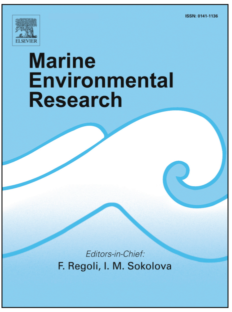

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DNA metabarcoding to assess prey overlap between tuna and seabirds in the Eastern Tropical Atlantic: implications for an ecosystem-based management

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Abstract

Overfishing has been drastically changing food webs in marine ecosystems, and it is pivotal to quantify these changes at the ecosystem level. This is especially important for ecosystems with a high diversity of top predators such as the Eastern Atlantic marine region. In this work we used high-throughput sequencing methods to describe the diet of the two most abundant tuna species, the Skipjack tuna (*Katsuwonus pelamis*) and the Yellowfin tuna (*Thunnus albacares*), highly targeted by fisheries off west Africa. We also explored prey diversity overlap between these tuna species and the seabird species breeding in Cabo Verde that are most likely to share prey preferences and suffer from bycatch, the Brown booby (*Sula leucogaster*) and Cape Verde shearwater (*Calonectris edwardsii*). Overall, the diet of both tuna species was more diverse than that of seabirds. Skipjack tuna diet was dominated by prey from lower trophic levels, such as krill, anchovies, and siphonophores, while the Yellowfin tuna diet was mainly based on epipelagic fish such as flying and halfbeak fishes. Some of the most abundant prey families detected in the Yellowfin tuna diet were shared with both seabird species, resulting in a high prey diversity overlap between this tuna species and seabirds These results have implications for the management of tuna fisheries in the Eastern Tropical Atlantic, because a large decrease of both tuna species might have cascading effects on both primary and secondary consumer levels, and the decrease of these underwater predators may have implications on the viability of tropical seabird populations.

Keywords: Diet, DNA metabarcoding, Tropical ecosystem, *Katsuwonus pelamis*, *Thunnus albacares*, *Sula leucogaster*, *Calonectris edwardsii*, Cabo Verde

1. Introduction

Overfishing around the globe has been drastically reducing populations of target and non-target marine species, with major effects on ecosystem structure and food web functions (Jennings et al., 2001; Pauly et al., 1998). These effects need to be quantified under an ecosystem-based approach, especially incorporating top predators, such as tunas (also overfished) and seabirds (Fowler, 1999; Karpouzi et al., 2007; Piatt et al., 2007). As predators on the top of marine food chains, seabirds and tunas have a major role in food web structure at the ecosystem level, because they contribute to the stability and persistence of marine ecosystems through top-down control (Estes et al., 2011). Information about their ecological niche and trophic dynamics is therefore necessary, for instance, to understand and predict potential cascading effects caused by the decrease of large predatory fishes due to overfishing (Cairns, 1992; Myers et al., 2007).

This is especially important for highly diverse ecosystems of tropical waters. On one hand, these ecosystems harbour a great diversity of both aerial (such as seabirds) and underwater (such as tunas, dolphins and sharks) top predators. On the other hand, these waters are usually less productive than their temperate counterparts, with scarcer and more patchily distributed prey for top predators (Longhurst and Pauly, 1987). To overcome this, predators tend to adopt foraging strategies which allow the detection of these prey patches, whilst minimizing energy spent foraging. For instance, seabirds can adopt foraging strategies that take into account sub-surface predators, especially tunas (Spear et al., 2007). More specifically, tunas often chase prey to the ocean surface, which facilitates prey availability for seabirds (Ashmole and Ashmole, 1967). This kind of facilitated foraging has not only been directly observed in the environment (e.g. Au & Pitman 1986, Hebshi et al. 2008, Correia et al. 2019), but has also been inferred from the overlap between seabirds' and aquatic predators' foraging areas (e.g., Catry et al. 2009), of their trophic niches (e.g., Kojadinovic et al. 2008) and prey species (e.g., Ménard et al. 2012). For example, in the Eastern Tropical Pacific facilitated foraging is the single most important strategy for seabirds' successful foraging, accounting for a total of 76% of prey mass made available to seabirds (Spear et al. 2007). It seems that these interactions might also be beneficial for tuna because seabirds might prevent prey such as flying fish from leaping out of the water, containing the prey at the surface (Burger 1988). Furthermore, it is expected that climate change may lead to the expansion of ocean hypoxic zones, which may limit pelagic tuna to the thinner oxygenated surface layer (Stramma et al., 2012; Vedor et al., 2021), and thus such interactions with seabirds might be more beneficial than expected (although competition for food between tuna and seabirds might also arise from such interactions). Several studies have shown, however, that while tunas and seabirds forage in the same schools, the two groups tend to forage at different trophic levels, on different prey or different prey sizes (Ashmole and Ashmole, 1967; Bertrand et al., 2002b; Cherel et al., 2008; Young et al., 2010). Therefore, the decrease of tunas on tropical waters due to overfishing may not benefit seabirds due to competition release, instead, it may have the opposite negative effect if seabirds' prey capture efficiency is intrinsically linked with tunas' capture events.

The East Atlantic marine region centred around Cabo Verde archipelago (Fig. 1a) is an iconic example where overfishing may have a detrimental effect due to the large diversity of breeding seabirds and the presence of several tuna species. However, little data is available on the sub-surface predators' direct and indirect trophic interactions with seabirds. Indeed, the most captured sub-surface predators in these waters are tuna species, especially Skipjack tuna (*Katsuwonus pelamis*), Yellowfin tuna (*Thunnus albacares*) and Bigeye tuna (*Thunnus obesus*) (Monteiro, 2012; Thorpe et al., 2022). On the other hand, Cabo Verde hosts eight breeding seabird species (including four endemic species) with large colonies all over the archipelago (Semedo et al., 2021).

In Cabo Verde, the contribution of fisheries to the Gross Domestic Product (GDP) is relatively small (Almeida et al., 2004; FAO, 2022), but they play a strategic social and economic role, employing approximately 5.2% of the economically active population (González and Tariche, 2009; Silva, 2009). Tuna species are the main target, comprising 30.5% of the total artisanal catches and 65% of the industrial fisheries (INE-CV, 2017). Favourable sea conditions allow fishing activity all year round in Cabo Verde, however, the pressure due to unreasonable fishing quotas and the high level of Illegal Unregulated and Unreported (IUU) catches are concerning (Ramos and Grémillet, 2013). Understanding resource partitioning between the most caught tuna species and seabird species of Cabo Verde (Fig. 1b) will allow a better understanding of the effects that overfishing might have, because their trophic ecology might reflect changes in the condition and availability of prey stocks (e.g., Barrett & Krasnov 1996) or serve as indicators of changes in the ecosystem (e.g. Weimerskirch et al. 2003).

Several methods can be used to quantify the diet of both tunas and seabirds, and analysis of stomach contents has been used as a standard and reliable method for this purpose. However, the morphological identification of stomach contents is time-consuming, and most importantly, can be limited by the differential digestion of prey, with species composed of soft tissues often being missed, and thus greatly underestimated. Also, the retrieval of stomach contents from seabirds through stomach flushing, which involves pumping salt water through the animal's oesophagus (Barnett et al., 2010; Wilson, 1984), can be time-consuming and is considered an invasive method (Harris and Wanless, 1993). As an alternative, genetic-based tools on the stomach, regurgitates or faecal samples can be applied. Metabarcoding combines genetic sequence-based identification with high-throughput sequencing technology (HTS), which allows the identification of a broad range of taxonomic levels at the same time, even from very degraded samples (Pompanon et al. 2012). The quantification of the relative proportion of prey consumed using DNA Metabarcoding approach, on the other hand, is still not achievable, and is considered the main limitation of this method for dietary studies (reviewed in Ceia et al., 2022). Nonetheless, this method has proven to surpass morphological identification in diverse species, including seabirds (e.g., Oehm et al. 2017, Xavier et al. 2018) and fishes (e.g., Kodama et al. 2017, 2020, Matley et al. 2018, Günther et al. 2021, Trujillo-González et al. 2022). It has the additional advantage of minimizing disturbance to seabirds since it can be applied to regurgitates or faecal samples.

