

# Trophic ecology of dusky grouper *Epinephelus marginatus* (Actinopterygii, Epinephelidae) in littoral and neritic habitats of southern Brazil as elucidated by stomach contents and stable isotope analyses

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**Abstract** Groupers are large-bodied fishes with broad global distribution and commercial and ecological importance. Many grouper species are endangered, but information on trophic ecology of groupers outside the Mediterranean Sea is limited. We integrated stomach contents analyses (SCA) and stable isotope analyses (SIA) to test the hypotheses that diets of dusky grouper *Epinephelus marginatus* differ between littoral and neritic zones in the southwestern Atlantic, but exhibit similar ontogenetic shifts in prey size and consumption of fishes. SCA were dominated by fishes, crabs, and shrimp and differed significantly between habitats, but prey size and consumption of fishes increased with grouper body size at both sites. Grouper stable isotope ratios were similar between sites and among size classes, but integrating SCA and

SIA distinguished ingested versus assimilated dietary components, improved dietary resolution, and provided a more comprehensive assessment of grouper in the food webs. Dusky grouper integrate benthic and pelagic trophic pathways, as well as estuarine and marine food webs via consumption of migratory prey. Our findings have applications to other threatened grouper populations, for example, in managing fragmented populations or for reserve designs considering inclusion of artificial substrates as grouper habitat, and more broadly for approaches examining trophic ecology of generalist predators.

**Keywords** Food web · IUCN red list · Man-made habitats · Migration · Ontogenetic shifts · Subsidies

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## Introduction

Groupers are large-bodied fishes of the families Serranidae and Epinephelidae with a broad global distribution and large commercial and ecological importance (Sadovy de Mitcheson et al., 2013). Groupers are targeted by artisanal, industrial, and sport fishing throughout their ranges (Heemstra & Randall, 1993; Craig et al., 2011) and often have strong influences on food webs and ecosystems through their roles as top predators (Heemstra & Randall, 1993; Sluka et al., 2001). Alterations to grouper populations can thus have significant commercial and ecological

consequences (Parrish, 1987; Brulé et al., 2005). Most grouper species exemplify a periodic life-history strategy (Winemiller, 2005) with slow growth rates, late maturation and a complex mode of seasonal reproduction including spawning aggregations and sexual reversal (Manooch & Mason, 1987), that combined with high fishing pressure leads to vulnerability and/or over-exploitation of many grouper populations (Huntsman et al., 1999; Coleman et al., 2000). One such example is the dusky grouper *Epinephelus marginatus* (Lowe, 1834), that like many other species of the family Epinephelidae, is included in the International Union for the Conservation of Nature (IUCN) red list as “endangered” (EN a2d), probably due to its complex life-history strategy and increasing fishing pressure (Cornish & Harmelin-Vivien, 2004).

The dusky grouper occurs along the southwestern Atlantic coastline with Argentina, Uruguay, and southeastern Brazil (Figueiredo & Menezes, 1980; Irigoyen et al., 2005), throughout the British Isles and the Mediterranean Sea (Heemstra & Randall, 1993) and along the African coast to Mozambique in the extreme southeastern Atlantic (Fennessy, 2006). Dusky grouper typically inhabit rocky substrates up to 250 m depth but with a preference for shallower waters up to 50 m depth (Bruslé, 1985; Heemstra & Randall, 1993; Harmelin & Harmelin-Vivien, 1999). Similar to other grouper species (Beckley, 2000), dusky grouper juveniles are commonly found in the coastal zone, including tidal pools (Azevedo et al., 1995) and small bays in estuarine regions (MVC unpublished data). Rocky substrates are utilized as cover by this ambush predator and individuals may use different habitat structures and body positioning depending on grouper body size and what prey item is being sought (e.g., fishes vs. octopii; Barreiros & Santos, 1998). The dusky grouper is a monandric protogynous hermaphroditic species (Marino et al., 2001) and sexual transition can occur in individuals between 52 and 77 cm TL (Reñones et al., 2010). In extreme southern Brazil, individuals larger than 75 cm TL have usually undergone sexual transition (Condini et al., 2013, 2014).

