


A glimpse into the trophic ecology of deep-water sharks in an important crustacean fishing ground

Sofia Graça Aranha¹  | Alexandra Teodósio¹ | Vânia Baptista¹ | Karim Erzini¹ | Ester Dias²

¹CCMAR – Centre of Marine Sciences, Universidade do Algarve, Faro, Portugal

²CIIMAR/CIMAR – Interdisciplinary Centre of Marine and Environmental Research, Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Matosinhos, Portugal

Correspondence

Sofia Graça Aranha, CCMAR-Centre of Marine Sciences, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
Email: sgramos@ualg.pt

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Abstract

Deep-water sharks are among the most vulnerable deep-water taxa because of their extremely conservative life-history strategies (*i.e.*, late maturation, slow growth, and reproductive rates), yet little is known about their biology and ecology. Thus, this study aimed at investigating the trophic ecology of five deep-water shark species, the birdbeak dogfish (*Deania calcea*), the arrowhead (*D. profundorum*), the smooth lanternshark (*Etmopterus pusillus*), the blackmouth catshark (*Galeus melastomus*) and the knifetooth dogfish (*Scymnodon ringens*) sampled onboard a crustacean bottom-trawler off the south-west coast of Portugal. We combined carbon and nitrogen stable isotopes with RNA and DNA (RD) ratios to investigate the main groups of prey assimilated by these species and their nutritional condition, respectively. Stable isotopes revealed overall small interspecific variability in the contribution of different taxonomic groups to sharks' tissues, as well as in the origin of their prey. *S. ringens* presented higher $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values than the other species, suggesting reliance on bathyal cephalopods, crustaceans and teleosts; the remaining species likely assimilated bathy-mesopelagic prey. The RD ratios indicated that most of the individuals had an overall adequate nutritional condition and had recently eaten. This information, combined with the fact that stable isotopes indicate that sharks assimilated prey from the local or nearby food webs (including commercially important shrimps), suggests a potential overlap between this fishing area and their foraging grounds, which requires further attention.

KEYWORDS

bottom-trawl, diet, ecophysiology, north-east Atlantic, RNA/DNA, stable isotopes

1 | INTRODUCTION

Deep-water chondrichthyans (*i.e.*, sharks, skates, rays and chimaeras living below 200 m depth) are among the deep-water taxa that are most vulnerable to extinction due to their highly conservative life histories (*i.e.*, slow growing, late to mature and low reproductive

rates; Cortés, 1999) and thus slow population recovery rates (Simpfendorfer & Kyne, 2009). According to the European Red List of Marine Fishes, approximately 30% of the deep-water shark species with zero total allowable catch are threatened, facing an elevated risk of extinction, while approximately 5% are classified as 'data deficient' (IUCN; Nieto *et al.*, 2015; Regulation EC no 2021/91). Despite the

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existing regulations, they still compose a large portion of the bycatch in European fisheries, especially the deep-water crustacean bottom-trawl fishery (Borges *et al.*, 2001). However, little is known about their biology, ecology and population status (Cotton & Grubbs, 2015; Kyne & Simpfendorfer, 2007). This is primarily due to the inherent difficulties in studying species in inaccessible habitats, which require expensive equipment and rigorous logistical protocols (Brooks *et al.*, 2015). The scarcity of basic biological and ecological information compromises the development of proper management and conservation strategies for deep-water sharks (Kyne & Simpfendorfer, 2010), creating uncertainty regarding the potential effects of their removal on the structure and functioning of deep-water ecosystems.

Deep-water sharks have multiple feeding habits, varying with species (*e.g.*, Cortés, 1999), size (*e.g.*, Besnard *et al.*, 2022; Xavier *et al.*, 2012), space (*e.g.*, Ebert *et al.*, 1992; Mauchline & Gordon, 1983; Pethybridge *et al.*, 2011; Preciado *et al.*, 2009), season (*e.g.*, Anastasopoulou *et al.*, 2013) and resource availability (*e.g.*, Dunn *et al.*, 2013). In general, deep-water sharks are meso- to top predators (Churchill *et al.*, 2015; Cortés, 1999), which implies they can have top-down interactions in the marine food webs. Thus, the removal of these predators due to overfishing, for instance, has the potential to affect the overall structure of marine food webs due to changes in prey composition or availability, or by impacting communities lower down the food web (Brooks & Dodson, 1965; Heithaus *et al.*, 2008; Shipley *et al.*, 2017). Therefore, understanding the role played by sharks in the deep-water food webs is necessary to improve predictions of ecosystem responses to ongoing perturbations (*e.g.*, Stergiou & Karpouzi, 2002). The most widely used approaches to study sharks' diet and estimate their trophic position are the analyses of stomach contents and stable isotopes (Hussey *et al.*, 2012). Stomach content analysis can provide a precise assessment of sharks' diet (Hyslop, 1980). However, this method is time-consuming, highly invasive and requires a large number of samples, while only providing a snapshot of the recently consumed prey (Baker *et al.*, 2014; Simpfendorfer *et al.*, 2001). Moreover, given the opportunistic feeding behaviour of many sharks (*e.g.*, Jones & Geen, 1977; Olaso & Rodríguez-Marín, 1995), stomach content data are usually insufficient to adequately characterize trophic position, except in rare instances where regular and long-term stomach content data sets are available (Caut *et al.*, 2013). On the other hand, carbon and nitrogen stable isotopes provide time-integrated information about the prey assimilated, rather than recently ingested, by a given consumer (Peterson & Fry, 1987). Carbon isotope ratios ($\delta^{13}\text{C}:^{13}\text{C}/^{12}\text{C}$) are frequently used to distinguish among different autotrophs at the base of the food web because they differ among primary producers with respect to C source and fixation (Cloern *et al.*, 2002; Dias *et al.*, 2016; Smith & Epstein, 1971). Carbon isotopes are also useful for identifying general patterns of inshore/benthic vs. offshore/pelagic feeding preferences (France, 1995; Lawson & Hobson, 2000; McMahon *et al.*, 2013). Nitrogen isotope ratios ($\delta^{15}\text{N}:^{15}\text{N}/^{14}\text{N}$) are more commonly used to estimate trophic position in food webs (Vander-Zanden *et al.*, 1997) as they generally exhibit high trophic fractionation between prey and consumers (Caut *et al.*, 2009).

Monitoring the nutritional condition can provide additional information about prey consumption, indicating if a given individual has been feeding or not for the previous days (Buckley *et al.*, 1999). Among the most used condition indices at the organism level in marine ecology are the nucleic acid-derived indices, such as RNA/DNA values (RD). This ratio has been successfully applied as an indicator of growth (*e.g.*, Bulow, 1987; Caldarone *et al.*, 2003; Tavares *et al.*, 2006), nutritional condition (*e.g.*, Alves *et al.*, 2020; Chícharo *et al.*, 2003; Chícharo & Chícharo, 1995), productivity (*e.g.*, Cruz *et al.*, 2017), health status (*e.g.*, Tavares *et al.*, 2006) and as an indicator of natural or anthropogenic impacts in marine populations and communities (Chícharo & Chícharo, 2008; Müller *et al.*, 2020). The RD values provide a short-term measure of nutritional condition (1–3 days Buckley *et al.*, 1999), based on the fact that DNA concentrations within individual cells remain relatively constant (Wallace, 1992) while RNA concentrations increase as protein synthesis increases (Buckley *et al.*, 1999). The RD values vary with life stage, sex, size, disease state and environmental conditions (*e.g.*, Buckley, 1980; Bulow, 1970; Ferron & Leggett, 1994; Suthers *et al.*, 1996). Thus, a recently well-fed, active-growing individual should have a relatively high RD value compared to a starving individual of the same species (Bulow, 1987; Richard *et al.*, 1991; Robinson & Ware, 1988). This approach was used in different marine taxa such as fishes (*e.g.*, Baptista *et al.*, 2019; Morais *et al.*, 2017), crustaceans (*e.g.*, Gonçalves *et al.*, 2021), bivalves (*e.g.*, Chícharo *et al.*, 2007), cephalopods (*e.g.*, Sykes *et al.*, 2004), marine turtles (Vieira *et al.*, 2014) and marine mammals (Alves *et al.*, 2020). To the best of our knowledge, only Cruz-Ramírez *et al.* (2017) and Tavares *et al.* (2006) have used RD in chondrichthyans, recognizing that this technique could be essential to evaluate instantaneous growth and health status in shark species. Thus, obtaining information on elasmobranchs' nutritional traits such as resource uptake and use may help identify critical life stages or areas for conservation and management.

