearwater species endemic to the Mediterranean basin, focusing on individuals breeding and foraging in the central Mediterranean. Both species fed on several prey taxa, suggesting generalist and opportunistic foraging. These results are in line with previous work based on stable isotope analysis (Péron et al. 2013; Grémillet et al. 2014; Thabet et al. 2019), but through the application of DNA metabarcoding, we achieved a higher taxonomic resolution, especially for fish prey. Clupeiformes were one of the main fish groups consumed by both shearwaters and match previous studies utilising visual analysis of stomach content and regurgitates (Sarà 1993; Bourgeois et al. 2011), but we found lower FO for *Sardina pilchardus*, while similar FO for *Engraulis* sp. and also *Scomber* sp. (Perciformes). In addition to previously described fish prey, we identified *Trachurus* sp. (60% FO), *Macroramphosus* sp. (25% FO) and *Mullus surmuletus* (20% FO) among others in Yelkouan shearwater diet and *Auxis rochei* (35% FO) and *Thunnus thynnus* (13% FO) in Scopoli's shearwater diet.

Table 3 Frequency of occurrence (%FO) for the prey consumed by Scopoli's shearwaters (N= 31) and Yelkouan shearwaters (N= 55) in the central mediterranean as identified by DNA metabarcoding of faecal and regurgitate samples. The primers used were metazoa COI (Leray et al. 2013), fish 12 S (F, Xavier et al. 2018) and Cephalopoda 16 S rRNA (C, berry et al. 2017)

Phylum	Class	Order	Family	Species	Common Name	Primer(s)	%FO SCSH	%FO YESH
Arthropoda	Branchiopoda	Diplostraca	Podonidae			M	–	4
				Evadne spinifera		C	–	2
	Malacostraca	Decapoda				M, C	10	13
			Benthesicymidae	*Gennadas elegans*	Graceful blunt-tail prawn	C	–	2
			Luciferidae			M	–	2
			Munididae		squat lobsters	M	10	9
		Euphausiacea	Euphausiidae			M	–	4
Mollusca	Cephalopoda	Octopoda				M, C	–	9
			Argonautidae		argonauts	M	–	5
				Argonauta argo	Greater argonaut	C	–	4
			Tremoctopodidae		blanket octopods	M	–	5
				Tremoctopus violaceus	Blanket octopus	C	–	4
		Oegopsida				M	6	5
			Ommastrephidae		flying squids	M	3	5
			Onychoteuthidae		hooked squids	M	3	–
Chordata	Thaliacea	Doliolida	Doliolidae		pelagic tunicates	M	–	2
	Actinopterygii	Atheriniformes	Atherinidae	*Atherina* sp.	Sand smelt	F	3	–
		Aulopiformes			aulopiforms	M, F	13	13
			Chlorophthalmidae		greeneyes	M	13	11
				Chlorophthalmus agassizi	Shortnose greeneye	F	3	4
			Synodontidae		lizardfishes	M	–	2
		Batrachoidiformes	Batrachoididae		toadfishes	M	–	2
		Beloniformes			needlefishes	M, F	6	7
			Belonidae	*Belone belone*	Garfish	F	6	–
			Exocoetidae		flying fishes	M, F	–	7
		Clupeiformes			anchovies	M, F, C	48	36
			Clupeidae		herrings	M	26	20
				Sardinella sp.	sardinellas	F, C	23	11
				Sardina pilchardus	European pilchard	F, C	-	7
			Engraulidae		anchovies	M	32	33
				Engraulis sp.		F	23	22
				Engraulis encrasicolus	European anchovy	F	3	–
		Gadiformes			cods	M, F, C	19	18
			Lotidae		rocklings	M	–	2
			Merlucciidae		hakes	M	6	15
				Merluccius merluccius	European hake	F	–	7
			Phycidae	*Phycis blennoides*	Greater forkbeard	F, C	13	2
		Lophiiformes	Lophiidae	*Lophius budegassa*	Blackbellied angler	F	3	–
		Mugiliformes	Mugilidae	*Chelon* sp.	grey mullets	F	–	2
		Myctophiformes	Myctophidae		lanternfishes	M, F	3	7
				Ceratoscopelus maderensis	Horned lanternfish	F	–	7
		Osmeriformes	Argentinidae	*Argentina sphyraena*	Argentine	C	–	4
		Perciformes			perch-like fish	M, F, C	61	85
			Carangidae		jacks	M	26	75

