Feeding habits of three Batoids in the Levantine Sea (north-eastern Mediterranean Sea) based on stomach content and isotopic data

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Understanding the diet of marine predators is essential to defining their trophic role in an ecosystem. Elasmobranchs (sharks and batoids) are considered pivotal components of marine food webs, and are often included in the top predator or mesopredator groups. However, in comparison with other Mediterranean areas, research focusing on marine predators inhabiting the Levantine Sea (eastern Mediterranean Sea) is very limited. Here, we examined the feeding habits (diet, trophic width and trophic position) of three endangered batoids (Gymnura altavela (Linnaeus, 1758), Raja asterias Delaroche, 1809 and Raja clavata, Linnaeus, 1758) coexisting in Iskenderun Bay (north-eastern Levantine Sea, Mediterranean Basin) by combining stomach content and stable isotope analyses. The results revealed clear differences in the trophic habits between them. Stomach contents showed differences in the diet between species, showing a clear feeding preference for teleosts in the case of G. altavela and a diet composed of fish and crustaceans in the case of R. asterias and R. clavata. In line with stomach content results, interspecific differences in the isotopic values and trophic levels were found. In particular, G. altavela was isotopically segregated from R. asterias and R. clavata, showing lower isotopic trophic width and higher trophic level. The results of this study provide new insights into the ecological role of these three endangered batoid species in the Levantine Sea and are of crucial importance for management and conservation of these species.

Keywords: Levantine Sea, stable isotopes, trophic ecology, elasmobranchs

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INTRODUCTION

Understanding the trophic ecology of a particular species is essential to determining its ecological role in marine ecosystems (Coll et al., 2013; Ferretti et al., 2013). Sharks and batoids are considered important components of marine food webs, often included in the top predator or mesopredator groups, although there are important differences in the diets among species (Cortés, 1999; Young et al., 2015). In fact, the high diversity of feeding strategies makes the ecology of this entire marine group particularly complex to understand (Cortés, 1999). For this reason, the trophic role of these species is often unclear. To unravel this limitation, more trophic studies are essential, as they can help inform conservation strategies for threatened species (Ferretti et al., 2013).

The Mediterranean Sea is considered a global hotspot of elasmobranch diversity, hosting ~7% of all elasmobranchs worldwide (Cavanagh et al., 2007; Dulvy et al., 2014). However, most of the batoids and shark species in the Mediterranean Sea have declined in abundance and distribution, mainly due to human impacts (Ferretti et al., 2010; Coll et al., 2013). In fact, around 40% of the elasmobranchs are considered threatened in the Mediterranean Sea by regional assessments of the International Union for Conservation of Nature (IUCN) (Abdul Malak et al., 2011; Bradai et al., 2012).

In comparison with other Mediterranean areas, research focusing on elasmobranchs inhabiting the Levantine Sea (eastern Mediterranean Sea) is very limited (Cavanagh et al., 2007), even though these waters host endemic, threatened and rare elasmobranchs. This is the case of the threatened batoids Gymnura altavela (spiny butterfly ray), with a vulnerable status, and the Mediterranean endemic Raja asterias (Mediterranean starry ray) and Raja clavata (thornback ray), both with a near threatened status based on the IUCN Red List (Vooren et al., 2007; Serena et al., 2015; Ellis et al., 2016). In the Levantine Sea, these three batoid fishes are highly impacted by the demersal fisheries operating in coastal and deep-sea waters (Dalyan, 2012; Yeldan et al., 2013; Yemisken et al., 2014).