In this work we described prey diversity of the most captured tuna species in Cabo Verde using metabarcoding and explored prey diversity overlap with seabird species breeding in the archipelago, which are most likely to interact with fisheries and potentially suffer bycatch (Montrond, 2020): the Brown boobies (*Sula leucogaster*) and Cape Verde shearwaters (*Calonectris edwardsii*). This also allowed us to critically evaluate the likelihood of tuna species to act as facilitators of prey availability to seabirds in Cabo Verde, and thereby contribute to a better understanding of this trophic network. Ultimately, this work will also allow us to understand how prey depletion by fisheries could trigger indirect trophic cascading effects and thus contribute to a better fisheries management in this region.

2. Materials and Methods

2.1. Tunas sample collection and processing

With the collaboration of local fish processing plants and authorities, we collected stomachs of the most representative underwater predatory species caught in fishing activities, the Skipjack (*Katsuwonus pelamis*), Yellowfin (*Thunnus albacares*) and Bigeye (*Thunnus obesus*) tuna (Monteiro, 2012; Thorpe et al., 2022). All the sampled stomachs belonged to tunas captured between July and early September of 2019, mostly by purse seine. Detailed data from all individuals, including the fishing method used, fishing vessel, and date of capture is described in the Supplementary Data A. Stomachs were immediately frozen at capture and preserved at a fish processing plant at the landing harbour of Mindelo (Cabo Verde). In October 2019, also in Mindelo, all stomachs were processed in laboratory conditions, in the Universidade Técnica do Atlântico (UTA). In more detail, each stomach was thawed in individualized sterilized trays at room temperature for two to three hours or in the fridge (4ºC) overnight. From each individual tissue samples for barcoding genetic identification of each tuna sample were also taken from other organs available (e.g., liver or heart) and stored in 2 mL tubes with 96% ethanol. Since incorrect assignment of the tuna species through morphology might occur at capture, this was done to genetically confirm the identification of the tuna species (further described in section 2.4). These misassignments can be related either to the presence of many related species on one single capture, or the ambiguous regional common names or fisherman’s slang that might encompass several species (Cawthorn and Mariani, 2017). Then each stomach was dissected, its content sampled, collected into sterile Petri dishes, homogenized and two subsamples per stomach were taken into 2 mL tubes with 96% (v/v) ethanol. All materials used between dissections were cleaned in running water, then sterilized using bleach and 96% (v/v) ethanol to avoid cross-contamination between samples. All samples were then refrigerated at 4°C as soon as possible, transported to CIBIO research centre in Portugal and then stored at -20ºC in the laboratory until DNA extraction.

2.2. Seabird sample collection and processing

In 2018 and 2019, Brown boobies (*Sula leucogaster*) and Cape Verde shearwaters (*Calonectris edwardsii*) were sampled in the Raso Islet (16º37’5” N, 24º35’15” W) and Boavista Island (15º59’15” N, 22°47’08” W, Fig. 1a) during the chick-rearing period, from July to September, to overlap with the tunas sampling period. These seabird species were selected based on the highest probability to suffer from direct mortality through bycatch in Cabo Verde waters (Montrond, 2020). Each individual was caught, processed, and released within 15 minutes. A faecal sample was collected from each bird directly into a 2 mL tube with 96% (v/v) ethanol, after an abdominal massage to facilitate defecation. These samples were refrigerated at 4°C as soon as possible, transported to CIBIO research centre in Portugal and then stored at -20ºC in the laboratory until DNA extraction.

2.3. Sequencing tuna tissue samples

Tuna muscle tissue samples undergone DNA extraction using the EZ-10 Spin Column DNA Gel Extraction Kit protocol (Bio Basic Inc., USA). A 645 bp fragment of the mtDNA cytochrome c oxidase subunit I (COI) was amplified with PCR using the primers LCOI 121 and HCOI 1199 (Paine et al., 2007). The PCR protocol consisted of 5 µL of QIAGEN Multiplex PCR Master Mix (Qiagen, USA), 0.4 µL of each primer (10 nM), 2 µL of DNA template and nuclease-free water for a total volume of 11 µL. The PCR conditions were 15 min at 95ºC, 40 cycles of 30 s at 95ºC, 30 s at 57ºC and 60 s at 72ºC, followed by 10 min at 72ºC. All PCR products were sequenced on a 3730xl Genetic

Analyzer (Applied Biosystems, USA), and results were edited and assembled in Geneious Prime 2022.0.2 (Biomatters, New Zealand).

Species assignment was validated using both Phylogenetic tree and BLAST search for more robust results. First, for each mtDNA region, sequences were aligned and an approximately-maximum-likelihood algorithm was used to build a phylogenetic tree in FastTree 2 (Price et al., 2010) to taxonomically assign our sequences. For this approach, we used reference sequences for all species of tuna and related species known to occur in the study area. Second, using the BLAST algorithm (Ye et al., 2006), each sequence was compared (1,000 hits) against the NCBI Genbank Nucleotide online data repository to further confirm the assignment of each sequence to a single species.

We found that a miss-assignment occurred in 33 % (19 out of 57) of the individuals. This did not impact the sample size for Skipjack (final n = 28) and Yellowfin (final n = 23) but drastically decreased our sample size for Bigeye (final n = 6), which prevented a robust statistical analysis of Bigeye metabarcoding data. Thus, the present study focused on the statistical analysis of the other two species, and data on metabarcoding on Bigeye tuna is made available in Supplementary Data B as valuable data for future research.

2.4. Diet determination

To avoid contaminations, all samples were extracted in a non-invasive laboratory using the Stool DNA Isolation Kit (Norgen Biotek, Canada), following the manufacturer's protocol. For each batch of samples to be extracted, a negative control prepared with distilled water was included. We chose to amplify two different mitochondrial DNA (mtDNA) fragments not only to duplicate our probability of correctly assigning the taxonomical identity of Osteichthyes – bony fishes prey items, but also to cover a wider taxonomical range of potential prey (especially macro-invertebrates). The first set, ‘MiFish-U’ amplifies the 12S fragment, preferentially from Osteichthyes (Table 1). The second set, mlCOIintF-XT and jgHCO2198, referred to as ‘Leray-XT’ hereafter, amplifies the COI fragment from metazoan sources, including therefore not only fish but also cephalopods and crustaceans (Table

1). Preliminary analysis of a small subset of seabird and tuna samples showed that both primer sets amplified the predator's DNA. Tuna's stomach content samples can contain tuna blood, and thus high concentrations of predator's DNA. Therefore, blocking primers had to be designed for decreasing the amplification of Tuna DNA in these samples (Table 1). On the other hand, seabird's faecal samples also may have epithelial cells from the predator digestive system, but these preliminary tests proved that blocking primers were not necessary for these faecal samples since they contained less predator DNA, and enough reads from prey items (more than 50%) were always obtained in these samples.

Library preparation followed the MiSeq protocol for 16S Metagenomics (Illumina, USA). For tuna samples, four libraries were prepared, three with 'MiFish-U' primer set and one with 'Leray-XT'. For the 'MiFish-U' the three libraries prepared were 1) without blocking primer, 2) with blocking primer at 20x, and 3) with blocking primer at 100x. The 'Leray-XT' library was prepared with 100x blocking primer. For seabirds' samples, two libraries were prepared, one for each primer set without blocking primer. For each sample, PCR reactions were carried out in volumes of 10 to 12 µl, comprising 5 µl of Multiplex PCR Master Mix (QIAGEN, Valencia, CA, USA), 0.2 µl of each forward and reverse primers (10 nM), the proper volume of the blocking primer depending on the library (100 nM), 1 µl of DNA extract and filled the left-over volume with ultra-pure water. All PCRs included two negative controls to test for possible contamination, one from the DNA extraction process, as well as a PCR negative control prepared with distilled water. PCR cycling conditions are described in detail in Appendix A.