Table 3 (continued)

Phylum	Class	Order	Family	Species	Common Name	Primer(s)	%FO SCSH	%FO YESH
				Trachurus sp.	saurels	F, C	16	60
				Trachurus trachurus	Atlantic horse mackerel	C	–	2
				Caranx crysos	Blue runner	F	–	9
			Centrolophidae		medusafishes	F	–	2
			Gobiidae		gobies	M	–	4
				Lesueurigobius suerii	Lesueur's goby	F	–	2
			Mullidae		goat fishes	M	3	24
				Mullus surmuletus	Red mullet	F	–	20
			Nomeidae		driftfishes	M, C	–	11
			Scombridae		mackerels and tunas	M	45	29
				Auxis rochei	Bullet tuna	F	35	2
				Scomber sp.	mackerels	F	6	18
				Thunnus sp.	albacores & tunas	F	13	2
				Thunnus thynnus	Atlantic bluefin tuna	F	13	–
			Sparidae		porgies	M, F, C	6	33
				Boops boops	Bogue	C	–	2
				Dentex maroccanus	Morocco dentex	C	–	2
				Pagellus acarne	Axillary seabream	F	–	4
				Pagellus bogaraveo	Blackspot seabream	F	–	2
				Pagellus erythrinus	Common pandora	F	–	4
				Spicara maena	Blotched picarel	C	–	2
				Spicara smaris	Picarel	C	–	4
				Spondyliosoma cantharus	Black seabream	F	–	4
			Trichiuridae		cutlassfishes	M	3	4
		Pleuronectiformes	Citharidae		cithartids	M	–	2
		Scorpaeniformes	Sebastidae		rockfishes	M	–	4
				Helicolenus sp.	rockfishes	F	–	2
		Stomiiformes	Sternoptychidae		hatchetfishes	M	–	4
				Maurolicus muelleri	Silvery lightfish	F	–	2
		Syngnathiformes	Centriscidae		snipefishes	M	3	31
				Macroramphosus sp.	snipefish	F	3	25
				Macroramphosus scolopax	Longspine snipefish	C	–	7

% Frequency of occurrence was calculated for as the number of samples in which each specific prey MOTU was detected from the total of samples with at least one positive detection of a prey MOTU (SCSH = 31; YESH = 55)

We identified four Cephalopod families, all at 3–5% FO, which is in line with previous indications that cephalopods are present in the diets of both shearwater species (Lago and Metzger 2020), but are secondary in occurrence compared to pelagic fish (Thabet et al. 2019). However, in the stomach contents of Yelkouan shearwaters breeding on islands in SE France and sampled in 2004 to 2007, cephalopod beaks were completely absent (Bourgeois et al. 2011). In both Scopoli's and Yelkouan shearwaters, we found low contributions of crustaceans to the diet, but amplification with only one universal primer might not be suitable to entirely detect marine invertebrates in faecal samples (Leite et al. 2021; Zhao et al. 2021; de Leeuw et al. 2024). Decapoda and Euphausiacea, identified by metabarcoding in our study, were also found in the stomach contents of Yelkouan shearwaters, albeit at decreasing abundance from the pre-laying to the chick-rearing period (Bourgeois et al. 2011). In Scopoli's shearwaters from Zembra, Tunisia, however, using stable isotope