Because fisheries are expanding to deeper waters worldwide (Cotton & Grubbs, 2015), and given the vulnerability of sharks to anthropogenic pressures (García *et al.*, 2008; Simpfendorfer & Kyne, 2009), more information on the biology and ecology of deep-water sharks is urgently needed. Thus, the present study combined stable isotopes and nucleic acids to investigate the feeding ecology of free-ranging deep-water sharks coexisting in a crustacean bottom-trawling fishing ground on the south-west coast of Portugal. This study area was selected because it is one of Portugal's most important fishing grounds for crustacean bottom-trawl fisheries, where high levels of bycatch of deep-water sharks have been reported (Borges *et al.*, 2001). The goals of this study were to (1) identify the main groups of prey (teleost, crustaceans and cephalopods) assimilated by deep-water shark species commonly found on the south-west coast of Portugal (ICES 2020) as well as their origin (bathyal or bathy-mesopelagic), (2) determine their trophic position and (3) estimate their short-term nutritional condition. We hypothesized that sharks are tertiary/quaternary consumers (trophic position ≥ 4 ; Cortés, 1999; Barriá *et al.*, 2015), feeding on a variety of deep-water prey from the local or

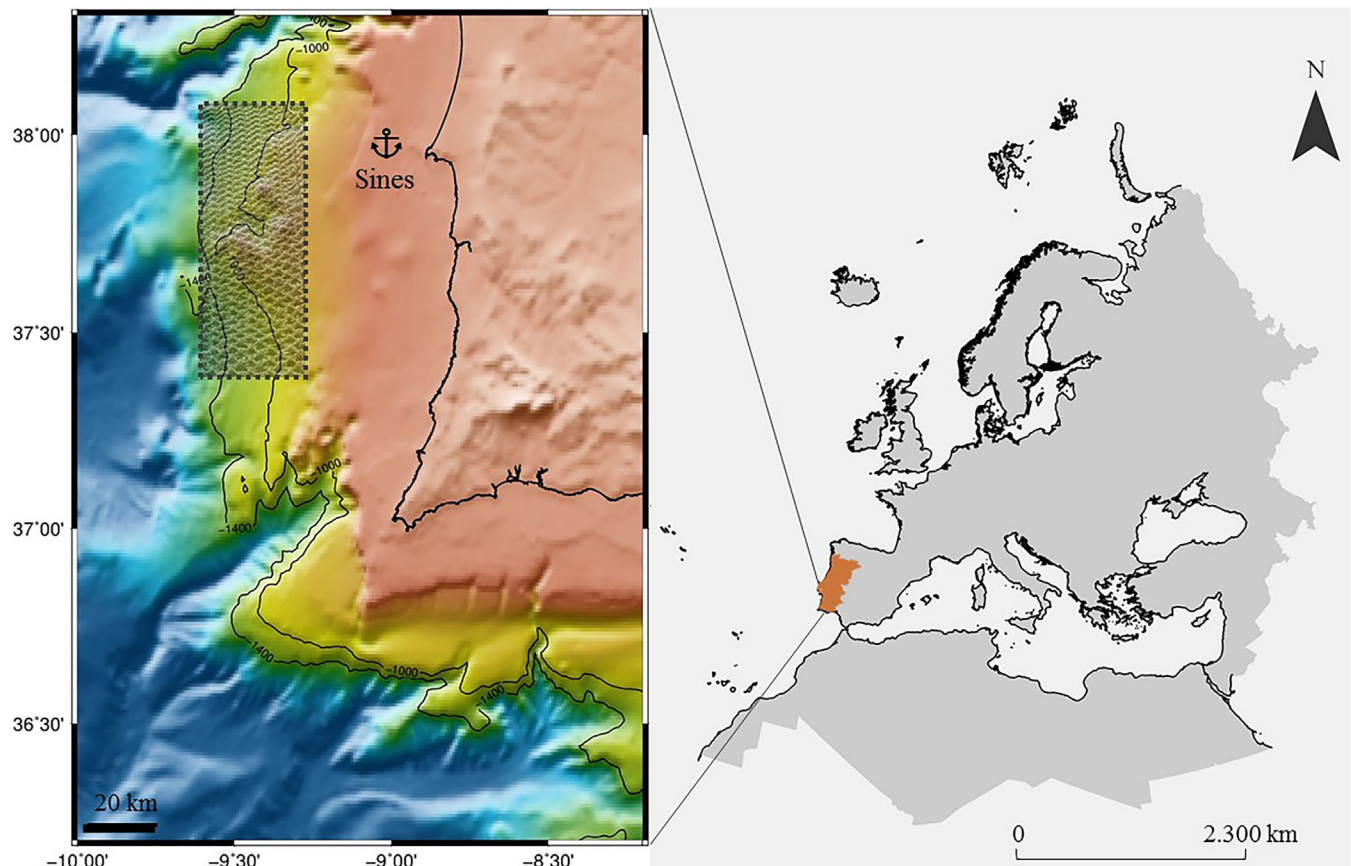


FIGURE 1 Study area off the south-west coast of Portugal (south-west Europe) showing the fishing port of Sines and the isobaths (black lines) of the sampling area (1000–1400 m) (created with Mirone software)

nearby marine food webs (Dunn *et al.*, 2013), including commercially important shrimps (Santos & Borges, 2001).

2 | MATERIALS AND METHODS

2.1 | Field sampling

Sampling took place off the south-west coast of Portugal (Figure 1) in February 2018 during a 4-day commercial fishing trip onboard a crustacean bottom-trawler targeting the giant red shrimp *Aristaeomorpha foliacea* (Risso 1827) and the scarlet shrimp *Aristaeopsis edwardsiana* (Johnson 1868) and operating at depths beyond the continental shelf (1107–1350 m). A total of six hauls were conducted with an average duration of 6 h. At the end of each haul, deep-water sharks (hereafter sharks) and their potential prey species were immediately separated from the remaining catch. Sharks that were alive were immediately placed inside three containers of 80 × 40 × 30 cm filled with flowing seawater. Each individual was identified (following Compagno *et al.*, 2005), measured [total length (TL): from the tip of the snout to the tip of the caudal fin, ±0.1 cm], weighed (±0.1 g) and sexed (male-claspers present, female-claspers absent). Life stage was identified (adult or juvenile) for all species based on Coelho and Erzini (2005),

Paiva *et al.* (2012) and Ebert *et al.* (2021) except for *Scymnodon ringens*, for which there is no available information to conduct such identification.