Amplification success was checked by visually inspecting 2 µl of each PCR product on a 2% gel-stained agarose (GelRed, Biotium, USA). Then, for individual identification of each amplified product before pooling, the PCR products were amplified again in a PCR with P5 and P7 indexes, each containing a unique 7 bp long barcode that differed at least 3 bp from any other index. PCR reactions and cycling conditions were similar to the previous PCR, however, only 10 cycles of denaturing, annealing and extension were done, with an annealing temperature of 55ºC. Indexing success was also verified on gel-stained agarose, then indexed PCR products were purified using

Agencourt AMPure XP beads (Beckman Coulter, USA), and subsequently quantified using an Epoch Microplate Spectrophotometer (BioTek, USA). All PCR products from each marker were pooled and normalized at equimolar concentrations (15 nM). Pools were then purified again and quantified into libraries using qPCR with a KAPA Library Quant Kit qPCR Mix (KAPA Biosystems, USA) on the iCycler Real-Time PCR Detection System (Bio-Rad, USA), and further diluted to 4 nM. The final library was run in a MiSeq sequencer (Illumina) using a v3 MiSeq reagent kit (Illumina) for an expected average of 24,000 paired-end reads per sample.

2.5. Bioinformatic analysis

First, paired-end reads were aligned using PEAR (Zhang et al., 2014), discarding both unassembled reads and alignments with overlapping quality scores < 26. Further processing of sequencing reads was done using OBITools (Boyer et al., 2016), where reads were assigned to samples and primer sequences were removed using ‘ngsfilter’, allowing a total of four mismatches. Using ‘obiuniq’ reads were collapsed into amplicon sequence variants (ASVs) and error-corrected using ‘cluster_unoise’ from VSEARCH (Rognes et al., 2016). Only for the ASVs obtained from the COI fragment, additionally we used VSEARCH to perform a 99% identity clustering step into Molecular Operational Taxonomic Units (MOTUs). The retained sequences were further filtered using LULU (Frøslev et al., 2017) by discarding sequences with over 84% similarity and 95% co-occurrence for ‘Leray-XT’, and 95% similarity and 95% co-occurrence for ‘MiFish-U’. Finally, a further filtering process excluded PCR products that exhibited less than 100 reads in total from the analysis, as well as all ASVs/MOTUs (hereafter all named MOTU for the sake of simplicity) that had a read count <1% of the total number of reads of that PCR (Mata et al., 2019).

Prey items were identified by comparing the final MOTUs against online databases (BOLD and NCBI Nucleotide Database) using BLAST algorithms. Haplotypes were assigned to the lowest possible taxonomic level (e.g., family, order, species) for which 1000 hits in BLAST, with the highest identity and identical match, clustered monophyletically. A curation step was performed to assess if each MOTU was described as occurring in the study area, consulting online databases such as GBIF (GBIF.org, 2022) or FishBase (Froese and Pauly, 2022). Identifications that failed this curation step and also were not found to be present on the Eastern Atlantic Ocean using the same online databases were conservatively assigned to the following taxonomic level. Finally, every MOTU that belonged to other taxa than the possible prey species (e.g., human, other mammals, parasites or bacteria) were discarded and a final matrix of MOTUs per sample was built.

2.6. Data Analysis

After bioinformatic filtering, an average of 6876 diet reads per sample was obtained and a total of 48 tuna samples (20 of yellowfin tuna, 28 of skipjack tuna) and 43 seabird samples (21 for Brown boobies and 22 for Cape Verde Shearwaters) were used for analysis. From the three 'MiFish-U' libraries, and to avoid overrepresentation over the 'Leray-XT' library, for each sample it was chosen the library that presented the highest proportion of diet DNA, which in most cases was the library built with blocking primer at a concentration of 100 x (Appendix B). Data was shown at the MOTU and family level and inferential analysis was done only at the family level. Our rationale is that many prey MOTUs will share the same trophic biologically relevant phenotypic characteristics (behaviour, size/energy ratios) and the analysis of MOTUs may have a lower statistical power due to the high number of MOTUs detected between tunas and seabirds (79 MOTUs). All MOTU-level visualizations can be consulted in Supplementary Data C.

For each predator species, and independently of the primer set for which it was amplified, the frequency of occurrence (%FO) of each MOTU and MOTU family was estimated and ranked %FO curves were plotted. The occurrence of MOTU and MOTU families between tunas and seabirds was visualized using the 'upset' command from the *UpSetR* package (Conway et al., 2017). To visualize MOTU and MOTU family richness variation taking into account sample size, for each of the four predators, an accumulation curve was calculated at both levels using 'specaccum' command from the *vegan* package (Oksanen et al., 2020). Observed and extrapolated MOTU and MOTU family richness was calculated using 'iNEXT' command from the *iNEXT* package (Hsieh et al., 2020). The extrapolated richness and its standard error were calculated for an endpoint of twice the sample size of the predator species with the lower sample size. We compared MOTU family observed richness estimates between both tuna and seabird species using a chi-squared goodness-of-fit test followed by pairwise comparisons, calculated using the 'chisq.multcomp' command from the *RVAideMemoire* package (Herv, 2022).

The Pianka niche overlap index (O_{jk}, Pianka 1973) was calculated at the family level to understand the niche overlap between the prey diversity of the four species using 'niche.overlap' command from the *spaa* package (Zhang and Ma, 2014). Null models were used to test whether the extent of niche overlap is greater than expected by chance, where 1,000 simulated MOTU matrices were built using 'niche_null_model' with the 'ra3' algorithm, from *EcoSimR* package (Gotelli and Ellison, 2013). The observed niche overlap was then compared to the simulated niche overlap values, which were considered significant when the observed value was greater than 95% of the simulated values ($p < 0.05$).

To explore differences in diet composition, a multivariate analysis of the MOTU family occurrence matrix was performed with tunas and seabirds' data, to test for interspecific (Species factor) and temporal (Month factor: June to September) differences. Generalized linear models for multivariate presence/absence data were fitted using the *mvabund* package (Wang et al., 2012) with the 'manyglm' command. Since seabird samples were collected in two different years, interannual differences in the diet composition of seabirds were tested prior to this analysis. This factor was found to be not significant, and thus we continued the analysis with samples from both years to obtain a more balanced sample size for each predator species. To test for each factor's significance, as well as their interactions, a model-selection approach was performed using the Akaike Information Criterion (AIC) and deviance using the 'anova.manyglm' command. The p-values for the effect of each variable in the alternative model were obtained by Bootstrap resampling (1000 x) of a log-likelihood ratio under the null model. To understand which MOTU families expressed significant effects, univariate statistics were calculated, and resampling-based univariate p-values were obtained. Finally, a non-metric multidimensional scaling (nMDS) was used to visualize diet composition dissimilarities, built with the function 'metaMDS', also from the *vegan* package. All analyses and visualizations were made under the statistical environment R 4.1.3 (R Core Team, 2022).

3. Results

The 'MiFish-U' identified MOTUs families in most of the tuna samples (38 out of 48) and also in the seabird samples (38 out of 43). In the remaining samples, MOTUs families were only amplified with the 'Leray-XT'. Both sets of primers identified MOTUs families in 21 and 8 samples from tunas and seabirds, respectively. The use of the designed blocking primers notably reduced the proportion of tuna DNA, enhancing the proportion of diet DNA per sample (Appendix B). However, the designed blocking primers were more effective on Yellowfin and Bigeye tuna samples than in Skipjack tuna, especially at the concentration of 100X.

Overall, both tuna species preyed on a higher number of families in comparison with seabirds (Fig. 2, Appendix C). Both tunas presented the highest family richness, both observed (Yellowfin = 30; Skipjack = 20) and extrapolated (Yellowfin = 47.04 ± 12.42; Skipjack = 25.66 ± 6.94). Seabirds showed the lowest observed family richness (Cape Verde shearwater = 14, Brown booby = 10), however, Cape Verde shearwater presented similar extrapolated family richness to Skipjack (21.00 ± 10.72), while Brown booby presented the lowest values (12.44 ± 4.66). These differences in the overall observed family richness were significant ($\chi^2 = 12.27$, df = 3, $p = 0.007$), where Yellowfin tuna family richness was significantly higher than Cape Verde shearwater ($p = 0.048$) and Brown booby ($p = 0.009$). Even at the same sampling completeness (extrapolated family richness at $n = 40$), the same tendency was found, with significant differences found in the overall extrapolated family richness ($\chi^2 = 27.75$, df = 3, $p < 0.001$), where Yellowfin tuna family richness was significantly higher than all the other predators (all $p < 0.009$).