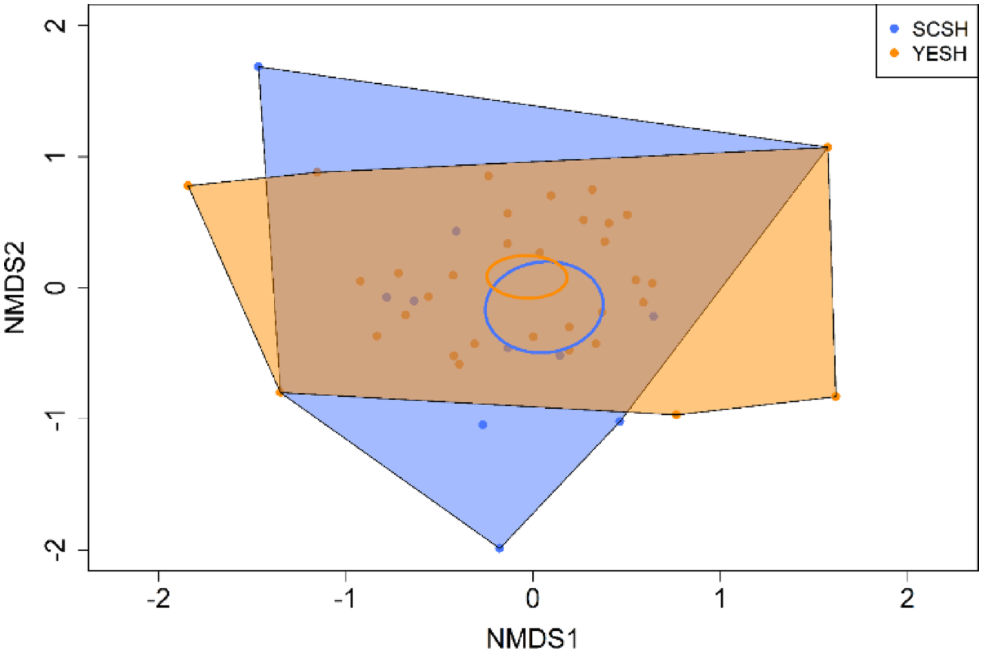

Fig. 1 Diet composition on order level of Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. NMDS stress level was 0.115

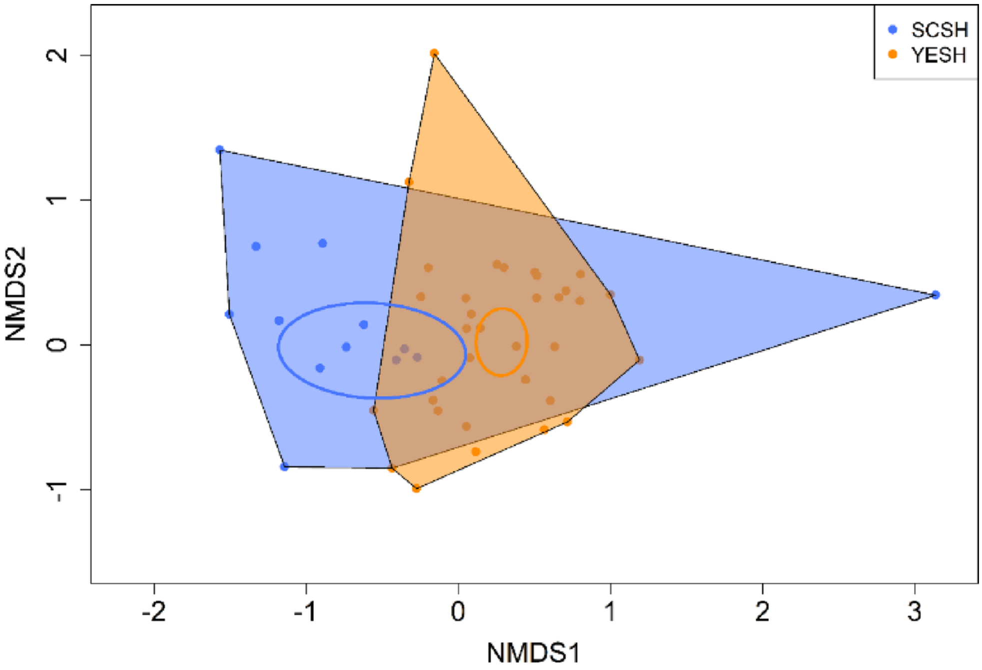

Fig. 2 Diet composition on genus level for fish prey consumed by Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. The NMDS stress level was 0.112

mixing models, Thabet et al. (2019) determined crustaceans to make up around half or even the entirety of the adult diet during chick-rearing with variations between years.