Studies on the trophic ecology of dusky grouper are relevant for basic understanding of the ecology of the species, as well as insights into the trophic organization of the ecosystem and mechanisms affecting interactions among species (Herrán, 1988). Furthermore, such information is relevant for applications such as fisheries management and selection and monitoring of marine

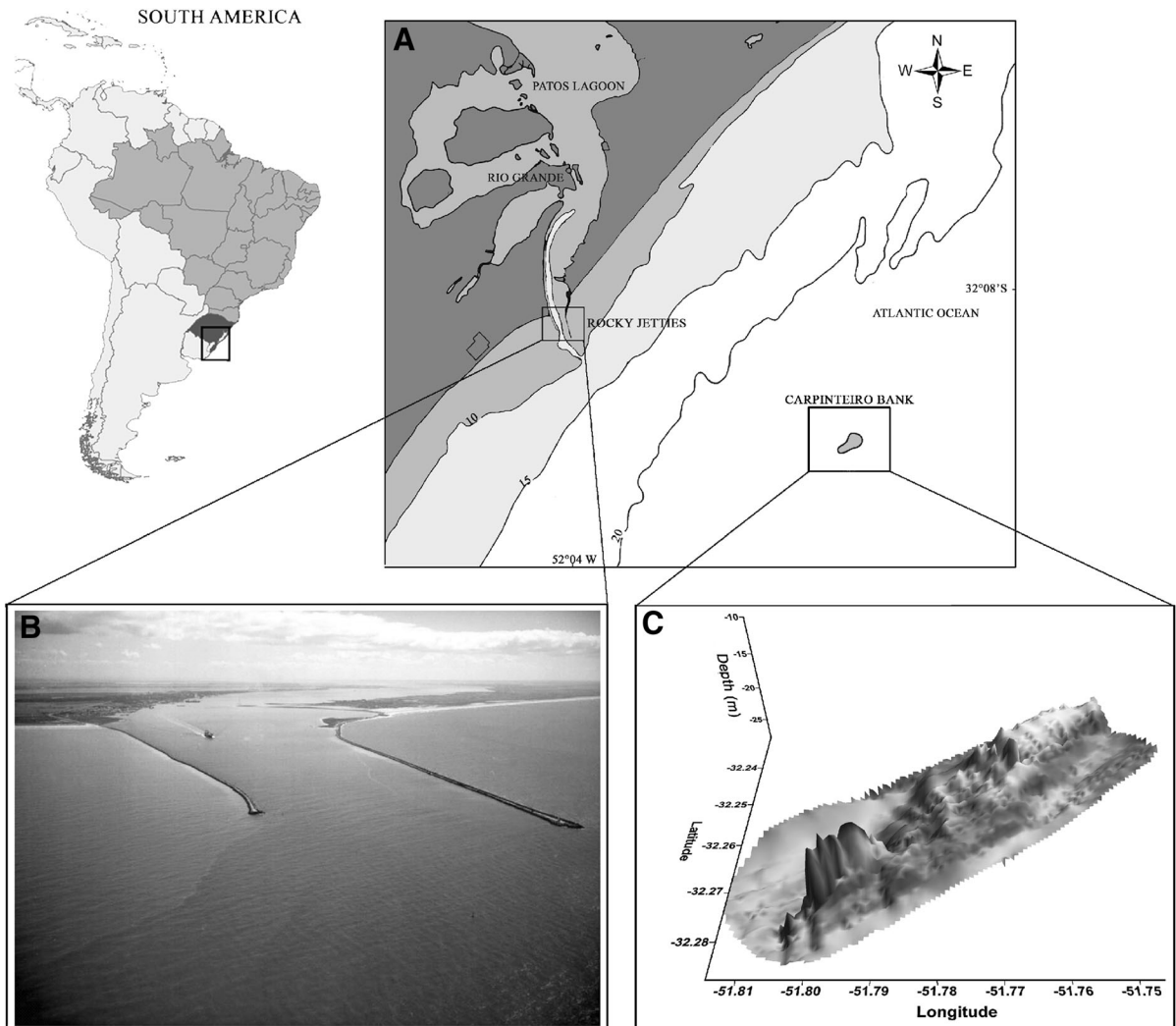
protected areas (e.g., Reñones et al., 2002). That being said, there is very limited information on the trophic ecology of dusky grouper outside of the Mediterranean Sea (e.g. Machado et al., 2008; Begossi & Silvano, 2008; Condini et al., 2011). This is especially true for the southern Atlantic coast of Brazil, even though the dusky grouper is an important high-value species harvested by small-scale fisheries (Condini et al., 2007) and is broadly recognized for its cultural and conservation importance (e.g., gracing the Brazilian R\$100 banknote; Fig. S1). For groupers in general, most trophic ecology studies are restricted to stomach contents analyses (Grover, 1993; Brulé et al., 2005; Condini et al., 2011) and few studies utilize stable isotope analyses (Reñones et al., 2002; Nelson et al., 2012).