A total of 12 families were shared between tuna species (Fig. 2), including 10 fish families, one decapod family, and one krill family. Skipjack tuna diet was mainly dominated by three families (Fig. 3a), Euphausiidae (53.57% FO), Engraulidae (42,86% FO) and Diphyidae (28.57% FO), while the other 17 prey families were present in much lower frequencies of occurrence (equal or lower than 17.86%). Yellowfin tuna presented a more generalist diet, with a higher number of prey families but all with a low frequency of occurrence (Fig. 3a). The prey families with the highest frequencies of occurrence were Hemiramphidae (30% FO) and Exocoetidae (25% FO).

From the total of prey families detected on the diet of both seabirds (17), four were shared between the two predator groups: Exocoetidae, Hemiramphidae, Coryphaenidae and Serranidae (Fig. 2). Other five families were shared between at least one species of each predator group. Only five prey families were exclusively detected in seabirds' diet: Congridae, Derichthyidae, Mullidae, Muraenidae and Scombridae. The overlap index between seabird and tuna diets (Fig. 3b) was significant between Yellowfin tuna and Brown booby ($p = 0.004$), and between both seabird species ($p = 0.022$). The overlap index between Yellowfin tuna and Cape Verde shearwater, as well as between Skipjack and both seabird species was not significant (all $p > 0.127$).

The multivariate data model analysis showed significant differences in diet composition between all species (Species factor: $p = 0.001$) as well as significant temporal differences (Month factor: $p = 0.003$). The interspecific differences could also be visualized on the nMDS plot by the low overlap and distance of centroids (Fig. 4), for which the multivariate data model showed the significant contribution of six prey families (Table 2). Temporal differences were mainly explained by the contribution of Gonostomatidae and Phosichthyidae families in August, when compared with the other two months (Table 2, Supplementary data D). The interaction between both factors was also significant ($p = 0.001$), mainly due to the differences between Skipjack tuna samples from August with the other groups, with Carangidae and Exocoetidae contributing significantly to these differences (Table 2, Supplementary data D).

4. Discussion

4.1. Tunas prey diversity

Both tuna species presented a diet with high species diversity, in line with the consensual view of tunas as generalists and opportunistic predators (e.g., Ménard et al. 2006, Romero et al. 2021). Yellowfin tuna exhibited a higher prey diversity than Skipjack tuna, corroborating previous studies (Setyadji et al., 2012; Trujillo-González et al., 2022) which describe Yellowfin tuna as generalists, also presenting a general low abundance of each prey type (Olson et al., 2014). This can be explained by the fact that true tuna species have high metabolic rates and require higher energy intake for continuous swimming activity (Magnuson, 1978; Olson and Boggs, 1986). They are also limited by oxygen levels, due to their physiological adaptations, especially concerning heart rate (Pecoraro et al., 2017; Stramma et al., 2012). With the increasing deoxygenation levels in tropical waters, prey availability is further limited to a smaller range of depths. Since prey tend also to present a patchy distribution at tropical latitudes, they must adopt a generalist, more opportunistic diet to ensure this energy intake.

On the other hand, Skipjack tuna also presented high prey diversity, but some prey families were more dominant, particularly krill (Euphausiidae), anchovies (Engraulidae) and siphonophores (Diphyidae). Most studies on tropical waters describe the Skipjack tuna diet as mainly composed by fish of the families Acanthuridae, Clupeidae, Scombridae, Carangidae and Serranidae (e.g. Dragovich & Potthoff 1972, Setyadji et al. 2012, Romero et al. 2021). Other studies have described other prey of lower trophic levels as the main components of Skipjack tuna's diet, namely krill (Alatorre-Ramirez et al. 2017) and anchovies (Varela et al., 2019). These differences between Skipjack and Yellowfin tuna diet composition can be mainly explained by differences in their body size. Not only the smaller size and different physiology of Skipjack may limit the maximum prey size it can consume (Graham et al., 2007), but also its anatomically smaller gill raker apparatus might retain a higher abundance of small prey, such as euphausiids (Ankenbrandt, 1984; Magnuson and Heitz, 1971). Because these two tuna species are present in these waters in higher numbers compared with other tuna species (Monteiro, 2012), such consumption of prey of different sizes might reduce competition and facilitate coexistence. From a trophic perspective, since DNA metabarcoding does not allow a quantification of the relative proportion of prey consumed, and considering that our results are at the family level, it is not possible to calculate a numerical average of the trophic levels of both tuna prey diversity. However, both krill and anchovies, i.e., main Skipjack prey, are widely known prey of lower trophic levels, since both families are comprised mainly of planktivorous species (Cabrol et al., 2019; Chouvelon et al., 2014; Cleary et al., 2012; Quetin and Ross, 1991). In the other hand, some of the most frequent prey of Yellowfin tuna are omnivorous fish which forage on different trophic levels, such as fish from the Hemiramphidae family (Collette, 2016), or squid from the Ommastrephidae family (Lipiński and Linkowski, 1988; Merten et al., 2017). Our results show that these two tuna species forage on prey of different trophic levels, and such trophic level differentiation between Yellowfin and Skipjack tuna was also observed in the tropical Pacific Ocean (Alatorre-Ramirez et al., 2017).

Differences in diet in relation to earlier studies may be partially explained by methodological biases because previous studies used traditional methodologies of stomach content analysis. Although Romero et al. (2021) used genetic methods, they were only used to assist in the identification of hard parts. When using stomach content analysis, stomachs that seem empty or have highly digested prey are often discarded from the analysis, and depending on the experimental design, the majority of the stomachs can be empty (e.g. 61% of empty stomachs in Ménard et al. 2000, 85% of empty stomachs in Karakulak et al. 2009). The use of metabarcoding enables to overcome most of these problems and biases, because the whole stomach content, including highly digested material, is used. Therefore, not only it allows to extract prey DNA from empty stomachs that would have been discarded in conventional studies, but also detects DNA of soft-bodied prey that would rapidly be digested (Diaz Briz et al., 2017; Magnuson, 1969) and will not be detected using conventional stomach content analysis. Inclusively, metabarcoding has recently revealed the importance of gelatinous prey on the diet of Atlantic Bluefin tuna (*Thunnus thynnus*) (Günther et al., 2021), which would otherwise be nearly impossible to detect.

4.2. Prey overlap with seabirds

Overall, when looking only to the values of the overlap index (all lower than 0.58), the prey diversity overlap between seabird and tuna species was relatively low, especially between Skipjack tuna and both seabird species. The greater frequency of krill (Euphausiidae) and siphonophores (Diphyidae) on the diet of Skipjack tunas contributes greatly to this low overlap, while Brown boobies and Cape Verde shearwaters prey mainly on epipelagic fish, and some of the fish species consumed are of commercial interest (Almeida et al., 2021; Carreiro et al., 2022; Rodrigues, 2014; Vieira, 2018). Another explanation for the low overlap between tunas and seabirds can be the higher prey availability for tunas. Because some tuna species perform vertical migrations (Bertrand et al., 2002a), they have a wider range of habitats to forage, which contributes to their higher prey diversity. However, when we focus on prey from the epipelagic fraction of the ocean, that both tunas and seabirds can explore, we observe a higher overlap of prey diversity.

On the other hand, from all prey diversity detected, some of the most abundant families were shared between both tuna and seabird species. Of the ten families that Brown Boobies prey upon, nine are shared with at least one of the tuna species. Indeed, this resulted in a significant overlap index between Brown boobies and Yellowfin tuna, especially due to the higher frequency of Flying fish (Exocoetidae) and Halfbeak fish (Hemiranmphidae) in the diet of these predators. Carreiro et al. (2022) analysed the diet of Brown boobies with a more comprehensive range of samples and showed that these prey are the most important in their diet. Flying fishes inhabit tropical and subtropical waters (Lewallen et al., 2017, 2016; Parin and Shakhovskoy, 2000) and historical fishery landings in Cabo Verde indicate that flying fishes are present on Cabo Verde waters throughout the year (Almeida, 2021). Both flying and halfbeak fishes are largely described as one of the main prey of marine top predators in tropical regions, including tunas (da Silva et al., 2019; Vaske Júnior et al.,

2003) and seabirds (Ashmole and Ashmole, 1967; Correia et al., 2021; Mancini and Bugoni, 2014). Furthermore, the fact that Yellowfins also prey on these two prey groups further hints that Brown boobies in Cabo Verde may take advantage of facilitated foraging provided by tunas, at least from Yellowfin tuna. Concerning temporal differences, as well as differences between Skipjack tuna samples from August with the other groups, it is very likely that these were due to the patchy distribution of prey and the foraging strategies of tunas, highly focused towards feeding on prey which tends to aggregate in schools.