Muscle samples were collected following a modified procedure developed for teleosts (Henderson *et al.*, 2016), which consisted of an incision in the base of the first dorsal fin on the left side of each individual's body. A subsample was collected for stable isotope analysis and another for RNA/DNA (RD) analysis, which were stored frozen at –20°C and in RNA Riboreserve™, respectively, for subsequent analysis. The entire procedure lasted a maximum of 2 min for the live sharks, which were returned to the sea.

The main groups of sharks' potential prey were selected based on previous studies on stomach content analysis, including in nearby areas (Table S1). Potential prey included crustaceans (shrimps, prawns and squat lobsters, Scarlet lobsterette and crabs), teleosts and cephalopods (squid and octopus), which were frozen prior to laboratory analysis. Additionally, zooplankton samples were collected to characterize the isotopic baseline of the local marine food web. For that, a plankton net (500 µm mesh size) was towed vertically from a maximum depth of 80 m during the night, considering diel vertical migration of zooplankton from deeper waters, and samples were immediately preserved in 70% ethanol after collection.

2.2 | Laboratory analysis

Shark muscle samples were dried at 60°C for at least 48 h and ground to a fine powder with a mortar and pestle. Urea and lipids were removed following Carlisle *et al.* (2017) as both compounds are known to impair $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, respectively.

Potential prey were thawed and identified to the lowest taxonomic level possible (Bauchot, 1986; Froese & Pauly, 2019; Gibbs, 1984; Jereb *et al.*, 2016; Whitehead *et al.*, 1987). Muscle was collected from the dorsal region of each fish. From crustaceans, muscle tissue was collected from the tail of shrimps and lobster, and from the crabs' appendages. Cephalopod muscle samples were collected from the mantle (squids) or from the appendages (octopuses). All samples were dried at 60°C for at least 48 h and ground to a fine powder with a mortar and pestle.

Zooplankton was sorted, and copepods were selected and dried (60°C) for 24 h.

Stable isotope ratios were measured using a Thermo Scientific Delta V Advantage IRMS *via* ConFlo IV interface (Marinova, University of Porto). The raw data were normalized by three-point calibration using the international reference materials IAEA-N-1 ($\delta^{15}\text{N} = +0.4\text{‰}$), IAEA-NO-3 ($\delta^{15}\text{N} = +4.7\text{‰}$) and IAEA-N-2 ($\delta^{15}\text{N} = +20.3\text{‰}$) for nitrogen isotopic composition, and two-point calibration using USGS-40 ($\delta^{13}\text{C} = -26.39\text{‰}$) and USGS-24 ($\delta^{13}\text{C} = -16.05\text{‰}$) for carbon isotopic composition. Stable isotope ratios are reported in δ notation, $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3$, where X is the C or N stable isotope and R is the ratio of heavy/light stable isotopes. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are expressed in units per mil (‰) relative to Vienna Pee Dee Belemnite and air, respectively. The analytical error, the mean standard deviation (SD) of the replicate reference material, was 0.1 ‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

The nutritional condition was determined using RNA and DNA ratios from a microplate fluorescent assay (Caldarone *et al.*, 2001). Samples were cleaned with distilled water, dried on a paper sheet, placed in a new vial and frozen at -80°C prior to lyophilization. Samples were freeze-dried under a pressure of -10 atm at -40°C for about 36 h. Nucleic acids were chemically and mechanically extracted and determined following procedures described in Esteves *et al.* (2000).

To reduce the differences in RD values among protocols and allow for future comparisons among studies, we used the standardized RD (hereafter RD) values to evaluate the nutritional condition of sharks, which was based on the DNA and RNA standard's slope ratio of 3.73 and the reference slope ratio of 2.4, as described in Caldarone *et al.* (2006). Then, to ensure that the RNA digestion was complete and that no DNA degradation occurred, 'only-DNA' and 'only-RNA' control samples were run in each plate, where the samples were previously analysed, and a RNAase digestion was applied to all the samples (except for the 'only-RNA').

2.3 | Data analysis

Interspecific differences in the sharks' $\delta^{13}\text{C}$ values were tested using a nonparametric Kruskal-Wallis test followed by a pairwise

multicomparison Dunn's test; differences between the $\delta^{15}\text{N}$ values were tested using a one-way analysis of variance (ANOVA one-way) with a Tukey's HSD *post hoc* test for paired contrasts.

When dealing with predators that feed on multiple species, a reduced set of prey species or consolidating prey species is necessary due to overlapping isotopic values (Phillips *et al.*, 2005). In this case, prey were grouped according to their taxonomic group (teleosts, squids, octopus, crabs, lobsters and shrimps) and habitat (bathyal and bathy-mesopelagic). Because Myctophids are considered important prey for some deep-water sharks (Supporting Information Table S1), and since they were not collected during this study, estimates from the Mediterranean were used instead ($8.4 \pm 0.2\text{‰}$ $\delta^{15}\text{N}$ and $-20.6 \pm 0.8\text{‰}$ $\delta^{13}\text{C}$; Barria *et al.*, 2015). Although stable isotope values may vary between geographic areas, zooplankton (Myctophid's main prey; Hulley, 1990) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ estimates are similar between these two areas (McMahon *et al.*, 2013). Therefore, small differences are expected between the stable isotope values of Myctophidae from the Mediterranean and the south-west coast of Portugal. Groups of the most likely prey for each shark species were identified using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ biplots where sharks' $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were compared to each potential prey's $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, after adjusting for trophic fractionation (Phillips *et al.*, 2014). The relative contribution of the most likely prey to the diet of sharks was quantified using the Bayesian stable isotope mixing model MixSIAR v3.1.12 (Stock & Semmens, 2016). To run the models, the stable isotope values of sharks and their most likely prey groups were input as raw data, using noninformative priors because of the general lack of dietary information for the species in the study area. Model convergence was assessed *via* Gelman-Rubin and Geweke diagnostics (Gelman *et al.*, 2013; Geweke, 1991). Posterior distributions obtained from the MixSIAR analyses are expressed as median and 95% credibility intervals. For the mixing model, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were adjusted to one trophic level using the trophic fractionation estimates from Hussey *et al.* (2010) ($2.3 \pm 0.2\text{‰}$ $\delta^{15}\text{N}$, $0.9 \pm 0.3\text{‰}$ $\delta^{13}\text{C}$), which were obtained from controlled experiments with lemon sharks (*Negaprion brevirostris*, Poey 1868) and sand tiger sharks (*Carcharias taurus*, Rafinesque 1810). Because the resulting isospace was narrow when compared to ecosystems like estuaries or shallower areas of the ocean, we ran three models for each shark species: all prey groups, prey groups combined according to their position in the isospace (squids, lobsters, octopus+tel1, crabs+shrimps+tel2) and prey group selection based on the proximity of prey in relation to a given consumer. Models were compared using the function `compare_models` available in the package `loo` (Vehtari *et al.*, 2017). The best model, in this case, model 3, was the one presenting $d\text{LOOic} = 0$ and the resulting errors were lower or close to 1 (Stock & Semmens, 2016). The errors obtained for the species *Deania calcea* and *Deania profundorum* were greater than 1, indicating that there was a structure in the data that was not resolved with the available information, sources could be missing, or due to isotopic routing (Stock & Semmens, 2016). For that reason, only a qualitative analysis was conducted for these species.

Copepods and teleosts (with C:N > 3.5) $\delta^{13}\text{C}$ values were corrected for lipid content according to the mass balance correction

models of Smyntek *et al.* (2007), equation 5 and Hoffman and Sutton (2010), equation 6, respectively. Copepods were also corrected for ethanol preservation (0.4‰ $\delta^{13}\text{C}$, 0.6‰ $\delta^{15}\text{N}$; Feuchtmayr & Grey, 2003).