Regarding their trophic habits, previous studies based on stomach contents conducted in the western and central Mediterranean Sea indicated that these three batoids act as mesopredators in the ecosystem, exploiting a wide variety of...
resources including crustaceans, demersal fish and cephalopods (Cuoco et al., 2005; Romanelli et al., 2007; Valls et al., 2011; Santic et al., 2012; Navarro et al., 2013). Although stomach content methodology permits a high level of taxonomic resolution in the identification of prey, batooids and sharks show a high frequency of empty stomachs. Moreover, the prey species that are often found in the stomachs are those of slower digestion rates, which could cause biases in the diet estimation (Hyslop, 1980). In addition, this conventional method requires a large number of stomachs to quantify diet. This can be difficult to obtain, especially for endangered species. The use of stable isotopes of nitrogen (δ¹⁵N) and carbon (δ¹³C) has been used as a complement to stomach content analysis to study the trophic ecology of elasmobranch species (e.g. Shiffman et al., 2012). This approach is based on the fact that δ¹³C and δ¹⁵N values are transformed from dietary sources to consumers in a predictable manner (Shiffman et al., 2012). δ¹⁵N values show a predictable increase from one trophic level to the next (Jennings et al., 1997; Layman et al., 2012). δ¹³C values show little change due to trophic transfer, but are useful indicators of dietary sources of carbon (Layman et al., 2012).

In this study, we investigated the feeding ecology (diet, trophic width and trophic position) of three batooids (G. altavela, R. asterias and R. clavata) coexisting in Iskenderun Bay (north-eastern Levantine Sea, Mediterranean Basin) by combining stomach contents and stable isotope analyses. Our study provides new insights into the ecological role of these three endangered batoid species in the Levantine Sea.

MATERIALS AND METHODS

Study area and sampling procedure

The north-east Levantine Sea (Figure 1) has a wide continental shelf and shows high marine productivity influenced by local wind effects, upwelling movements and rich terrestrial nutrient inputs from the Ası River (Polat & Piner, 2002). The area includes a high richness of marine species within the eastern Mediterranean Sea (Bilecinoğlu, 2016). The oceanographic conditions, such as the environmental conditions similar to tropical and sub-tropical regions, has promoted the colonization by invasive species from the Red Sea (Bilecinoğlu, 2016). A total of 13 G. altavela, 46 R. asterias and 26 R. clavata individuals were collected between September 2014 and April 2016 at a depth ranging from 33 to 450 m, by commercial trawl vessels in the Iskenderun Bay (Figure 1). All individuals were accidentally captured as by-catch of the fishing fleet. Individuals were taken to the lab in a freezer where body size (disc width length; DW, to the nearest mm) and weight (nearest g) were recorded.

Stomach content analysis

All prey items presented in the stomachs were identified at the lowest taxonomic levels (copepods, decapods, cephalopods and teleosts). Per cent of number (%N), weight (%W) and frequency of occurrence (%F) of prey items were calculated and these values were utilized for calculating the Index of Relative Importance (IRI) of each prey item (IRI = %F(%N + %W)). The IRI was standardized using the formula: %IRI = (IRI/ ΣIRI) x 100 (Cortés, 1997). The vacuity index (v; the percentage of empty stomachs) and the percentage of fullness of stomachs (Fullness %) were also calculated. Levin’s and Pianka’s measures were used to determine niche breadth (Bv) with the standardized niche breadth (Bsv), and niche overlap between the three batoiid fishes (Colwell & Futuyma, 1971). Prey-specific abundance was calculated according to the following: Pi = (ΣSi/ΣSi) x 100, where Pi is the prey-specific abundance of prey i, Si is the stomach contents (number) including prey i, and S is the total stomach contents among those individuals with prey in their stomach (Amundsen et al., 1996).

Stable isotope analysis

We obtained a muscle sample from the pectoral fin of seven spiny butterfly rays, seven Mediterranean stary rays and nine thornback skates. Before stable isotope analysis, we extracted lipid from muscle samples using a chloroform-methanol solution (Kim & Koch, 2011). Samples were subsequently freeze-dried and powdered and 0.28–0.4 mg of each sample was packed into tin capsules. Isotopic analyses were performed at the Stable Isotopes Laboratory at the Estación Biológica de Doñana CSIC (Seville, Spain). Samples were combusted at 1020°C using a continuous flow isotope ratio mass spectrometry system (Thermo Electron) by means of a Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer. Stable isotope ratios were expressed in the standard δ-notation (%) relative to Vienna Pee Dee Belemnite (δ¹³C) and atmospheric N₂ (δ¹⁵N). Based on laboratory standards, the measurement error was ±0.1 and ±0.3 for δ¹³C and δ¹⁵N, respectively (Cabana & Rasmussen, 1996).