5. Conclusions

In Cabo Verde, we found that Skipjack tuna also explores prey of lower trophic levels, while Yellowfin tuna prey mostly on mid-level trophic levels. It is well known that overfishing large predatory fishes can have cascading effects through top-down perturbations (Casini et al., 2012; Steneck, 2012; Verity et al., 2002). Besides local fisheries, countries like Spain, Japan, Taiwan, North Korea and China operate in Cabo Verde waters, with or without fishing agreements (Kroodsma et al., 2018). Because national authorities are not able to control all these foreign fleets, some countries may use illegal and destructive fishing methods, which contribute to the decline of fish stocks and local fish landings (Benchimol et al., 2009). Therefore, the results of this study can have implications for the management of tuna fisheries in Cabo Verde. Regarding seabirds, we show that the overlap is higher with tunas that prey on pelagic fish, namely the Yellowfin tuna. Although we cannot assert if such overlap has negative effects on the viability of these seabirds populations, these results should not be overlooked because the overexploitation of these tuna species may have a direct effect on the availability of prey to seabirds.

Finally, the results of this study also confirm that ecosystem-based management is needed for this study region, through the inclusion of other top predators such as seabirds. Because we detected temporal differences, future research should focus on studying the diet of both tunas and seabirds throughout the year, and include other seabirds that are present year-round in the archipelago and also known to be a target of bycatch, such as the Red-billed tropicbird (*Phaethon aethereus*) (Montrond 2020).

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Declarations of interest: none

Data Availability

Data analyzed during the current study are available publicly on Biostudies: https://www.ebi.ac.uk/biostudies/studies/S-BSST918

Compliance with Ethical Standards

This project was authorized by the "National Directorate of the Environment" of Cabo Verde (DNA) to be carried out at Raso Islet, Desertas Islands Natural Reserve. All sampling procedures and/or experimental manipulations were reviewed and specifically approved as part of obtaining the field license.

Author Contributions

Ana R. Carreiro: investigation; Ana Carreiro, Vitor H. Paiva, Jaime A. Ramos and Ricardo J. Lopes: conceptualization, methodology, resources; Ana R. Carreiro and Ricardo J. Lopes: formal analysis, data curation, writing - original draft; Vitor H. Paiva, Jaime A. Ramos and Ricardo J. Lopes: funding acquisition, supervision, writing – review & editing; Vanessa A. Mata: software, writing – review & editing; Nathalie M. Almeida, Isabel Rodrigues, Ivo dos Santos, Diana M. Matos, Pedro M. Araújo, Teresa Militão, Jacob González-Sólis: resources, writing – review & editing. All authors read and approved the final manuscript.

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Tables

Table 1 - Primers used for prey DNA screening and blocking primers designed for this study.

Genetic target	Taxa target	Pipeline/Aim	Primer set	Primer names	Sequence 5’- 3’	Amplicon size (bp)	Reference
mtDNA 12S	Osteichthyes	Screening	MiFish-U	MiFish-U-F	GTCGGTAAAACTCGTGCCAGC	165-185	Miya et al. 2015
				MiFish-U-R	CATAGTGGGGTATCTAATCCCAGTTTG		
	Thunnus sp.	Blocking	*Thunnus* 12S BP	MiFish-blkThunnus	CTAATCCCAGTTTGTGTCATAGCTTTCGTGGGGTCAGGGT[SpC3]	-	This study
	Katsuwonus sp.	Blocking	*Katsuwonus* 12S BP	MiFish-blkKatsuwonus	CTAATCCCAGTTTGTGTCATAGCTTTCGTGGGGTCAGGGG[SpC3]	-	This study
mtDNA COI	Metazoa	Screening	Leray-XT	mlCOIintF-XT	GGWACWRGWTGRACWITITAYCCYCC	~313	Wangensteen et al. 2018
				jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA		Geller et al. 2013
	Scombridae	Blocking	Tuna COI BP	Leray-blkTuna	AAGAATCAGAATAGGTGTTGGTAAAG[SpC3]	-	This study

Table 2 – Results of the multivariate analysis of the prey MOTUs family occurrence matrix between species (CE = Cape Verde shearwater, SL = Brown booby, SKJ = Skipjack tuna, YFT = Yellowfin tuna) and between months (Jul = July, Aug = August, Sep = September). Only significant ($p < 0.05$) pairwise and univariate comparisons are presented.

Factor	p	Significant pairwise comparisons	p	Significant univariate comparisons	p
Species	0.001	CE ≠ SL ≠ SKJ ≠ YFT	all < 0.003	Engraulidae	0.002
				Exocoetidae	0.001
				Diphyidae	0.002
				Euphausiidae	0.001
				Ommastrephidae	0.040
				Scombridae	0.002
Month	0.002	Aug ≠ Jul	0.002	Gonostomatidae	0.017
		Aug ≠ Sep	0.031	Phosichthyidae	0.017
Species : Month	0.001	SKJ : Aug ≠ YFT : Jul	0.001	Carangidae	0.049
		SKJ : Aug ≠ SL : Sep	0.012	Exocoetidae	0.037
		SKJ : Aug ≠ CE : Aug	0.027		
		SKJ : Aug ≠ SL : Jul	0.033		
		YFT : Jul ≠ YFT : Aug	0.027		
		YFT : Aug ≠ SL : Sep	0.022		

Figures

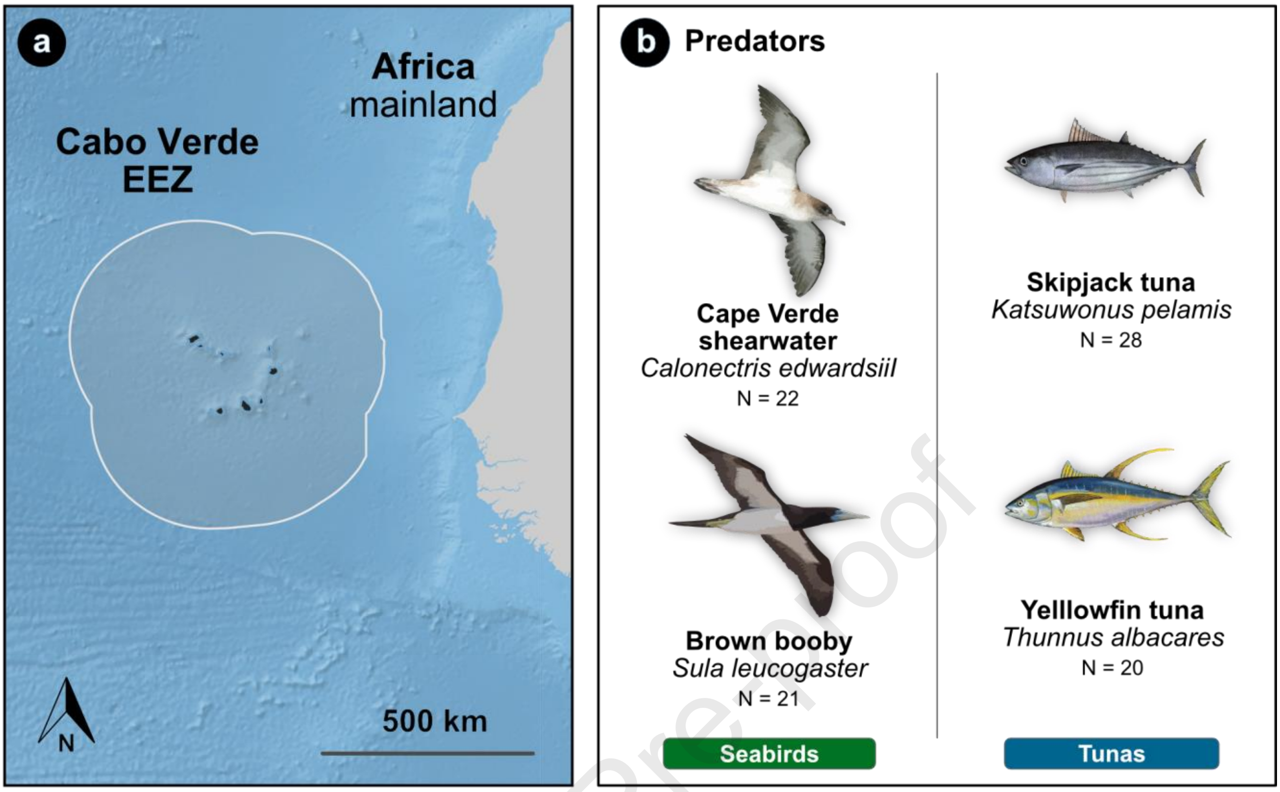
Figure 1. a) Location of the Cabo Verde Archipelago in the eastern Atlantic Ocean and its Exclusive Economic Zone (EEZ); b) The species highlighted in this article, as representative members of two groups of top marine predators (Seabirds and Tunas), likely to interact at the trophic level.