Shark trophic position (TP) was determined following the scaled framework proposed by Hussey *et al.* (2014b):

$$\text{TP} = \frac{\log(\delta^{15}\text{N}_{\text{lim}} - \delta^{15}\text{N}_{\text{copepod}}) - \log(\delta^{15}\text{N}_{\text{lim}} - \delta^{15}\text{N}_{\text{sharks}})}{k} + \text{TP}_{\text{copepod}}$$

where $\delta^{15}\text{N}_{\text{lim}} = -\beta_0/\beta_1$ and the intercept β_0 and slope β_1 were 5.92 and -0.27 , respectively (Hussey *et al.*, 2014a). The $\delta^{15}\text{N}_{\text{copepod}}$ was 4.7‰, which is the direct measurement of the $\delta^{15}\text{N}$ values for the baseline organisms, in this case copepods, which are assumed to belong to the $\text{TP}_{\text{copepod}}$ 2, $\delta^{15}\text{N}_{\text{sharks}}$ is the direct measurement of the $\delta^{15}\text{N}$ values for each shark and k is the rate at which $\delta^{15}\text{N}_{\text{TP}}$ approaches $\delta^{15}\text{N}_{\text{lim}}$ per TP step, *i.e.*, $k = -\log(\beta_0 - \delta^{15}\text{N}_{\text{lim}} / -\delta^{15}\text{N}_{\text{lim}})$ (Hussey *et al.*, 2014b).

The nucleic acid-derived indices were calculated in relation to dry weight ($\mu\text{g RNA mg}^{-1}\text{ DW}$, $\mu\text{g DNA mg}^{-1}\text{ DW}$ and RD). The RD was obtained by the ratio of the RNA mg^{-1} and the DNA mg^{-1} resulting in the RD value. Since the effect of size (TL) may influence these indices, a linear regression model was conducted and no significant relationships were found between those indices and size (Supporting Information Figure S2; Suthers *et al.*, 1996; Chicharo *et al.*, 1998). A one-way ANOVA was conducted to test for differences in the RD between shark species.

Because there are no estimates of critical RD values for sharks (*i.e.*, threshold values to determine if sharks are in a good or poor condition), the nutritional condition was evaluated using the percentile approach (Alves *et al.*, 2020; Meyer *et al.*, 2012). The percentile approach shows that if the RD mean values are closer to the 75th percentile, then the individuals' samples from a certain species are in an adequate nutritional condition and have fed in the last 1–3 days (Alves *et al.*, 2020; Meyer *et al.*, 2012). On the other hand, if the RD mean values of the individuals' samples are closer to 10th percentile, individuals are considered to be in poor nutritional condition (Alves *et al.*, 2020; Meyer *et al.*, 2012).

To test for the differences (at $P < 0.05$) between the stable isotope values between sharks or in the RD values, various parametric and nonparametric analyses were used depending on whether normality and homoscedasticity hypotheses were verified. All statistical analyses were conducted with the open-source statistical language R (R Core Team, 2021).

3 | RESULTS

3.1 | Stable isotope analysis

A total of 34 sharks were collected belonging to five species (Table 1). The mean $\delta^{13}\text{C}$ (Kruskal–Wallis: $H(4) = 16.5$, $P = 0.002$) and $\delta^{15}\text{N}$ values (ANOVA: $F_{(3,24)} = 15.78$, $P = 8.98 \cdot 10^{-7}$) were different between

species. *S. ringens* was ^{15}N -enriched when compared to all the other species (pairwise tests, $P < 0.001$) and more ^{13}C -enriched than *D. profundorum* (pairwise tests, $P = 0.009$), which was ^{15}N -depleted when compared to *D. calcea* (pairwise tests, $P = 0.02$) (Table 2).

The teleosts, crustaceans and cephalopods collected during this study (57 individuals) included a variety of species occupying different habitats (mesopelagic to bathyal), habits (pelagic and demersal) and with different migratory behaviours (*i.e.*, migratory, nonmigratory, diel vertical migrations) (Table 3). Overall, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the sharks collected, after adjusting for trophic fractionation, were intermediate between the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of several prey groups, indicating reliance on different sources (Figure 2). The low $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of *E. pusillus*, *Galeus melastomus*, *D. calcea* and *D. profundorum* indicate they assimilated ^{13}C - and ^{15}N -depleted sources, such as shrimps, squids and lobsters (Figure 2). *S. ringens* assimilated ^{13}C - and ^{15}N -enriched prey than the previous shark species, indicating a higher contribution of bathyal prey, including teleosts (Tel1, Table 3) and octopus (Figure 2).

The dual-stable isotope mixing model results indicate that overall, cephalopods and crustaceans were the main prey contributing to the tissues of the analysed sharks, followed by bathyal teleosts (Figure 3). Bathyal squids were the main prey assimilated by *G. melastomus* and *E. pusillus*, which also relied on mesopelagic and bathyal crustaceans (Figure 3). *S. ringens* assimilated mostly bathyal species of cephalopod, crustacean and teleost groups (Figure 3).

Overall, sharks from this study were classified as tertiary consumers (TP close to 4). Trophic position (TP) values varied between 3.8 for *D. profundorum* and 4.5 (± 0.2) for *S. ringens* (Table 2).

3.2 | Nutritional condition

A nonsignificant positive relationship was found between the nucleic acid-derived indices and size (TL) for *D. profundorum*, *E. pusillus* and *S. ringens*. Contrarily, a nonsignificant negative relationship was found for *D. calcea* and *G. melastomus* (Supporting Information Figure S2). Likewise, the values of RD did not vary significantly between the shark species (ANOVA: $F_{(6, 30)} = 1.5$, $P = 0.2$).

G. melastomus presented the highest mean RD values among the species studied and the one with the lowest was *D. profundorum* (Table 2). The interquartile analysis showed that mean RD values were generally close to the 75th percentile for all species except for *G. melastomus*, where the mean RD value was close to the 10th percentile (Figure 4).

4 | DISCUSSION

The present study combined, for the first time, stable isotopes and nucleic acids to investigate the feeding ecology of free-ranging deep-water sharks coexisting in a crustacean bottom-trawling fishing ground on the south-west coast of Portugal. The stable isotopes revealed that sharks are tertiary consumers and the mixing models

TABLE 1 Shark species and number of individuals (*n*) collected in February 2018 off the south-west coast of Portugal

Species	<i>n</i>	Sex (<i>n</i>)	Life stage (<i>n</i>)	TL (cm)	Weight (g)	Condition (<i>n</i>)
<i>Deania calcea</i>	8	M (3)	A (3)	84.3 ± 3.1	2067 ± 152.8	P (1) D (2)
		F (5)	J (5)	70.3 ± 14.8	1514 ± 1112	P (3) D (2)
<i>Deania profundorum</i>	4	F (4)	J (4)	44.3 ± 6.1	287.4 ± 98.3	P (1) D (3)
<i>Etmopterus pusillus</i>	5	M (2)	A (2)	41	315 ± 35	G (1) D (1)
		F (3)	A (2) J (1)	41.8 ± 4.1	343.3 ± 89.9	D (3)
<i>Galeus melastomus</i>	5	F (5)	A (5)	61.8 ± 5.6	678 ± 168.7	P (2) D (3)
<i>Scymnodon ringens</i>	12	M (4)	n/a	51.9 ± 2.2	957.5 ± 316.7	P (2) D (2)
		F (8)	n/a	59.5 ± 11.7	1382.5 ± 958.7	P (6) D (2)

Note: Mean (±S.D.), total length (TL) and weight of the individuals collected from each species by sex, male (M) or female (F), life stage [adults (A), juveniles (J) or not available (n/a)]. The overall condition of each individual was determined as good (G), poor (P) or dead (D).