Application of DNA metabarcoding

Through the application of DNA metabarcoding, we have broadened the knowledge of prey consumed by shearwaters in the Mediterranean. However, faecal samples of shearwaters, especially those collected by cloacal swabs, yielded low amounts of prey DNA, possibly due to effects of varying digestion during different breeding-stages (McInnes et al. 2017a; Komura et al. 2018). Adult shearwaters revert between short and long foraging trips that are chick- and self-provisioning, respectively, and during the short trips have been shown to use endogenous lipid reserves and limit digestion (Cherel et al. 2005). Moreover, during incubation birds fast at the nest for several days, resulting in decreased body mass (Colominas-Ciuró et al. 2022) and decreased amounts of food DNA in faecal samples (McInnes et al. 2017a). This can result in a high proportion of samples that do not yield prey DNA, driving up sequencing costs without

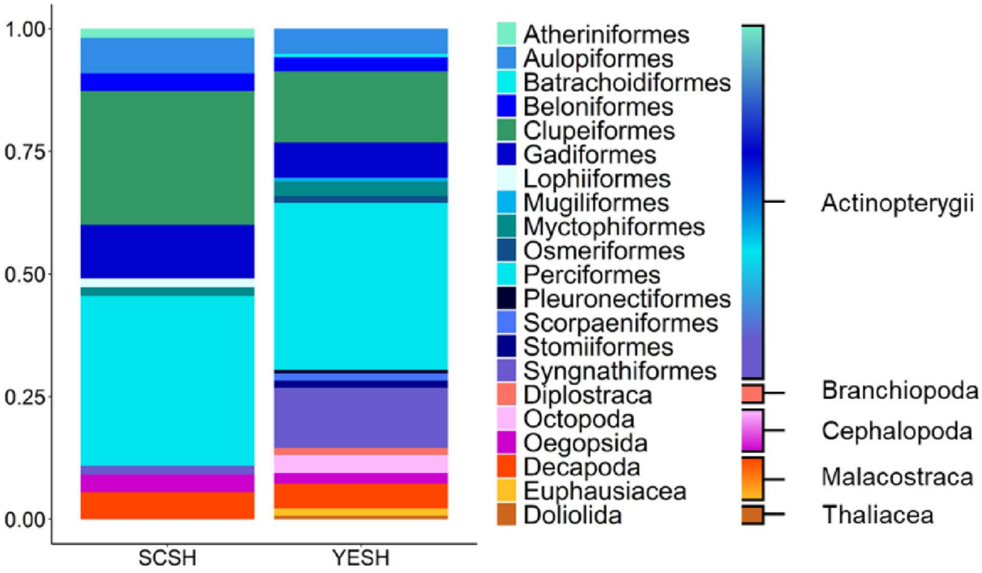

Fig. 3 Diet Composition of Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean on the level of taxonomic order. The percent stacked barplot is showing the percentage contribution of each dietary item to the whole. The taxonomic order of dietary items is listed alphabetically and separated by class