Figure 2. Visual characterization of the number of prey MOTUs families in the diet of the studied seabirds and tunas, ordered by taxonomic similarity. Colour classifies each MOTU family according to its frequency of occurrence.

Figure 3. a) Line plots of ranked frequency of occurrence, highlighting the most frequent MOTUs families for each predator species; b) Pianka's niche overlap index for the tuna and seabird predator species. Significant values ($p < 0.05$) are highlighted with an asterisk.

Figure 4. Non-metric multidimensional scaling ordination (nMDS) of prey MOTU family composition (Jaccard dissimilarity) in the diet of the studied seabirds and tunas. Convex hull polygons delineate the four predator species. The nMDS scores for all prey MOTUs families are shown as points. The name of the prey MOTUs family is only shown when their frequency of occurrence is high (>20%).

843 **Figure 1**



844

Figure 2

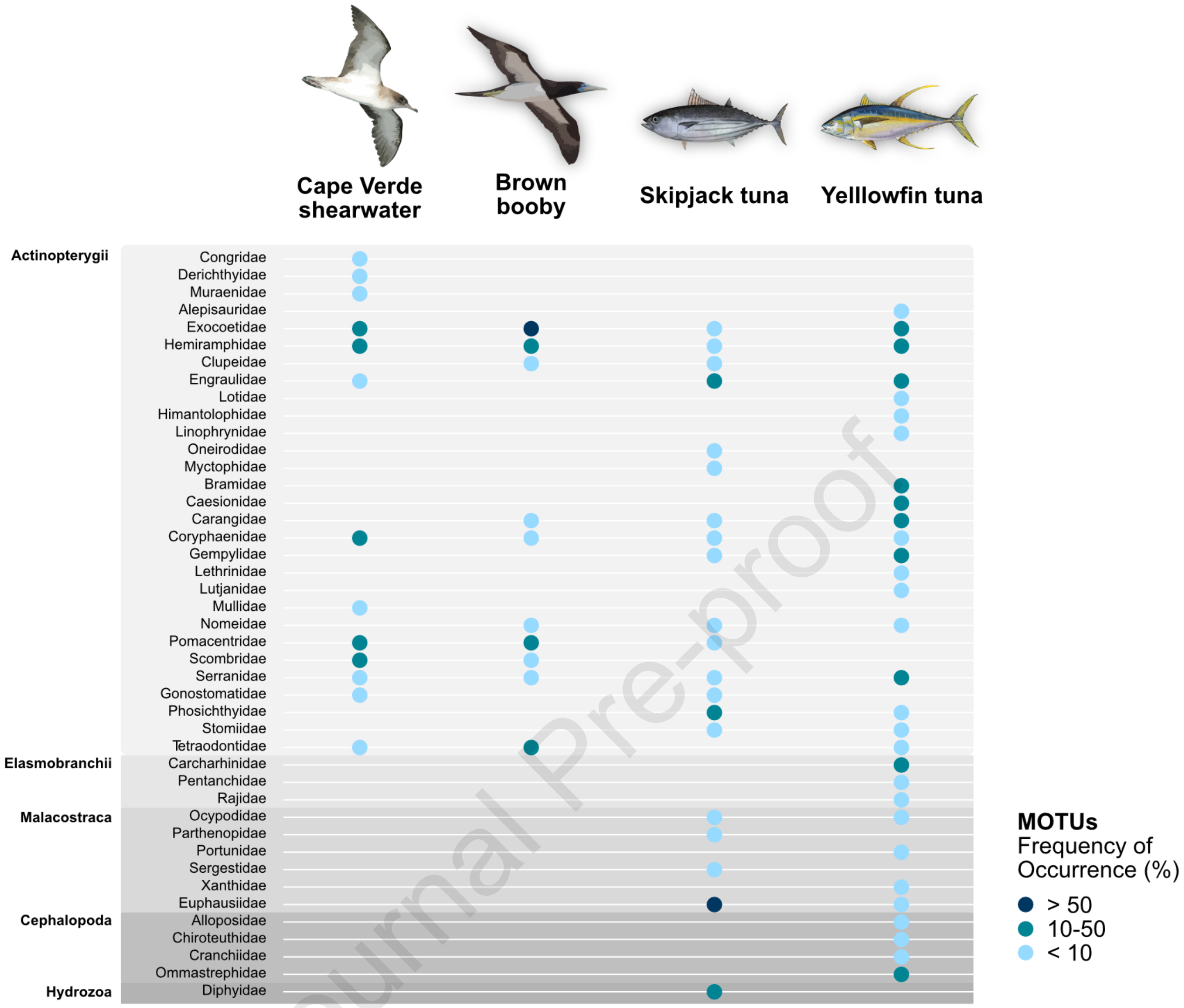
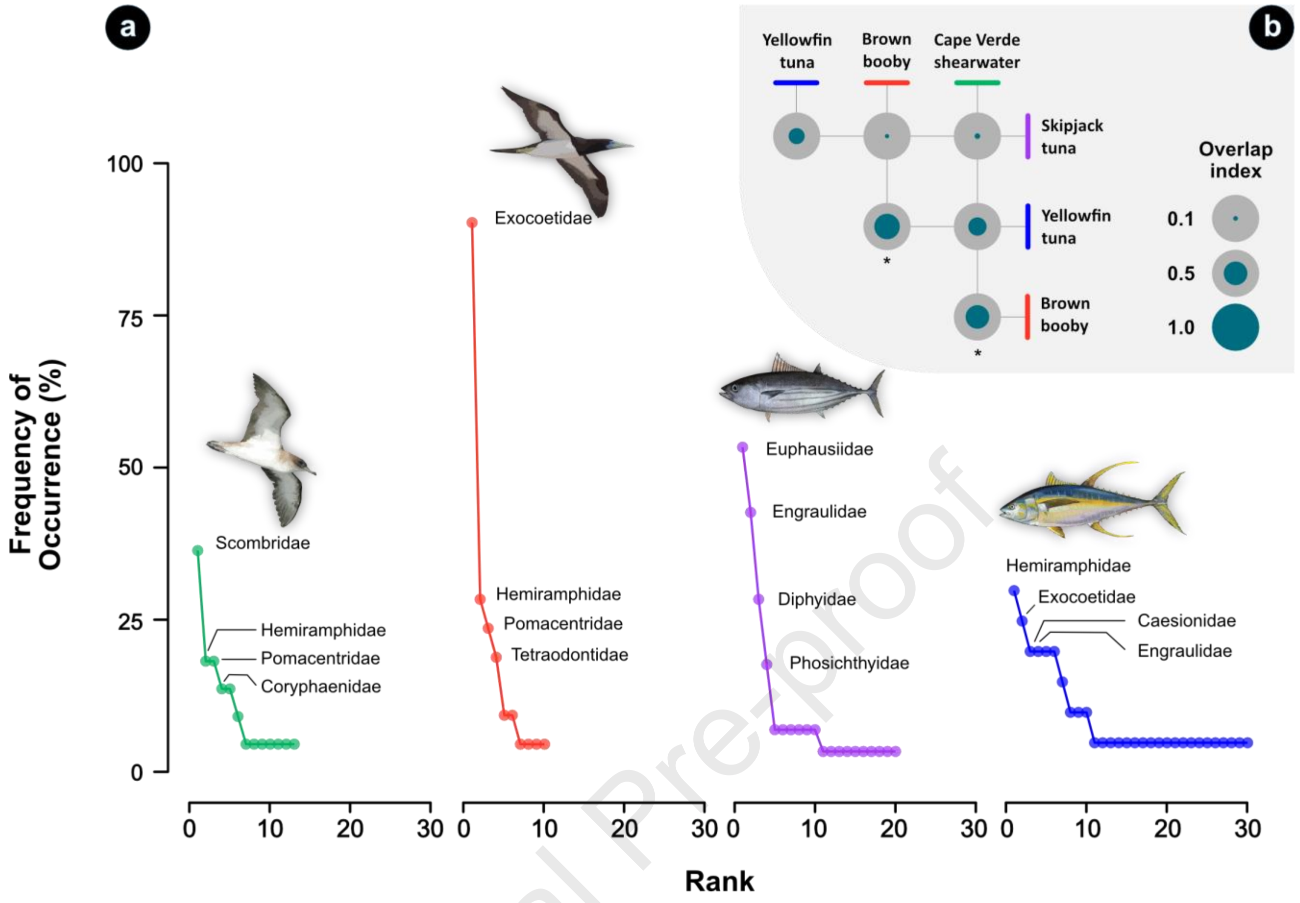
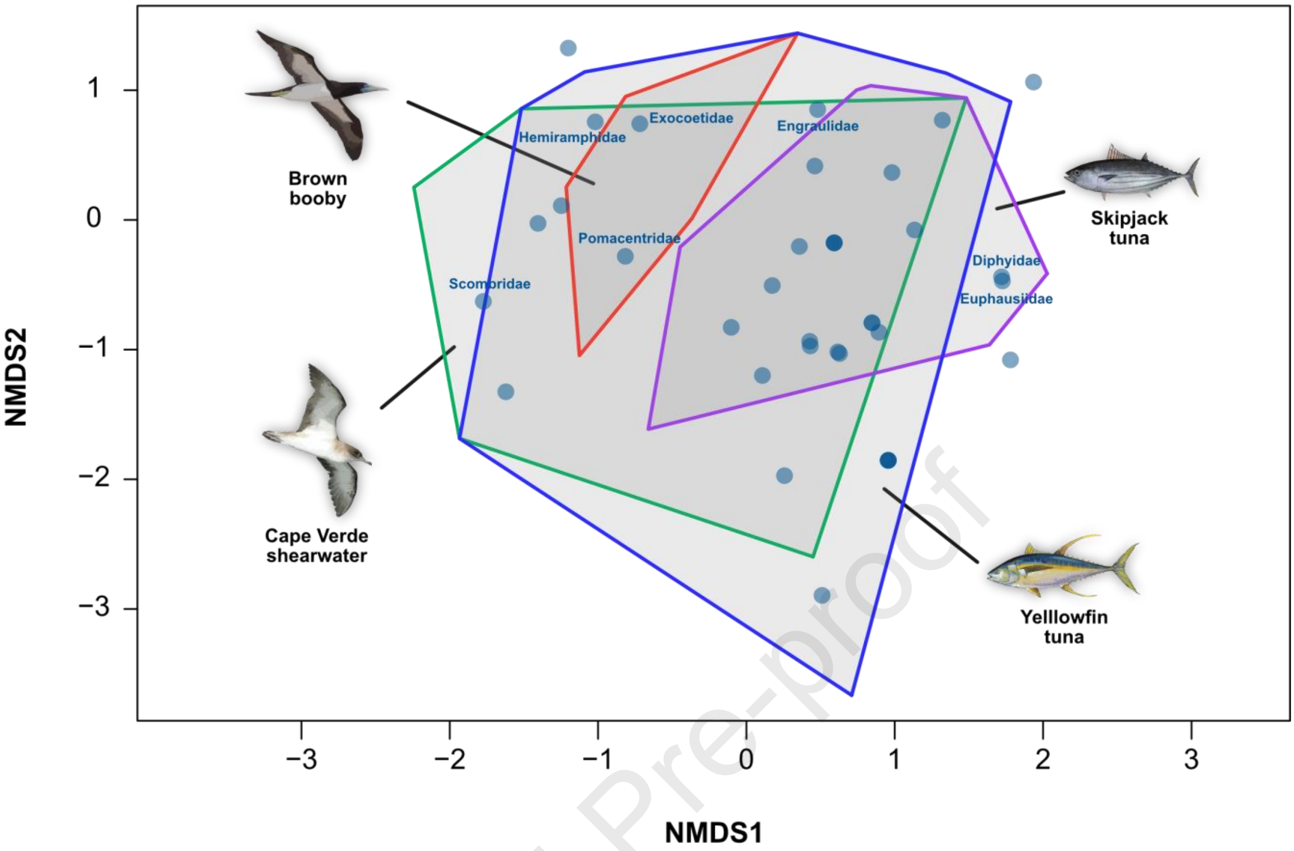