Species	δ ¹⁵ N (‰)	δ ¹³ C (‰)	TP	RNA/mg	DNA/mg	RD
<i>Deania calcea</i>	10.8 ± 0.6	-19 ± 0.7	4.2 ± 0.2	2.2 ± 1.1	3.3 ± 0.7	0.4 ± 0.2
<i>Deania profundorum</i>	9.8 ± 0.1	-19.8 ± 0.2	3.8 ± 0.0	1.5 ± 0.3	4.1 ± 0.9	0.3 ± 0.1
<i>Etmopterus pusillus</i>	10.5 ± 0.4	-19.2 ± 0.2	3.9 ± 0.1	3.1 ± 0.6	4.8 ± 0.9	0.4 ± 0.1
<i>Galeus melastomus</i>	10.6 ± 0.2	-19.3 ± 0.1	4.1 ± 0.1	2.6 ± 0.6	2.8 ± 0.4	0.6 ± 0.2
<i>Scymnodon ringens</i>	11.8 ± 0.4	-18.5 ± 0.2	4.5 ± 0.2	2.0 ± 1.3	3.5 ± 0.8	0.4 ± 0.2

TABLE 2 Mean (±S.D.) δ¹⁵N and δ¹³C values (‰) after correction for lipids and urea, trophic position (TP), RNA, DNA and standardized RNA/DNA values (RD) values of each shark species collected in February 2018 off the south-west coast of Portugal

showed low interspecific variability in the prey groups assimilated and their origin. The RD analysis suggests that overall most sharks were in a good condition and had recently fed, most likely in the area where sampling occurred or nearby areas.

4.1 | Stable isotopes analysis and trophic position

Bathyal cephalopods (pelagic and demersal) presented the highest relative contribution to the tissues of *E. pusillus*, *G. melastomus* and *S. ringens*, followed by bathy-mesopelagic crustaceans and teleosts, although with some variability between species. This pattern of prey assimilation does not mirror the relative importance of each prey group derived from stomach content analysis; in general, shrimps and teleosts are identified as the most frequent and abundant prey groups (e.g., Barría *et al.*, 2018; Muñoz, 2015; Neiva *et al.*, 2006; Xavier *et al.*, 2012). Nonetheless, a previous study conducted in the Mediterranean showed that the relative importance of cephalopods in *G. melastomus* stomachs can be as high as the one found in this study (close to 50%) (Barría *et al.*, 2018). Also, they compared the relative contribution of each group of prey derived from stomach contents and stable isotope analysis, which suggests that in some cases stomach content analysis alone might underestimate the relative importance of cephalopods to sharks' tissues. Whether the results in our study reflect prey availability or isotopic routing (i.e., differential allocation of isotopically distinct dietary components to different tissues; Schwarcz, 1991) is unclear because we did not analyse the stomach contents of the sampled sharks. Moreover, stomach contents studies may fail to characterize the entire prey spectrum of a given consumer due to the inherent limitations associated with such studies: (i) they

provide a snapshot of what the individual ate in the last hours, thus a high number of stomachs and a good temporal resolution is necessary to capture the diversity of all their prey due to the imbalance between easy-to-digest and difficult-to-digest, (ii) deep-water sharks generally present empty stomachs (e.g., Mauchline & Gordon, 1983; Preciado *et al.*, 2009) and may regurgitate food when brought to the surface (Bowman, 1986) and (iii) prey items from deep-water communities often are fragile and difficult to identify (Cailliet *et al.*, 1999; Drzen *et al.*, 2001; Robinson *et al.*, 2007). Nonetheless, we cannot exclude the possibility that we have not sampled all the possible prey groups. This seems to have been the case for *D. profundorum*, given their position in relation to that of the sampled prey, which suggests that ¹⁵N- and ¹³C-depleted prey may be missing in this dataset. Thus, future studies in this area should combine stable isotopes with traditional stomach content analysis or more recent metabarcoding approaches (e.g., Dunn *et al.*, 2010; van Zinnic Bergmann *et al.*, 2021) to identify the predator-prey links to improve the quantification of their importance.

The similarity in the stable isotope values of *E. pusillus* and *G. melastomus* suggests they likely share the same trophic niche. Although estimating trophic niche size and overlap was out of the scope of this study (low number of individuals per group; Jackson *et al.*, 2011), the stable isotope mixing models indicate they assimilated mostly bathyal squids, followed by bathy-mesopelagic shrimps and teleosts. *S. ringens*, on the other hand, showed higher δ¹⁵N and δ¹³C values than the remaining species, indicating the assimilation of ¹³C- and ¹⁵N-enriched prey such as bathyal octopus and bathyal teleosts. The fact they rely on different groups of prey suggests some degree of resource partitioning between *S. ringens* and the other

TABLE 3 Mean (\pm S.D.) $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values (‰) of each species collected in February 2018 off the south-west coast of Portugal, grouped according to their taxonomic group, stable isotope values and/or habitat (group codes)

Group	Species	n	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	Diet	Habitat
Tel1	<i>Gadomus</i> sp.	4	12.6 \pm 1.4	-18 \pm 0.4 ^a	Copepods, amphipods	Bathyal
	<i>Aldrovandia phalacra</i>	3	12.4 \pm 0.2	-18.1 \pm 0.2 ^a	Copepods, amphipods	Bathyal
	<i>Trachyrincus scabrus</i>	2	13.7 \pm 0.6	-17.5 \pm 0.7 ^a	Copepods, mysids, shrimps, cephalopods, fish, polychaets	Bathyal, nonmigratory
	<i>Nezumia sclerorhynchus</i>	4	14.0 \pm 0.2	-18.1 \pm 0.5 ^a	Copepods, amphipods, decapods, mysids, polychaets	Bathyal, nonmigratory
	<i>Cetonurus globiceps</i>	1	12.5	-18.9	Small fishes, planktonic crustaceans	Bathyal
	<i>Chaunax pictus</i>	1	12.7	-18.9 ^a	Shrimps, crabs	Bathyal
	<i>Bathypterois dubius</i>	3	12.8 \pm 0.1	-18.5 \pm 0.1 ^a	Mysids, benthopelagic copepods	Bathyal
	<i>Alepocephalus rostratus</i>	3	11.4 \pm 0.2	-19.2 \pm 0.1	Euphausiids, decapods, mysids	Bathyal
	<i>Anoplogaster cornuta</i>	2	11.6 \pm 1.1	-19.8 \pm 0.8	Crustaceans, shrimps, fishes, cephalopods	Bathyal
	<i>Serrivomer beanii</i>	1	9.3	-19.3	Euphausiids, decapods, mysids, cephalopods, fishes	Bathyal, DVM
	Total	24	12.5 \pm 1.2	-18.5 \pm 0.7		
Tel2	<i>Chauliodus sloanii</i>	1	10.5	-19.6	Mid-water crustaceans and fishes, mainly mictophids	Bathy-mesopelagic, DVM
	<i>Melanonus zugmayeri</i>	3	10.7 \pm 0.3	-18.6 \pm 0.2	n/a	Bathy-mesopelagic
	<i>Rouleina maderensis</i>	1	11.4	-18.2 ^a	n/a	Bathy-mesopelagic
	<i>Hoplostethus mediterraneus</i>	2	11.2 \pm 0.6	-18.6 \pm 0 ^a	n/a	Bathy-mesopelagic
	<i>Omosudis lowii</i>	1	10.4	-20	Cephalopods, fishes	Bathy-mesopelagic
	Myctophidae ^b	2	8.4 \pm 0.2	-20.6 \pm 0.8	Copepods, euphausiids	Mesopelagic, DVM
		Total	10	10.6 \pm 0.9	-19.1 \pm 0.8	
Lobster	<i>Nephropsis atlantica</i>	2	8.9 \pm 0.2	-18.5 \pm 0.3	n/a	Bathyal
Crab	<i>Geryon longipes</i>	3	11.5 \pm 0.3	-18.8 \pm 0.2	n/a	Bathyal
Shrimp	<i>Aristaeopsis edwardsiana</i>	1	10.9	-17.6	n/a	Bathy-mesopelagic
	<i>Aristaeomorpha foliacea</i>	3	10.8 \pm 1.3	-18.8 \pm 0.8	Crustaceans, fishes	Mesopelagic
	<i>Polycheles typhlops</i>	4	10.8 \pm 0.3	-18 \pm 0.4	n/a	Bathy-mesopelagic Bathyal
	<i>Dichelopandalus bonnieri</i>	1	11.3	-18.7	n/a	n/a
		Total	9	10.9 \pm 0.8	-18.3 \pm 0.8	
Octopus	Octopodidae	1	12.4	-18.3	n/a	Bathy-mesopelagic
	<i>Opisthoteuthis</i> sp.	2	11.9 \pm 0.2	-19.1 \pm 0.1	n/a	Bathyal
		Total	3	12.2 \pm 0.3	-18.7 \pm 0.6	
Squid	<i>Mastigoteuthis</i> sp.	2	11.5 \pm 0.2	-20.6 \pm 0.3	n/a	Bathyal
	<i>Histioteuthis</i> sp.	1	10.5	-20.1	n/a	Bathyal
		Total	3	11 \pm 0.7	-20.3 \pm 0.4	
COP	Copepods		4.7 \pm 0.3	-20.7 \pm 0.1	Phyto- zooplankton or organic matter	Epipelagic, mesopelagic