In this study, we integrate stomach contents and stable isotope analyses to describe the trophic ecology of dusky grouper at two locations along the southern Brazilian coast. Our objectives were to identify key prey species, test for ontogenetic shifts in diet and between males and females, and finally to compare trophic ecology of dusky grouper populations between natural and man-made habitats. We expect grouper diets to differ between sites, specifically with greater importance of estuarine-dependent prey for littoral grouper and marine-dependent prey for neritic grouper, but exhibit similar ontogenetic diet shifts of increasing prey size and greater dependence on fishes with increasing grouper body size at both sites. Carbon and nitrogen stable isotope values should complement the stomach contents data, specifically, we expect carbon stable isotope values of grouper to differ between sites due to differences in carbon sources supporting littoral and neritic food webs and grouper nitrogen stable isotope values to increase with increasing body size as larger individuals feed higher in the food web (i.e., consume greater biomass of fishes). This study contributes to the limited knowledge currently available for this endangered species in the southern Atlantic and has direct application to management and conservation activities for this region and elsewhere.

## Materials and methods

### Study area and field collections

This research was conducted at two locations on the southern Brazilian coast. The first location is in the



**Fig. 1** Patos Lagoon (10,360 km<sup>2</sup>) and its estuarine zone in the state of Rio Grande do Sul, southern Brazil (A), showing the locations of the two sampling sites, the pair of rocky jetties at the

mouth of the lagoon (B), and Carpinheiro Bank in the adjacent marine area (C). The lines along the coast (A) denote 10, 15, and 20 m isobaths, respectively

littoral zone along a pair of rocky jetties (Molhes da Barra de Rio Grande) located at the mouth of Patos Lagoon Estuary (Fig. 1; 32°09'S, 52°05'W). The jetties were constructed at the beginning of the twentieth century to maintain the channel connecting Patos Lagoon with the southern Atlantic Ocean for navigation purposes. In addition to their logistical and strategic importance for the regional economy, the jetties constitute an important rocky habitat for a diverse benthic fauna (Capítoli, 1996), an area of reproduction for some crustacean species (Alaniz et al., 2011) and also serve as cover for some fish species (Condini et al., 2007). Due to its position at the

mouth of the estuary, this study location is characterized by varying hydrological conditions, primarily in regard to salinity (Möller et al., 2001). The second study location (Carpinteiro Bank) is a group of elevated submarine beach rocks in the neritic zone approximately 16 nautical miles from the coast and between 20 and 30 m depth (Fig. 1; 32°16'S, 51°47'W; Abreu & Calliari, 2005). The rocks are highly cemented by re-crystallized calcium carbonate and superficially colonized by bryozoans, sponges, crustaceans, and polychaetes (Buchmann et al., 2001). This study location experiences minor, if any, influence from the estuarine plume of Patos Lagoon and

average salinity is nearly constant year-round (Möller et al., unpublished dataset). Both locations represent rocky habitats within a coastal landscape that is otherwise dominated by homogeneous sandy substrates.