Cape Verde shearwater
Brown booby
Skipjack tuna
Yelllowfin tuna
Actinopterygii
Congridae
Derichthyidae
Muraenidae
Alepisauridae
Exocoetidae
Hemiramphidae
Clupeidae
Engraulidae
Lotidae
Himantolophidae
Linophrynidae
Oneirodidae
Myctophidae
Bramidae
Caesionidae
Carangidae
Coryphaenidae
Gempylidae
Lethrinidae
Lutjanidae
Mullidae
Nomeidae
Pomacentridae
Scombridae
Serranidae
Gonostomatidae
Phosichthyidae
Stomiidae
Tetraodontidae
Elasmobranchii
Carcharhinidae
Pentanchidae
Rajidae
Malacostraca
Ocypodidae
Parthenopidae
Portunidae
Sergestidae
Xanthidae
Euphausiidae
Cephalopoda
Alloposidae
Chiroteuthidae
Cranchiidae
Ommastrephidae
Hydrozoa
Diphyidae
MOTUs
Frequency of Occurrence (%)
> 50
10-50
< 10

Figure 3


a
b
100
75
50
25
0
Frequency of Occurrence (%)
Scombridae
Hemiramphidae
Pomacentridae
Coryphaenidae
Exocoetidae
Hemiramphidae
Pomacentridae
Tetraodontidae
Euphausiidae
Engraulidae
Diphyidae
Phosichthyidae
Hemiramphidae
Exocoetidae
Caesionidae
Engraulidae
0
10
20
30
0
10
20
30
0
10
20
30
0
10
20
30
Rank
Yellowfin tuna
Brown booby
Cape Verde shearwater
Skipjack tuna
Yellowfin tuna
Brown booby
*
*
Overlap index
0.1
0.5
1.0