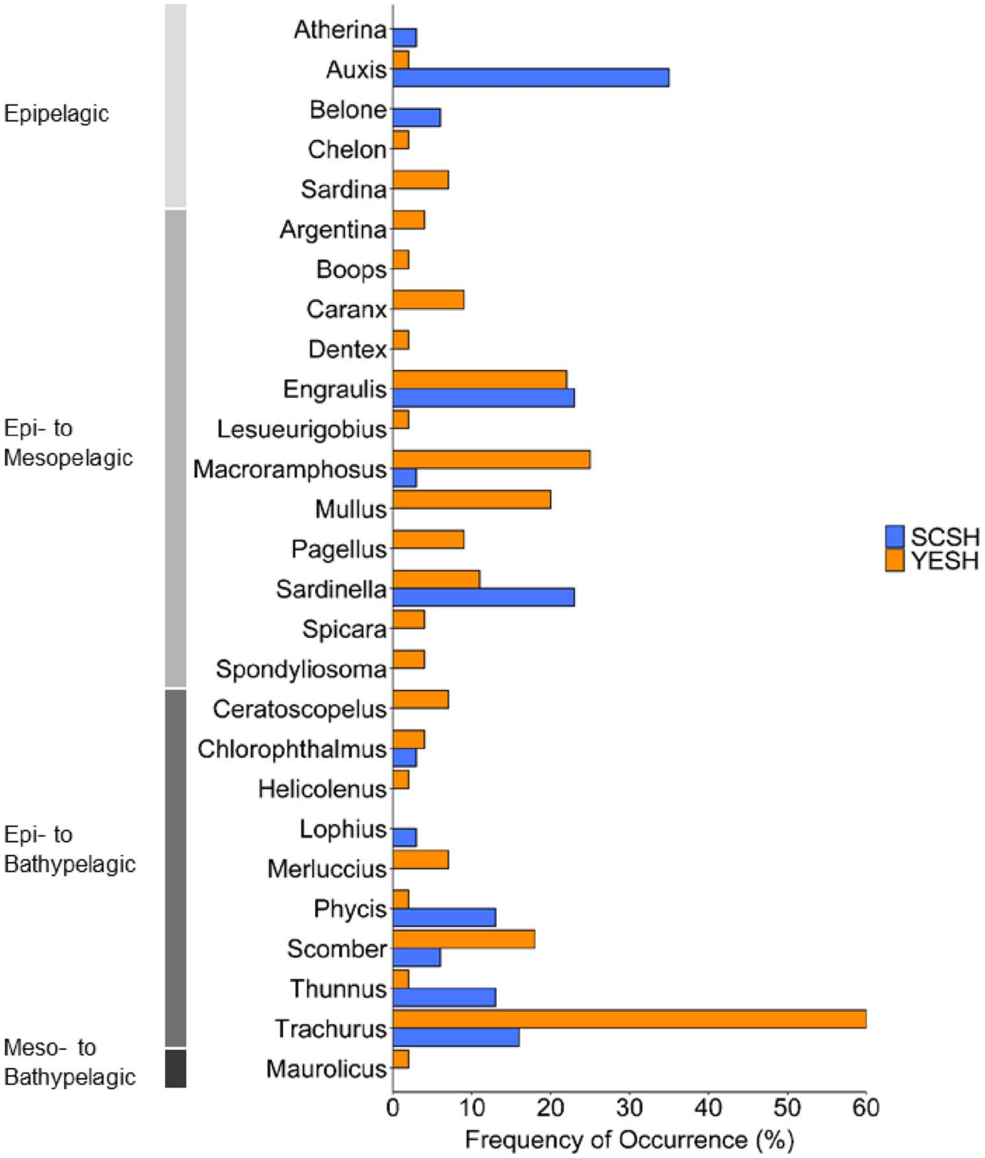

Fig. 4 Composition and frequency of occurrence (%) of fish genera in the diet of Scopoli's shearwaters (SCSH, in blue) (*Calonectris diomedea*) and Yelkouan shearwaters (YESH, in orange) (*Puffinus yelkouan*) from the central Mediterranean revealed by DNA Metabarcoding of faecal and regurgitate samples. We categorised the fish genera into their pelagic zone according to the depth-range information from fishbase.se. (Froese and Pauly 2024). If several species within a genus were geographically relevant prey, we adapted the range to include the minimum and maximum depth range for all relevant species. We then assigned a pelagic zone, where a depth range between 0–200 m falls in the category epipelagic, 200–700 m mesopelagic, 700–1000 m bathypelagic. The order of the fish genera represents their preferred pelagic zone where depth increases from top to bottom

Table 4 Mean stable isotope values of δ^{13}C and δ^{15}N (± SD) from blood cells in Scopoli's shearwater and Yelkouan shearwater collected from adult shearwaters during the breeding seasons of 2020 and 2021 (N =Number of samples). δ^{13}C and δ^{15}N values from Scopoli's shearwaters sampled at La Maddalena were adjusted for latitudinal gradient by Subtracting 0.25 and 1.3 respectively. Trophic level (TL) was calculated using the formula and base values presented in Thabet et al. 2019)

	Year	*N*	Mean ADδ^{13}C	SD	Mean AD$\delta^{15}N$	SD	TL
Scopoli's shearwater	2020	92	−19.10	0.26	8.57	0.80	3.93
	2021	33	−18.94	0.27	8.35	0.62	3.87
Yelkouan shearwater	2020	11	−19.22	0.21	7.87	0.53	3.73
	2021	36	−19.16	0.56	8.78	0.44	3.99