Field collections were conducted at both locations to obtain dusky grouper for stomach contents and stable isotope analyses, as well as potential diet items of dusky grouper and other components of the food web for stable isotope analyses. Dusky grouper were collected from the littoral zone between January 2008 and April 2009 in collaboration with regional artisanal fishermen using baited lines. Dusky grouper from the neritic zone were collected with the fishing fleet primarily between January 2010 and May 2011, with eight additional individuals collected in 2008 and 2009. An additional expedition to each study location was conducted in December 2010 to collect potential diet items of dusky grouper and other key components of the food web for stable isotope analyses. Stable carbon and nitrogen isotope values of the large-bodied consumers in this study have slow tissue turnover rates and thus reflect long-term feeding trends rather than episodic events, and as such we are confident in our use of samples from multiple expeditions. We also used conservative values for variability in our mixing models (see below) which should further reduce potential bias due to temporal variability in isotope values of diet items. In the littoral zone, fishes and crustaceans were collected using beach seines and minnow traps, mollusks and anemones were collected by hand, a beam trawl grab was used to collect polychaetes, and zooplankton were collected using a 30-cm-diameter plankton net with 500  $\mu\text{m}$  mesh. Fishes and mollusks were captured from the neritic zone using benthic trawls, crustaceans, and polychaetes were captured using a van Veen grab, and zooplankton were collected using a 50-cm plankton net with 500  $\mu\text{m}$  mesh. Samples from the littoral zone were transported on ice and frozen in the lab for subsequent processing, whereas samples from the neritic zone were frozen on the vessel during transport (Jardine et al., 2003; Garcia et al., 2007; Hoeninghaus et al., 2011).

#### Laboratory methodology and sample preparation

In the laboratory, each individual dusky grouper was measured for total length (TL, mm), weighed (g), and

eviscerated. The entire stomach was fixed in 10% formaldehyde for a period of 7 days then stored in 70% alcohol until analyzed. See Conдини et al. (2011) for detail on methodology employed for stomach contents analyses. Briefly, each stomach was weighed before and after removing the contents. Numerical abundance, weight (0.01 g) and total length (TL, mm) were recorded for all diet items recovered, with prey TL measured only for items that were whole and not overly digested. Items encountered in the stomach contents were identified to the lowest taxonomic level and assigned to one of the following five broad categories: fishes, shrimps, non-blue crabs (all of the anomurans and brachyurans, but excluding portunids), blue crabs (all of the portunids), and mollusks. The distinction between non-blue crabs and blue crabs from stomach contents is facilitated by significant differences in size and weight of individuals between the two groups.

All samples for stable isotope analyses were processed in the laboratory following standardized procedures (Garcia et al., 2007; Hoeninghaus & Davis III, 2007; Hoeninghaus et al., 2011). Samples were from individuals, though combined samples comprising multiple individuals were used for zooplankton (Garcia et al., 2007; Hoeninghaus et al., 2011). Because isotopic values of crustacean exoskeletons reflect assimilated calcium carbonate derived from the environment, only pure muscle tissue was used for stable isotope analyses. Using a sterile scalpel, muscle tissue was dissected from the anterior-dorsal region from fishes, the abdominal cavity of crustaceans, the adductor muscle for bivalves, and tentacles of cephalopods. Polychaetes were processed whole. Zooplankton individuals were separated into taxonomic groups and processed whole as combined samples for each group. All samples were inspected to remove any non-muscle material (e.g., bone or scales in fish tissue), rinsed with distilled water, and dried in sterile Petri dishes in an oven at 60°C to constant weight (minimum of 48 h). Dried samples were ground to a fine powder with a mortar and pestle and stored in sterile glass vials. Sub-samples were weighed to the nearest 0.01 mg using an analytical balance, pressed into Ultra-Pure tin capsules (Costech Analytical, Valencia, California), and sent to the Analytical Chemistry Laboratory of the Institute of Ecology, University of Georgia, for analysis of carbon and nitrogen isotope ratios. Results are expressed in delta

notation (parts per thousand deviation from a standard material):

$$\delta^{13}\text{C} \text{ or } \delta^{15}\text{N} = \left[ \left( R_{\text{sample}} / R_{\text{standard}} \right) - 1 \right] \times 1000,$$

where  $R = {}^{13}\text{C}/{}^{12}\text{C}$  or  ${}^{15}\text{N}/{}^{14}\text{N}$ . The standard material for carbon is Pee Dee Belemnite (PDB) limestone, and the nitrogen standard is atmospheric nitrogen. Standard deviations of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  replicate analyses of internal standards were 0.08 and 0.11‰, respectively.