As a measure of trophic width, for each species a Bayesian isotopic ellipse area (SEA) was calculated from the stable isotope values (Jackson et al., 2011). This metric represents a measure of the total amount of the isotopic niche exploited by a particular predator and is thus a proxy for the extent of trophic diversity (or trophic width) exploited by the species (high values of isotopic standard ellipse areas indicate high trophic width). This metric uses multivariate ellipse-based Bayesian metrics. Bayesian inference techniques allow for robust statistical comparisons between datasets with different sample sizes. Isotopic standard ellipse areas were calculated using the routine Stable Isotope Bayesian Ellipses incorporated in the SIAR library (SIBER, Jackson et al., 2011).

Trophic position

The trophic position (TP) of each species was estimated by using isotopic values (TPsia) and stomach content analysis (TPstomach). TPsia was performed according to Zanden & Rasmussen (2001):

$$TP_{consumer} = TP_{basal} + (\delta^{15}N_{consumer} - \delta^{15}N_{basal})/\Delta\delta^{15}N,$$

where δ¹⁵Nconsumer is the value for each batoiid species and δ¹⁵Nbasal is that of the crab, Monodactylus couchii (7.1‰) sampled from the north-eastern Levantine Sea. We used 1.95 for Δ¹⁵N values (Hussey et al., 2010), defined as the trophic enrichment factor between organism and diet.

$$TP_{stomach} = \frac{1 + \sum_i IRI_i \times TP_j}{\sum_i IRI_i},$$

where j is the predator of prey i, IRIi is the fraction of prey i in the diet of predator j, and...
TLᵢ is the trophic position of prey i (Cortés, 1999). Trophic positions of prey categories were based on Ebert & Bizzarro (2007).

Statistical analysis of stomach content data
Data analysis was performed with multivariate techniques (PERMANOVA). The diets of the three batoid species were analysed using the Bray–Curtis resemblance matrix of log(x + 1) transformed, with prey abundance data. The comparison similarities of prey groups among the three batoids were determined by SIMPER and appeared as vector overlay on the principal coordinates analysis (PCO) plot by PRIMER v6 (Clarke & Warwick, 2001). One-way analysis of covariance (ANCOVA) was used to reveal relationships between body size and the fullness index in stomach contents with SPSS 21 software.

RESULTS

Stomach content analysis
A total of 85 individual stomachs were analysed belonging to three batoids. We found that 71 of these individuals had food in their stomachs (coefficient of vacuity: 32% for R. asterias, 15% for R. clavata and 31% for G. altavela). We identified 15 different prey species belonging to three different taxonomic groups (Table 1). The minimum average of percentage of fullness was estimated at 14% for the spiny butterfly ray and 43% for the thornback skate and Mediterranean starry ray. There were no significant differences between the fullness index and body size in any of the three batoids (P > 0.05).

Although teleosts were the main prey group for all three batoids, we found significant differences in the diet composition (PERMANOVA tests, Pseudo-F₁₆₀ = 28.01; P < 0.001). In particular, pairwise tests indicated that stomach contents differed between G. altavela and both R. asterias and R. clavata. The PCO analysis showed that the horizontal axes explain separation with 57.7% total variation because of the contribution of cephalopods and decapods to the diet of the batoids. The vertical axes explained separation with 29.8% total variation in accordance with the contribution of the teleost species to the diets (Figure 2). While the teleost group was common in all batoids, the main differences among the batoid species were found in decapod and cephalopod groups.

Champsodon sp. was a common prey fish species in the stomach of G. altavela and R. clavata, whereas Equulites klunzingeri (IRI = 21.13%) was only found in the stomach of G. altavela (Table 2). In addition, Chlorophthalmus agassizi was found in the stomachs of both R. asterias and R. clavata. Argentina sphyraena (IRI = 0.2%), Bregmaceros atlaticus (IRI = 0.4%) and Trachurus sp. (IRI = 0.5%) were identified only in the stomach contents of R. asterias. Decapoda was the second main prey group for R. asterias and R. clavata (IRI = 18.68% and IRI = 44.40%, respectively). Copepods were only found in the stomach of R. asterias (IRI = 1.7%). Although it was somewhat common to find cephalopods in the batoid stomachs, they were not represented in a high percentage of the stomach contents (IRI % between 2.15 and 10.64).