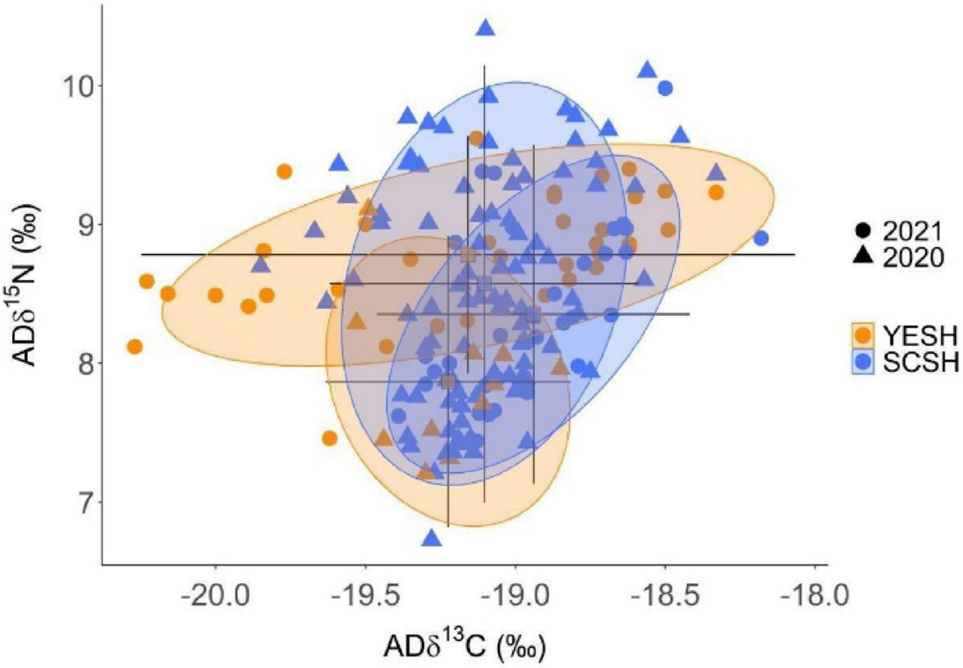

Fig. 5 Stable isotope values for δ^{13}C and δ^{15}N in red blood cells of Scopoli's shearwaters (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) in the sampling years 2020 and 2021. Values are adjusted for a latitudinal gradient. The ellipses were set to cover 80% of the points, assuming a multivariate normal distribution, while the error bars were 1.96 times the standard deviation

adding to the dataset unless screened at the initial PCR stage (Deagle et al. 2007).

To avoid sequencing samples with non-target DNA only, initial PCR amplification should not be carried using a single universal primer pair, or include a blocking oligonucleotide primer for the specific host species (Vestheim and Jarman 2008; Deagle et al. 2010) and inspection of differential amplicon length (Kleinschmidt et al. 2019). The use of blocking primers can increase the amplification of rare prey DNA found at lower concentrations compared to host DNA (Vestheim and Jarman 2008), but should be considered with caution since they can affect the amplification of prey DNA too (McInnes et al. 2017a). Moreover, future studies can focus on obtaining samples large enough to extract the non-uric acid portion from samples (McInnes et al. 2017a), and consider using additional DNA extraction kits such as the Invitrogen PureLink Microbiome Purification kit (de Leeuw et al. 2024). However, even low prey DNA yields provide evidence of the prey taxa in the diet, albeit at underestimated dietary diversity (Figs. S8 and S9). The regurgitates collected opportunistically in this study gave proportionally higher prey DNA yields compared to faecal samples, and extraction could probably be improved further following other protocols (see Nimz et al. 2022).

DNA metabarcoding can be further subject to bias caused by different prey digestibility (Deagle et al. 2010; Pompanon et al. 2012) or secondary predation (Nimz et al. 2022; Marcuk et al. 2024). Therefore, final interpretations must account for the plausibility of the results in the context of known aspects of the food chain. Nevertheless, metabarcoding allows for identification of ingested items and offal as well as spawn or juvenile prey that are generally underrepresented in studies of hard parts such as otoliths, vertebrae and cephalopod beaks (Alonso et al. 2014; McInnes et al. 2017b). On the other hand, identified diet taxa without associated morphological information, poses difficulties in interpreting how prey items were foraged (Komura et al. 2018). In the absence of morphological information in dietary samples (i.e. faecal samples), we recommend including isotopic analysis as done in the current study.