### Data analyses

A total of 348 dusky grouper, 133 from the littoral zone and 215 from the neritic zone, were examined for stomach contents analyses. Of these, 78 littoral zone and 113 neritic zone individuals had non-empty stomachs and are included in data analyses. Diet items and categories were quantified using frequency of occurrence (%F; the percent of non-empty stomachs in which the diet item or category was encountered), relative abundance (%N; total number in percent of a diet item or category in relation to the total number of items or categories encountered in non-empty stomachs), and relative weight (%W; total weight in percent of a diet item or category in relation to the total weight of items or categories encountered in non-empty stomachs) following Hyslop (1980). The above parameters were subsequently used to calculate the index of relative importance (%IRI; Pinkas et al., 1971) as:  $\%IRI = \%F * [\%N + \%W]$ . To evaluate size-related effects on diet, dusky grouper were separated into four size classes (<351 mm TL, 351–500 mm TL, 501–650 mm TL, and >650 mm TL) and %IRI was calculated for all diet categories for each size class for both study locations. In addition, composition and relative biomass of diet items in individual grouper stomach contents were compared between sites and among size classes using non-metric multidimensional scaling (NMDS) calculated with the Bray–Curtis index. Permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in diet between sites and among size classes based on the Bray–Curtis similarity index. Factors included in the PERMANOVA model were location, size class and the location\*size interaction and statistics were based on 999 permutations using the ‘adonis’ function in the R package ‘vegan’ (Oksanen et al., 2013). Average prey size and biomass were compared between sites and

among size classes using ANOVA, with pairwise comparisons performed using Tukey’s post hoc test. Normality and homogeneity of variance were assessed using Kolmogorov–Smirnov and Cochran tests (Zar, 1994), respectively, and data were log-transformed prior to analyses to meet statistical assumptions. Diet data for the littoral zone were previously analyzed by Conдини et al. (2011), though not for the same size classes used in this comparison with the new neritic zone data.

Stable isotope analyses are based on carbon and nitrogen isotopic ratios of 262 dusky grouper (72 from the littoral zone and 190 from the neritic zone) and 157 samples of various consumer taxa that represent potential dusky grouper diet items and other components of the food web (58 from the littoral zone and 99 from the neritic zone). Bi-plots of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of dusky grouper and all other samples were used to visualize patterns of isotopic variation within and between study locations. The relative importance of various sources of organic carbon assimilated by consumers is indexed by relative positions of consumers on the  $\delta^{13}\text{C}$  axis, whereas trophic position is indicated by relative position on the  $\delta^{15}\text{N}$  axis (Peterson & Fry, 1987; Fry, 2006). Analysis of variance (ANOVA) was used to compare  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of dusky grouper between sites and among size classes. When a significant main effect was observed, pairwise comparisons were performed using Tukey’s post hoc test.

High lipid content can affect  $\delta^{13}\text{C}$  (i.e., yielding less enriched values) and in such cases, chemical lipid extraction or mathematical normalization can be used to control this effect (e.g., Jardine et al., 2003; Post et al., 2007). Samples with less than 5% lipid content, corresponding to C:N ratio less than 3.5, do not need to be corrected for lipids (Post et al., 2007). We did not extract lipids from our samples because chemical lipid extraction can have undesired effects on  $\delta^{15}\text{N}$  values (especially when lipid content is low; Ingram et al., 2007), and mathematical correction was deemed unnecessary because >70% of our consumers had C:N <3.5 (the majority of those with higher C:N were not direct diet items of grouper and not included in mixing models, see below).