Regarding the niche width, G. altavela showed lower values (Bᵥ: 1.1, Bᵥ: 0.1), followed by R. asterias (Bᵥ: 3.32, Bᵥ: 0.8) and R. clavata (Bᵥ: 3.25, Bᵥ: 0.8). Diet overlap was lowest between G. altavela and R. clavata (0.46), and highest between R. asterias and R. clavata (0.98). The relationship between prey specific abundance and prey occurrence confirms a specialist feeding strategy on teleosts for G. altavela (Figure 3). In contrast, R. asterias and R. clavata displayed generalist feeding strategies.

Fig. 1. Study area (North-eastern Levantine Sea, eastern Mediterranean Sea), indicating the sampling locations (black points).
Table 1. Diet composition of Gymnura altavela, Raja asterias and Raja clavata in the Iskenderun Bay (DW, disc width; TL, trophic level estimated from stomach contents; N, number of stomach; %FO, frequency of occurrence; %N, percentage in number; %W, percentage in mass; %IRI, index of relative importance of prey).

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>𝛿¹³C (%)</th>
<th>𝛿¹⁵N (%)</th>
<th>TL SIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnura altavela</td>
<td>7</td>
<td>-16.44 ± 0.50</td>
<td>11.21 ± 0.52</td>
<td>4.82 ± 0.26</td>
</tr>
<tr>
<td>Raja asterias</td>
<td>7</td>
<td>-17.27 ± 0.41</td>
<td>8.81 ± 0.42</td>
<td>2.93 ± 0.22</td>
</tr>
<tr>
<td>Raja clavata</td>
<td>9</td>
<td>-17.13 ± 0.42</td>
<td>8.54 ± 0.85</td>
<td>2.74 ± 0.46</td>
</tr>
</tbody>
</table>

Stable isotope results

Combined values of stable nitrogen and carbon differed among batoid species (δ¹⁵N, F₃,₉₈ = 73.22, P < 0.001; δ¹³C, F₃,₁₀₀ = 24.38, P < 0.001). Specifically, R. asterias and R. clavata did not differ in their stable nitrogen and carbon values (Tukey post hoc tests, all P > 0.05; Table 2, Figure 4) but showed lower values of stable isotopes than G. altavela (post hoc test, P < 0.05; Table 3, Figure 4). The isotopic niche width based on the Standard Ellipse Area (SEA) clearly differed between batoid species (Figure 4), with the highest values for the thornback skate (SEA = 0.91%), followed by G. altavela (SEA = 0.91%) and R. asterias (SEA = 0.41%) (Figure 4).

Trophic level

The trophic position estimated from stomach contents (TPstomach) varied between 3.88 and 4.24 among the three batoids, with G. altavela having a higher value than R. asterias and R. clavata, which occupied a very similar trophic position. When we estimated the trophic level from nitrogen isotope values, we found that absolute values differed from those estimated by stomach contents, but the relative position of the three studied species remained similar (Table 2).

DISCUSSION

In this study, the trophic ecology of three batoids (G. altavela, R. asterias and R. clavata) inhabiting the Levantine Sea (East Mediterranean Sea).
Mediterranean Sea) was studied by combining stomach contents and isotope analyses. Stomach content results provide a snapshot of the diet of each species, and isotopic values identify the trophic width and trophic level integrating a long-term view (Peterson & Fry, 1987; Kim & Koch, 2011; Navarro et al., 2014). Based on the results of both stomach contents and stable isotopes, we found clear differences in the trophic habits among these three demersal predators.

Table 3. Main prey groups in the diet of Gymnura altavela, Raja asterias and Raja clavata from the Mediterranean Sea. NW, north-western; SC, south-central; C, central; W, western; SE, south-east.