Note: Bold values represent the mean (\pm S.D.) of each prey group. Teleosts are divided into two major groups: Tel1 are bathyal and Tel2 are bathy-mesopelagic. COP are copepods. For some species, information about their habitats and diet is not available (n/a) and others perform diel vertical migratory movements (DVM).

^aSpecies with $\delta^{13}\text{C}$ (‰) values corrected for lipid content.

^bMean values extracted from Barría *et al.* (2015) based on two individuals from the Mediterranean.

species. The reasons for that are unclear because little is known about the biology and ecology of *S. ringens* (Finucci *et al.*, 2021).

Groups of commercially important shrimps contributed to the diet of all the sharks studied, although in general only with a small

contribution (median < 31%). Despite the small contributions, the consumption of commercially important shrimps might be among the reasons why those sharks are the most frequently caught in the studied area, as observed by an ongoing study (unpublished data).

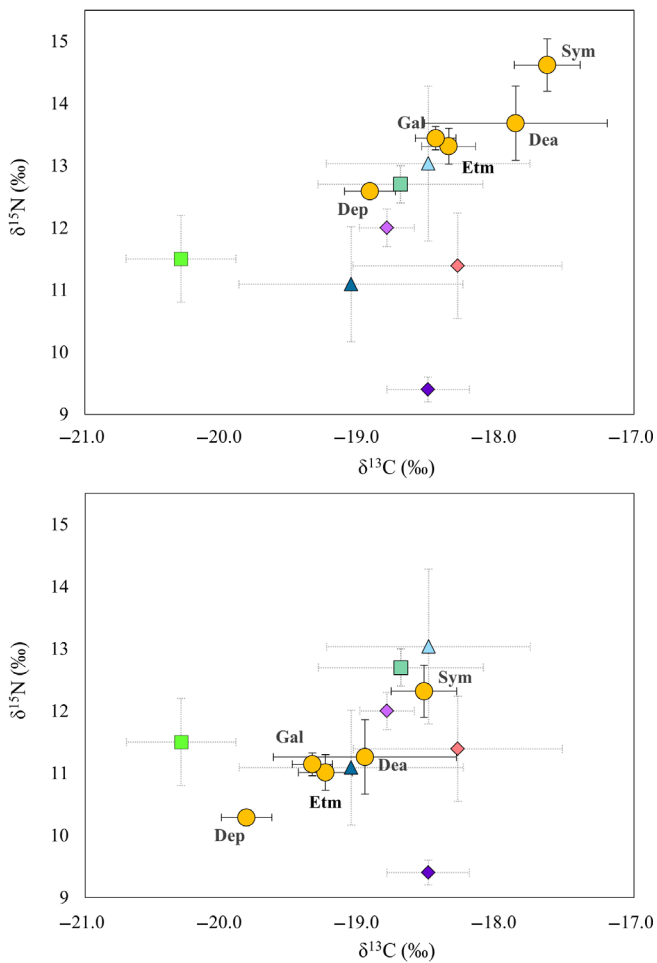


FIGURE 2 Mean (\pm s.d.) $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values (‰) of sharks collected off the south-west coast of Portugal (south-west Europe) not adjusted for trophic fractionation (upper) and adjusted for trophic fractionation (lower; $2.3 \pm 0.22\text{‰}$ $\delta^{15}\text{N}$, $0.9 \pm 0.33\text{‰}$ $\delta^{13}\text{C}$; Hussey *et al.*, 2010). Teleosts were grouped into bathyal (Tel1) and bathy-mesopelagic (Tel2) represented by the triangles, Crustaceans (lobster, crab and shrimp) represented by the diamonds and Cephalopods (octopus and squid) represented by squares. Sharks are represented by yellow circles: *Deania calcea* (Dea), *Deania profundorum* (Dep), *Etmopterus pusillus* (Etm), *Galeus melastomus* (Gal) and *Scymnodon ringens* (Sym). \blacktriangle , Tel1; \blacktriangle , Tel2; \blacklozenge , shrimp; \blacklozenge , crab; \blacklozenge , lobster; \blacksquare , octopus; \blacksquare , squid.

It is possible that demersal predators, such as deep-water sharks, exploit different depths gradients to help increase net energy gain, similarly to pelagic species (Schabetsberger *et al.*, 2000; Watanabe *et al.*, 1999). This was not seen in our study since we did not sample prey from habitats other than the bathyal, although some of the sampled prey are also from the mesopelagic habitat. Furthermore, the group Tel2, which also contains teleosts that perform diel vertical migrations, was not included as potential prey of the studied sharks (explanation below), although some authors have suggested that *E. pusillus* (Coelho & Erzini, 2007; Xavier *et al.*, 2012) and *D. calcea* (Clark & King, 1989) might perform diel vertical migrations to follow their prey.

The lack of a clear preference for any prey group (median contributions lower than 50%) suggests that these species may be generalists' predators. Although this study does not allow a conclusion to be drawn about their feeding behaviour, a generalist/opportunistic behaviour was previously reported for *G. melastomus* in the Cantabrian and Ionian Seas (Anastasopoulou *et al.*, 2013; Olaso *et al.*, 2005). Nonetheless, the narrow range in prey $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, which probably reflects the low number of available sources of productivity in the deep-sea environment, introduced some challenges to the quantitative analysis. The ranges of the stable isotopes of Tel2 overlapped with those from crabs and shrimps, which poses a limitation to the interpretation of mixing models (Phillips *et al.*, 2014). Although the models obtained in this study were robust (low errors and median values close to mean values), we cannot reject the possibility that they could also be assimilating fish from the group Tel2. In fact, previous stomach content studies reported the consumption of mesopelagic fish such as Myctophidae by the species *D. calcea*, *D. profundorum*, *E. pusillus* and *G. melastomus*. Thus, it is possible they also assimilate mesopelagic fish along with bathyal and bathy-mesopelagic crustaceans, as previously reported on stomach content studies (Supporting Information Table S1).