To estimate relative contributions of primary diet items to dusky grouper growth, we used the Bayesian mixing model SIAR (version 3.0.1; Parnell et al., 2010). Importantly, SIAR incorporates uncertainties associated with variability in trophic enrichment, even

in indeterminate systems (i.e., when  $n_{\text{sources}} > n_{\text{isotopes}} + 1$ ), and both variability (i.e., standard deviation) and trophic enrichment factors are user defined. Considering the strong influence that these factors may have on model outcomes (e.g., Bond & Diamond, 2011), as well as known variability in enrichment among trophic groups, tissue types, and other factors (Caut et al., 2009; Wyatt et al., 2010), we opted for conservative estimates of trophic enrichment factors for both carbon and nitrogen (1.0 and 3.0‰, respectively) and as well for variability (standard deviation of 0.5) that includes the range of enrichment estimates and variability across a diverse taxa representing various trophic guilds (Post, 2002; Vanderklift & Ponsard, 2003). We evaluated the sensitivity of our model results to different enrichment factors and variability estimates and found that our results were robust to reasonable variation in these parameters. Conservative enrichment factors and variability estimates also allow for potential temporal variability in isotope values of consumers and prey among our sampling periods. Mixing models were calculated for the same grouper size classes (<351 mm TL, 351–500 mm TL, 501–650 mm TL, and >650 mm TL) at both sites as in the stomach contents analyses, and the pool of potential diet items was restricted to include only those species recovered from grouper stomachs or identified in previous studies (Reñones et al., 2002; Linde et al., 2004; López & Orvay, 2005; Machado et al., 2008) and grouped into the same classes as used with IRI analyses (i.e., non-blue crab, blue crab, shrimp, and fish) with the exception of mollusks which were excluded due to low sample size and limited importance in the diet. Our isotope data for shrimp from the littoral zone were complemented with five additional samples from the same study area (unpublished data provided by Renata Mont'Alverne), and non-blue crab isotope values for the littoral zone were conservatively estimated as the average values for invertebrates with similar trophic ecologies collected from the same site. Mixing model results are presented as the 95, 75, and 50% credibility intervals of diet categories for each grouper size class at both sites.