<table>
<thead>
<tr>
<th>Species</th>
<th>Region</th>
<th>Main prey group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. altavela</td>
<td>NE</td>
<td>Teleosts</td>
<td>Present study</td>
</tr>
<tr>
<td>G. altavela</td>
<td>NW</td>
<td>Teleosts, Crustaceans</td>
<td>Barria et al. (2015)</td>
</tr>
<tr>
<td>G. altavela</td>
<td>C</td>
<td>Teleosts</td>
<td>Psomadakis et al. (2008)</td>
</tr>
<tr>
<td>G. altavela</td>
<td>C</td>
<td>Teleosts</td>
<td>Neifar et al. (2002)</td>
</tr>
<tr>
<td>R. asterias</td>
<td>NE</td>
<td>Crustaceans, Teleosts</td>
<td>Present study</td>
</tr>
<tr>
<td>R. asterias</td>
<td>NW</td>
<td>Crustaceans, Teleosts</td>
<td>Navarro et al. (2013)</td>
</tr>
<tr>
<td>R. asterias</td>
<td>C</td>
<td>Crustaceans</td>
<td>Romanelli et al. (2007)</td>
</tr>
<tr>
<td>R. asterias</td>
<td>C</td>
<td>Crustaceans</td>
<td>Cuoco et al. (2005)</td>
</tr>
<tr>
<td>R. asterias</td>
<td>C</td>
<td>Crustaceans</td>
<td>Serena et al. (2005)</td>
</tr>
<tr>
<td>R. asterias</td>
<td>SC</td>
<td>Crustaceans, Teleosts</td>
<td>Capapé &amp; Quignard (1977)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>NE</td>
<td>Crustaceans, Teleosts</td>
<td>Present study</td>
</tr>
<tr>
<td>R. clavata</td>
<td>NW</td>
<td>Crustaceans, Teleosts</td>
<td>Barria et al. (2015)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>NW</td>
<td>Crustaceans, Teleosts</td>
<td>Valls et al. (2011)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>C</td>
<td>Crustaceans</td>
<td>Santic et al. (2012)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>E</td>
<td>Crustaceans, Teleosts</td>
<td>Yığın &amp; İsmen (2010)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>E</td>
<td>Crustaceans, Teleosts</td>
<td>Yeldan et al. (2005)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>E</td>
<td>Crustaceans, Teleosts</td>
<td>Kabasakal (2001)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>C</td>
<td>Crustaceans, Teleosts</td>
<td>Bello (1997)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>C</td>
<td>Crustaceans, Cephalopods, Teleosts</td>
<td>Azouz &amp; Capape (1971)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>C</td>
<td>Crustaceans, Teleosts</td>
<td>Capapé (1975)</td>
</tr>
<tr>
<td>R. clavata</td>
<td>C</td>
<td>Crustaceans, Teleosts</td>
<td>Jardas (1972)</td>
</tr>
</tbody>
</table>

Stomach contents revealed that the diet of G. altavela was mainly composed of fish prey, a result that agrees with the very few studies conducted previously in this species in Mediterranean waters (Table 3; Neifar et al., 2002; Psomadakis et al., 2008; Barria et al., 2015). This indicates that this species is a predator with clear preferences for fish. Although R. asterias and R. clavata also included fish in their diet, crustaceans were important prey as well for these species, contributing to the diet in the same proportion as fish. These results contrast with those from other locations in the Mediterranean, where the diet of these two rajid species were composed mainly by crustaceans (Kabasakal, 2001; Vannucci et al., 2006; Valls et al., 2011; Navarro et al., 2013; Eronat & Özaydın, 2015; Fatimeou & Younes, 2016). For example, Navarro et al. (2013) found that crabs were the dominant prey for R. asterias in the western Mediterranean Sea. In the Ligurian Sea and Tyrrhenian Sea, similar results were found with R. asterias. Goneplax rhomboides and Liocarcinus sp. were reported mostly in stomach content of R. asterias from shallow water (Cuoco et al., 2005; Romanelli et al., 2007). Yeldan (2005) showed that crustacean species were the main prey in the diet of R. asterias along the east coast of the Iskenderun Bay (North Levantine Sea). The current study differs from Yeldan (2005) in its sampling area. Yeldan (2005) sampled the individuals in coastal waters, where the availability of crustaceans is high. Our samples of Raja spp. were captured mostly from deeper waters. Discrepancies in the diet of R. clavata between our study and those carried out previously are probably due to geographic and depth differences reported for this batoid (Kabasakal, 2001; Vannucci, 2006; Valls et al., 2011; Eronat & Özaydın, 2015). For instance, Eronat & Özaydın (2015) indicated the dominant occurrence of crustaceans in the diet of R. clavata between 120 and 350 m in the Aegean Sea, while Valls et al. (2011) showed that the contribution of teleosts was much more relevant for this species in...
deeper waters. In our study, the relative contribution of crustaceans and teleosts was nearly the same.