The TP estimates obtained during this study position the deep-water shark species analysed as tertiary consumers, with TP varying between 3.8 in *D. profundorum* and 4.7 in *S. ringens*. These estimated values agree with other studies that used stable isotopes (Chouvelon *et al.*, 2012; Colaço *et al.*, 2013) or stomach content analysis (Cortés, 1999). Furthermore, these TP values are close to the 4.5 obtained for deep-water top predators such as the *Hexanchus griseus* (Froese & Pauly, 2022) which also inhabits this same area and depths. This might indicate that these sharks are also top predators in this food web. The high trophic levels suggest that they might not sustain direct or indirect exploitation (Pauly *et al.*, 1998). While most deep-water sharks are protected in European waters (EC council regulation 2021/91), with zero total allowable catch, they are still frequently caught as bycatch in bottom-trawlers and longliners and most often discarded dead or in a poor condition (Rodríguez-Cabello & Sánchez, 2017) which calls for better fisheries management to avoid their catch in the first place.

However, caution is necessary when comparing TP between studies, since these estimates can vary with the input value for values used for trophic fractionation and also with the method applied to generate the TP estimates. The most used fractionation value is 3.4‰ (Post, 2002), which is usually assumed to be constant across trophic levels (e.g., Colaço *et al.*, 2013; Iitembu & Richoux, 2015; Pethybridge *et al.*, 2012). Nevertheless, experimental studies conducted under controlled situations proved that there is a wide variation in $\Delta^{15}\text{N}$ ($\Delta^{15}\text{N} = \delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{prey}}$) values among species and taxa (Caut *et al.*, 2009). Thus, we used the scaled $\delta^{15}\text{N}$ framework approach proposed by Hussey *et al.* (2014a) since it improves the ability to accurately measure absolute TP variation, extending the length of the food web in comparison to conventional constant fractionation frameworks.

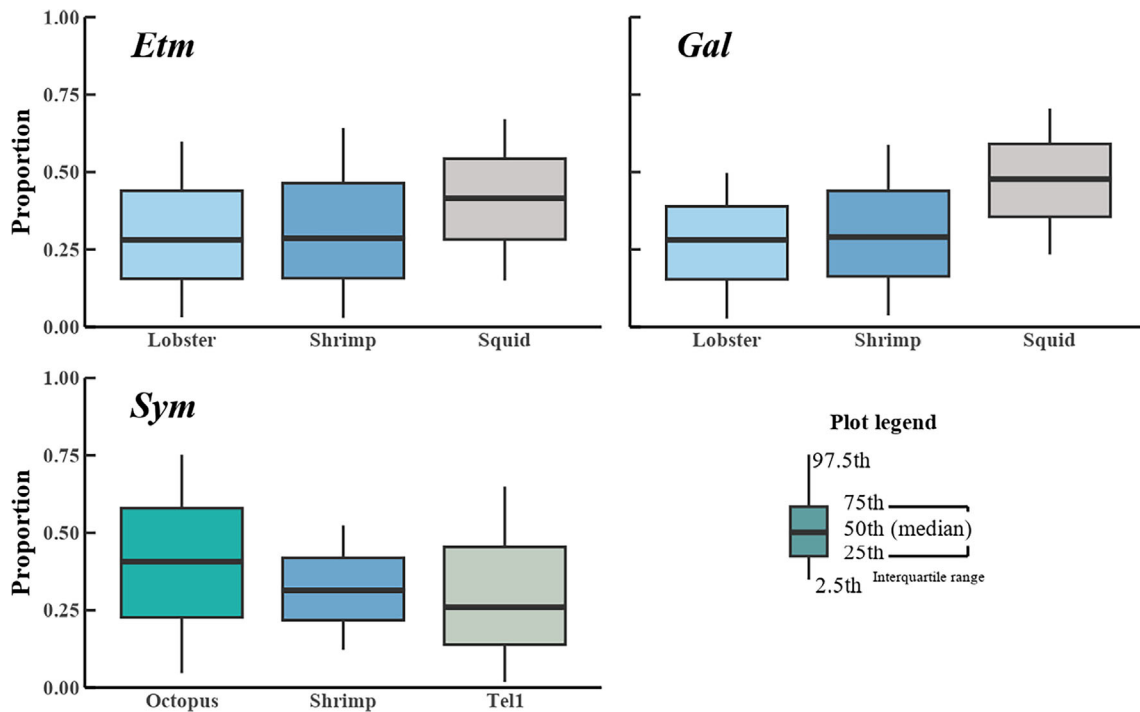
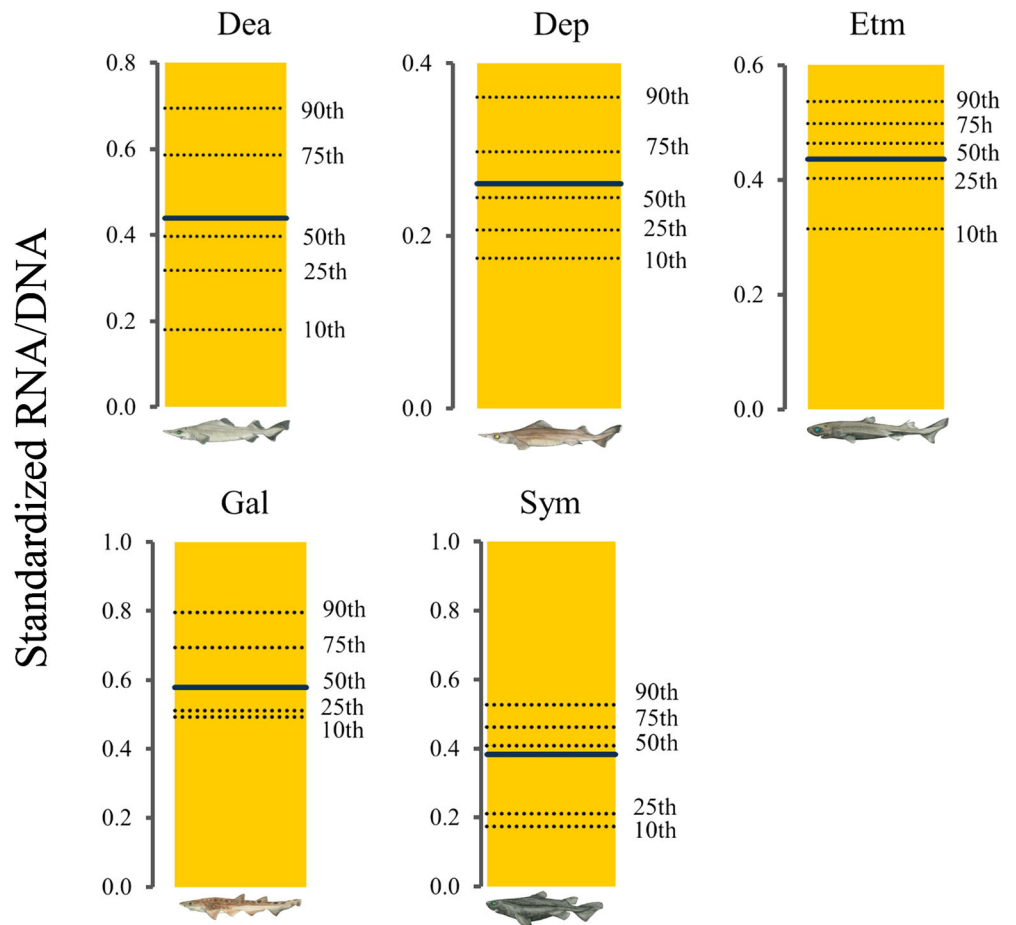