Fish composition and SI values

Fish species that are usually found at greater depths can become accessible to shallow divers due to vertical migration (Olivar et al. 2012), larval development in surface waters (D'Onghia et al. 2006), secondary predation (Nimz et al. 2022), fishery discards (Karris et al. 2018) or predator assemblies that drive prey fish to the surface (Michel at al. 2022). The identified depth ranges of the detected fish genera revealed that all fish genera consumed by shearwaters in our study, with one exception (*Maurolicus*), can occur in the epipelagic zone, meaning we found no obvious evidence of discard consumption.

Molecular analysis identified some taxa that preferentially occur at great depths, usually out of reach of both shearwaters (e.g. *Macroramphosus*, *Chlorophthalmus*, *Phycis*). However, low variation in δ^{13}C values found in blood samples does not indicate a prevalence of demersal fishes, as δ^{13}C is expected to increase in benthic species compared to pelagic ones (Hobson et al. 1994). Also, trophic levels of both shearwater species showed low variation even though they consumed some high trophic level taxa (e.g. *Thunnus*, TL: 4.5; *Auxis* TL: 4.4; *Merluccius* 4.4; *Lophius* TL: 4.4; Froese and Pauly 2024). Due to enrichment of ^{15}N up the food chain (Post 2002), we would expect a larger difference between the TL of shearwaters and their prey if shearwaters consumed these fish as adults (including offal). One explanation could be that these taxa were consumed as spawn or juveniles, which hold a lower trophic level and can occur closer to the surface than adults (D'Onghia et al. 2006; Laiz-Carrión et al. 2015). Generally, SI values increase with body size of fish (Estrada et al. 2005; Hirch and Christiansen 2010; Fanelli et al. 2018). Consumption of juvenile fish does not exclude that fish were consumed as discards, because a majority of fishery discards is made up of undersized commercial species (Karris et al. 2018). Another compatible explanation is that the quantity of prey consumed from fishery discards is low compared to other prey and while they are identified in DNA analysis, contribute less to the trophic status. Indeed, in shearwaters during the chick-rearing period, more natural foraging events were observed than foraging on discards (Michel et al. 2022), and foraging on discards might not be efficient overall (Cianchetti-Benedetti et al. 2018). However, our results are presence/absence observations and do not determine quantities in the sample.

Even though high overlap between the years 2020 and 2021 was found we still expect that seasonal variation in consumed prey species is likely to occur due to dynamics of the prey stocks (Romero et al. 2021; Thabet et al. 2019; Neves et al. 2012) and altered foraging behaviour during breeding (Cecere et al. 2013, 2014). Scopoli's shearwaters were sampled at three different breeding sites, and the SI values among colonies from the central Mediterranean did not differ after baseline adjustment, which supports our results on similar diet composition (Fig. S5). Trophic levels are also within estimates of Scopoli's shearwaters from Zembra (north Tunisia) during the chick-rearing period in 2016 (Thabet et al. 2019). However, unlike Campioni et al. (2022) who measured isotopic variations in feathers grown in the chick-rearing period of 2015, we did not find higher δ^{15}N in Linosa compared to La Maddalena.

Dietary and isotopic niche space in the two shearwater species

Albeit sampling from different populations, we found a high overlap in the diet and trophic level calculated from stable isotope values in the two shearwater species. The slight variation in the fish taxa consumed can be attributed to differences in foraging behaviour particularly a higher diving capacity in Yelkouan shearwaters (Péron et al. 2013; Grémillet et al. 2014; Cianchetti-Benedetti et al. 2017). Another reason for slight differences can be seasonal changes in fish communities from the earlier breeding season of the Yelkouan to the Scopoli's shearwater, which can be driven by the intensification of the summer thermocline (Zotier et al. 1999). Although diet across sample types, colonies and age groups was similar, unequal sample size and a third primer applied to Yelkouan shearwater samples could also contribute to the variation. Despite possible methodological effects on the DNA metabarcoding results, the widest isotopic niche width was identified in chick-rearing adult Yelkouan shearwaters supporting their broader diet.