The existence of interspecific differences in teeth morphology could explain differences in the diet (McEachran & Capapé, 1984; Jacobsen & Bennett, 2013). The presence of crushing teeth plates in the two *Raja* spp. probably confers a greater capacity to crush the carapace of crustaceans, whereas the cuspidate teeth of *G. altavela* facilitate the capture of fish (Vannucci et al., 2006; Motta & Huber, 2012; Ellis et al., 2016). Based on the principle of competitive exclusion, we expect that competing predators coexisting in the same waters segregate their exploitation of trophic resources (e.g. Papastamatiou et al., 2006; Follesa et al., 2010; Albo-Puigserver et al., 2015). For this reason, the three batoids partially segregate their main trophic resources as a mechanism that allows coexistence in the demersal habitat.

As expected from the stomach content results, interspecific differences in the isotopic values and trophic levels were found. In particular, *G. altavela* was isotopically segregated from *R. asterias* and *R. clavata*, showing a lower isotopic trophic width and higher trophic level. The trophic width estimated from SEAs was larger for *G. altavela* and *R. clavata* in comparison to *R. asterias*. Distribution of *R. clavata* shows variety from shallow to deep water in the area. This could be a result of the more generalized feeding strategy of *R. clavata*. On the other hand, previous studies on the feeding ecology of *R. asterias* show its specialized feeding strategy on crustacean species (Barria et al., 2015). The diversity richness of the coastal area in which *G. altavela* is mainly distributed (Emre Yemisken, unpublished data) probably explains the high trophic width of this species. Based on the trophic position of the species, both methodologies (stomach contents and isotopic values) revealed that *G. altavela* was at a higher position than the other two species. This pattern was previously found within demersal food webs in the western Mediterranean Sea where *G. altavela* shows a higher trophic position than coexisting batoids (Valls et al., 2011; Barria et al., 2015), probably related to its large body size.

Although we expected a similar estimation of trophic position using stable isotope analysis (SIA) and stomach contents, we found differences between the methods in both Rajidae species. The estimation of trophic level from stable isotopes was lower than from stomach contents. Differences between TP_{sia} and TP_{stomach} would be expected considering that the estimated trophic levels from isotopic data are vulnerable to the basic assumption of which basal sources are used (Olin et al., 2013). Discrepancies between the methodologies (TP_{sia} and TP_{stomach}) revealed the need for caution when values of trophic levels are compared (Albo-Puigserver et al., 2015). However, differences observed in the trophic position between the two methods in this study might be explained by long-term and short-term prey preference differences of Rajidae species in the region. When resources are restricted in the ecosystem, sometimes species may adapt and change their feeding behaviour after a while in the area. Although stomach content results have shown teleost and shrimp preferences in feeding behaviour, prey availability may not be sustainable on the same prey.

In conclusion, this study presents new information regarding the feeding ecology of three endangered batoids (*G. altavela*, *R. asterias* and *R. clavata*) in the Levantine Sea. The results indicate differences in the diet between species, showing a clear feeding preference for teleosts in the case of *G. altavela* and a diet composed of fish and crustaceans in the case of *R. asterias* and *R. clavata*. These results can be used by managers to conduct an appropriate assessment and inform conservation strategies for these species.

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