849 **Figure 4**



850

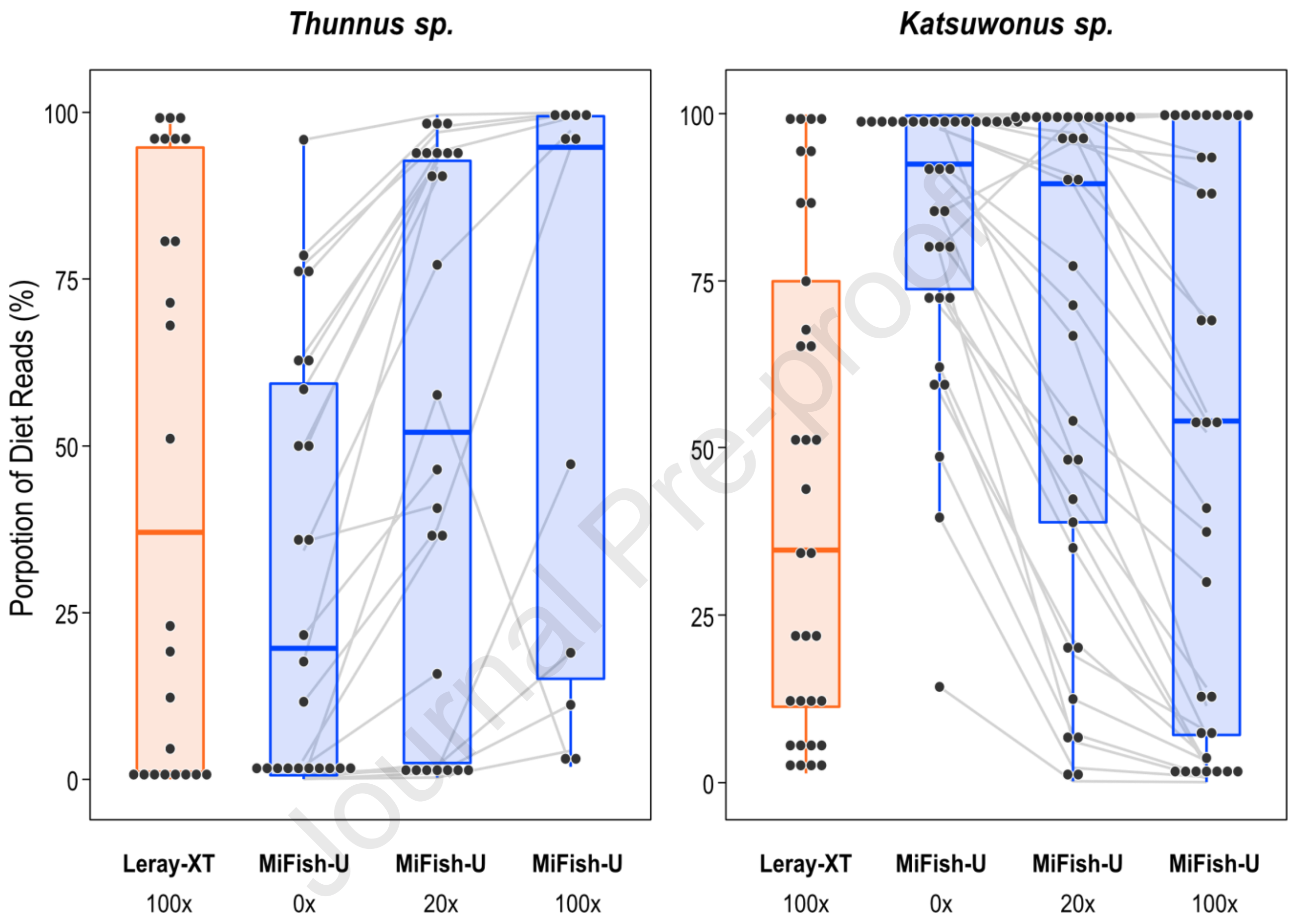
851

Appendices

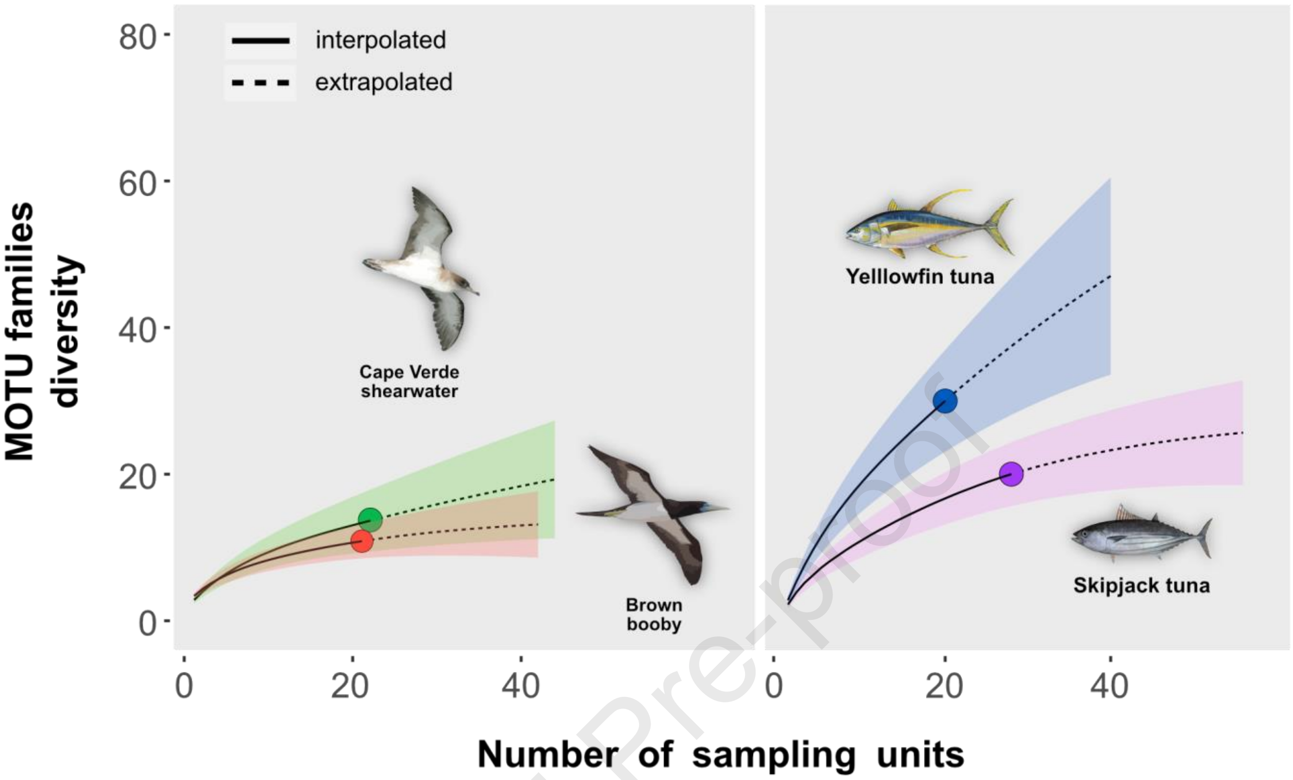
Appendix A. PCR conditions for each of the HTS libraries.

Library		Blocking primer concentration	Volume of reaction (µl)	Number of cycles	Denaturing	Annealing	Extension
Tuna	MiFish-U	0X	10	35	94ºC - 30s	60º - 30s	72º - 60s
	MiFish-U	20X	12	35	94ºC - 30s	60º - 30s	72º - 60s
	MiFish-U	100X	12	35	94ºC - 30s	60º - 30s	72º - 60s
	Leray-XT	100X	12	35	94ºC - 30s	45º - 30s	72º - 60s
Seabird	MiFish-U	0X	10	35	94º - 30s	60º - 30s	72º - 60s
	Leray-XT	0X	10	40	94º - 30s	45º - 45s	72º - 60s

Appendix B. Boxplots representing the proportion of diet reads per sample. Differences between libraries are shown for each of the blocking primers designed, for *Thunnus* sp. samples (left panel) and *Katsuwonus* sp. samples (right panel). Different concentrations of blocking primer were connected with a grey line to visualize the trend for each sample between MiFish-U libraries.



Appendix C. Prey MOTUs family's accumulation curves and extrapolated richness, with 95% confidence intervals, for each predator species.



DNA metabarcoding to assess prey overlap between tuna and seabirds in the Eastern Tropical Atlantic: implications for an ecosystem-based management

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Highlights

- DNA metabarcoding assess diet of tuna and seabirds of Eastern Atlantic Ocean.
- Skipjack tuna diet was dominated by prey from lower trophic levels.
- Significant diet overlap between Brown booby and Yellowfin tuna.
- Results have implications for the management of fisheries in this region.

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Author Contributions

Ana R. Carreiro: investigation; Ana Carreiro, Vitor H. Paiva, Jaime A. Ramos and Ricardo J. Lopes: conceptualization, methodology, resources; Ana R. Carreiro and Ricardo J. Lopes: formal analysis, data curation, writing - original draft; Vitor H. Paiva, Jaime A. Ramos and Ricardo J. Lopes: funding acquisition, supervision, writing – review & editing; Vanessa A. Mata: software, writing – review & editing; Nathalie M. Almeida, Isabel Rodrigues, Ivo dos Santos, Diana M. Matos, Pedro M. Araújo, Teresa Militão, Jacob González-Sólis: resources, writing – review & editing. All authors read and approved the final manuscript.

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Declaration of interest: None

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