FIGURE 3 Relative contribution of each prey group based on the stable isotope mixing models of the sharks *Etmopterus pusillus* (Etm), *Galeus melastomus* (Gal) and *Scymnodon ringens* (Sym). The prey groups include Tel1 (bathyal teleosts), squid, octopus, shrimp and lobster. The boxplots show the median (horizontal lines) with 50% (boxes) and 95% credible intervals (vertical lines)

FIGURE 4 Percentile approach of the standardized RNA/DNA of the shark species with $n > 3$, collected off the south-western coast of Portugal. The dotted lines are the 10th, 25th, 50th, 75th and 90th percentiles and the dark blue lines are the RD mean values of the species *Deania calcea* (Dea), *Deania profundorum* (Dep), *Etmopterus pusillus* (Etm), *Galeus melastomus* (Gal) and *Scymnodon ringens* (Sym). This approach shows that when the mean is closer to the 75th and far from the 10th percentile, the species has a high number of individuals with an adequate nutritional condition



Another critical aspect to consider when conducting diet reconstruction and estimation of TP through stable isotopes analysis is the use of proper trophic fractionation values. The trophic fractionation values are usually estimated by conducting controlled feeding experiments, where animals are fed a variety of prey types for an extended period until isotope values reach a plateau (Hussey *et al.*, 2010). However, there are a limited number of trophic fractionations estimates for this group of species because of the difficulties of keeping chondrichthyans in captivity for long periods (Shiple *et al.*, 2017). Moreover, the existing estimates, based on controlled experiments, which do not include deep-water species, are highly variable ($\Delta^{15}\text{N}$ 2.3–5.5‰, $\Delta^{13}\text{C}$ 0.9–3.5‰), varying according to the consumer species, prey species, prey tissue-type, consumer tissue-type, natural conspecific variation and species-specific metabolic rates (e.g., Hussey *et al.*, 2012; Kim & Koch, 2012; Malpica-Cruz *et al.*, 2012; McClain *et al.*, 2012). Because there are no trophic fractionation estimates derived from captivity studies with deep-water elasmobranchs, we used those obtained from muscle tissues of two reef-associated shark species (*Negaprion brevirostris* and *Carcharias taurus*) fed with a fish diet for over 2 years (Hussey *et al.*, 2010). In many fish species, there is a positive relationship between body size, trophic position (Romanuk *et al.*, 2011) and $\delta^{15}\text{N}$ values, where consumers feeding on prey at higher levels of the food web would be large animals (Hussey *et al.*, 2012), thus likely resulting in lower trophic fractionation values with increasing trophic position and body size (Hussey *et al.*, 2014a). However, Churchill *et al.* (2015) found no relationship between average body size and mean $\delta^{15}\text{N}$ values in deep-water sharks from the Gulf of Mexico, suggesting this might be due to reduced resource pathways in the deep-sea habitat along with high levels of scavenging contributing to compressed food webs.

4.2 | Nutritional condition

Based on the percentile RD analysis, we were able to conclude that the sharks analysed during this study were in an overall adequate nutritional condition (Alves *et al.*, 2020; Meyer *et al.*, 2012) and that their food likely came from the study area, or nearby areas, due to the short window of time provided by the RD analysis (Buckley *et al.*, 1999). The species that presented the highest values of RD was *G. melastomus*, but it was also the only species with individuals in a poor nutritional condition. If *G. melastomus* was a selective feeder, the poor nutritional condition could mean that their preferred prey was absent from the study area. Since they are considered generalist feeders, other reasons could have been the motive for their poor nutritional condition. Because RD values decrease with death and since the individuals collected were either in poor condition or dead, another possible explanation could be related to their condition upon arrival at the boat. However, if this was true, the same should have been observed in other specimens arriving dead or in poor condition, which was not the case. Thus, we consider that the most likely explanation is related to the fact that the individuals collected from *G. melastomus* were all adult females that may have recently given

birth, since these conditions are usually associated with low RD values because the energetic reserves of females are transmitted to the offspring (e.g., Chicharo *et al.*, 2003; Garrido *et al.*, 2007; Pérez-Camacho *et al.*, 2003).

Even though RD values seemed to increase with shark size for the species *D. profundorum*, *E. pusillus* and *S. ringens* and decrease for the species *D. calcea* and *G. melastomus*, none of those relationships were significant (Supporting Information Figure S2). Previous studies attempted to apply nucleic acid-derived indices to evaluate the nutritional condition of other elasmobranchs where an inverse relationship was found between RD values and size (Tavares *et al.*, 2006) and age (Cruz-Ramírez *et al.*, 2017). However, we cannot establish direct comparisons with the above studies because their RD values were not standardized as in this study. Another reason is that RD is an index of cellular protein synthesis capacity and might take days to weeks to change (Chicharo & Chicharo, 2008), thus it cannot be used as an instantaneous growth index, as done by Tavares *et al.* (2006), and cannot be biased by stress related to capture events, as stated by Cruz-Ramírez *et al.* (2017).

To the best of our knowledge this is the first attempt to evaluate the nutritional condition of deep-water sharks using this approach. Nevertheless, because there are no estimates for the critical RD values for the studied species and due to the small number of individuals analysed, these conclusions should be interpreted cautiously. Further studies are necessary to confirm the usefulness of RD values as indicators of the nutritional condition in deep-water sharks, which should include a higher number of individuals of different sex and maturation stages when compared to those used in this study.

5 | CONCLUSION

This study was the first to combine the dietary information and nutritional condition of deep-water shark species in an important Portuguese deep-water crustacean fishing ground. Despite the small sample size of these especially inaccessible organisms to trophic studies, we were able to show that the sharks studied here are tertiary consumers, assimilating cephalopods, crustaceans and teleosts with bathyal and mesopelagic origins from the local food webs or from nearby areas. The fact that they assimilated different groups of prey but showed no high relative contribution of any group to their tissues, suggests they could be generalist predators. The RD percentile approach indicated that most of the species were in an adequate nutritional condition and had recently eaten in the days before sampling. Thus, the fact they had recently eaten prey from the local or nearby food webs, which included groups of prey targeted by the crustacean bottom-trawl fisheries, suggests there is some potential for overlap between their foraging grounds and the most important fishing areas for deep-water crustaceans in this country. Further studies are necessary to determine the complete prey array and their feeding behaviour and estimate the actual overlap between sharks' foraging grounds and fishing areas, including a more exhaustive monitoring programme covering a greater number of species and using

complementary approaches that combine predator–prey relationships with shark habitat use.

AUTHOR CONTRIBUTIONS

S.G.A., K.E., A.T. and E.D. conceived and designed the study. S.G.A. collected the raw data. S.G.A. and E.D. performed the stable isotopes analyses and wrote the manuscript. S.G.A., V.B. and A.T. performed the nucleic acids analysis. K.E. provided funding. All authors gave final approval for publication.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All the study was conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of animals and enforced by CCMAR. CCMAR staff are certified to house and conduct experiments with live animals, and their facilities are also certified in accordance with the three “R” policy, national and European legislation, and with guidelines defined by the ethical committee ORBEA CCMAR-CBMR.

ORCID

Sofia Graça Aranha  <https://orcid.org/0000-0002-3664-6896>

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SUPPORTING INFORMATION

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