Niche segregation appears to be higher during the breeding stages that are simultaneous, while there is more overlap in niche space during the chick-rearing periods. The overlap in isotopic niche space occupied by two species during their chick-rearing periods could indicate that their staggered breeding seasons are a strategy to avoid competition. However, it is important to note that our sampling included individuals from diverse populations, meaning that some of the observed differences may stem from variations in prey availability across different seascapes.

Conclusion

Clupeiformes and Perciformes represent the main groups of fish found at high FO in the current and in previous diet studies of two shearwater species, while other taxa such as *Macroramphosus* sp. might reflect opportunistic foraging to cope with changing marine ecosystems (Romero et al. 2021). We did not detect any demersal fish species which were definitely consumed as offal from fisheries by the study species. However, forage fish such as Clupeiformes can be consumed as discards from trawling operations (Arcos and Oro 2002; Karris et al. 2018) and while discards might sustain scavenging seabird populations on the short-term (Genovart et al. 2016; Sherley et al. 2020), unsustainable fishing practices are depleting fish stocks including Clupeids and *Trachurus* sp. in the central Mediterranean region (Hattab et al. 2013; FAO 2023). Irrespective of whether pelagic species are consumed by natural predation or as discards, the breeding performance and abundance of shearwaters in the Mediterranean follows fish stocks, especially small pelagic species (Louzao et al. 2006; Martín et al. 2019), which in turn require ecosystem-based management (Colloca et al. 2017; Piroddi et al. 2020). Hence, we advocate for increased spatio-temporal or gear selectivity to avoid immature or spawning individuals (Tsagarakis et al. 2014; Basilone et al. 2021), and protection of spawning and nursery areas from bottom-trawling (Piroddi et al. 2020). Moreover, management of overfishing should especially be focussed in areas identified as important foraging areas for shearwaters taking into consideration population specific spatial structure and temporal heterogeneity (Morinay et al. 2022). Future research should further disentangle the relationship between fishery activity, seabird diet composition and demographic traits, important in regard to policy directed at discard bans (Bicknell et al. 2013; Genovart et al. 2016). Our results indicate a narrower niche space in chick-rearing Scopoli's shearwaters making them potentially more sensitive to further reductions in forage fish availability. Yelkouan shearwaters have a wider niche space, potentially due higher diving capability but further studies should investigate the cost of this behaviour. Ultimately, our findings underscore the complexity of shearwater diets but lay the foundation for further comparisons between seasons and seascapes in the region.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00227-025-04670-z.

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Author contributions Martin Austad, Lucie Michel and Petra Quillfeldt contributed to the study's conceptualization and design. Formal analysis and data curation were carried out by Martin Austad, Lucie Michel and Juan F. Masello. Martin Austad, Lucie Michel, Giacomo Dell'Omo, Federico De Pascalis and Jacopo G. Cecere carried out the fieldwork. Martin Austad and Lucie Michel contributed equally to writing the original draft, which was reviewed and edited by all co-authors. Investigations were led by Martin Austad and Lucie Michel. All authors read, commented and approved the final manuscript. Parts of the funding were acquired by Martin Austad. Supervision and project administration were carried out by Petra Quillfeldt. Paco Bustamante contributed also in the project administration.

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Data availability Sequences are deposited at the National centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) under the BioProject PRJNA1232430 and accession numbers SRR32585073 to SRR32585232. Stable Isotope Data is available: https://www.ncbi.nlm.nih.gov/genbank on Zenodo: https://doi.org/10.5281/zenodo.15221504.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval In Malta, all handling and sampling of shearwaters were carried out under permits from the Environment & Resources Authority (ERA) and the Wild Birds Regulation Unit (WBRU), Malta. In Linosa fieldwork was carried out according to the national legislation under the permit n. 2452 issued the Regional authorities (Regione Siciliana, Assessorato Regionale dell'Agricoltura, dello Sviluppo Rurale e della Pesca Mediterranea, Dipartimento Regionale dello Sviluppo Rurale e Territoriale) on February 1 st, 2018. The fieldwork was carried out with moral responsibility for the animals, with the final goal to preserve and enhance the future survival of that species and to provide knowledge vital to their conservation. No specimens were collected for this study.

Consent to participate Not applicable.

Consent for publication Not applicable.

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