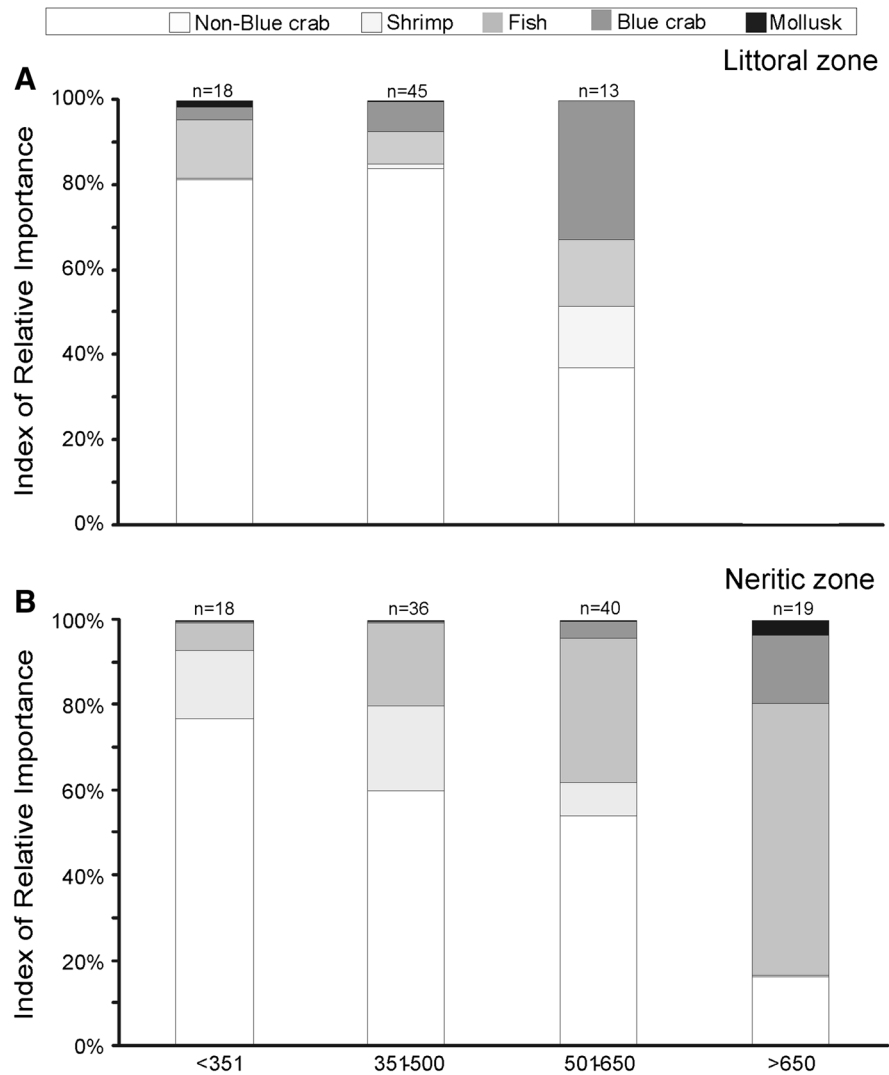
## Results

Fifty-eight prey types classified at various taxonomic levels were identified from the stomach contents of

dusky grouper (Table S1). Of those, 31 and 38 prey types were recorded from stomachs of littoral zone and neritic zone samples, respectively, with only 12 shared prey categories (Table S1). Large numbers of shells, vertebrae, and otoliths were encountered but not identifiable to lower taxonomic levels due to the high degree of digestion. Non-blue crabs and fishes were the most important prey items at both sites, with blue crabs and shrimp also frequently encountered (Table S1). Based on %IRI, dusky grouper from the littoral zone had diets dominated by non-blue crabs (%IRI = 75.29) and complemented by fishes and blue crabs (%IRI = 12.56 and 9.86, respectively). In contrast, diet of dusky grouper from the neritic zone had a lower relative importance of non-blue crabs (%IRI = 52.40), increased relative importance of fishes (%IRI = 32.32) and included shrimp rather than blue crabs as the third most important category (%IRI = 10.38). The Cuban stone crab *Menippe nodifrons* and unidentified Brachyura had the highest %IRI values for non-blue crabs in the littoral zone, whereas unidentified Brachyura, unidentified Caridae, and unidentified Xanthidae had the highest %IRI values for non-blue crabs in the neritic zone. Most of the fishes encountered in stomach contents were unidentified, but the demersal whitemouth croaker *Micropogonias furnieri* was an important prey item for dusky grouper in the neritic zone (%IRI = 4.60) that was not found in stomachs of dusky grouper from the littoral zone (Table S1). Cannibalism was also recorded, with a dusky grouper of 985 mm TL consuming an individual of approximately 250 mm TL.

For both locations, %IRI for the major diet categories changed with increasing body size, most notably a decrease in importance of non-blue crabs and increase in blue crabs for the littoral zone (Fig. 2A) and an increasing importance of fish in the diet of dusky grouper from the neritic zone (Fig. 2B). Prey size and biomass increased with increasing dusky grouper body size at both sites, but the rate of increase was higher for littoral dusky grouper (all ANOVAs  $P < 0.05$ ; Fig. 3). The large %N of shrimp in the diet of neritic dusky grouper contributed to a lower mean size and biomass of diet items for the 351–500 mm and 501–650 mm size classes compared with dusky grouper from those size classes in the littoral zone (Table S1; Fig. 2). NMDS of taxonomic composition and relative biomass of stomach contents distinguished dusky grouper primarily between sites (recognized as distinct groups along NMDS axis 1; Fig. 4)

**Fig. 2** Index of relative importance (% IRI) for each of the five major dietary categories found in the stomach contents of dusky grouper *Epinephelus marginatus* in the littoral zone (A) and neritic zone (B). Data are summarized for four size classes (<351 mm TL, 351–500 mm TL, 501–650 mm TL, and >650 mm TL), and the respective numbers of individuals analyzed in each size class are provided above the bars

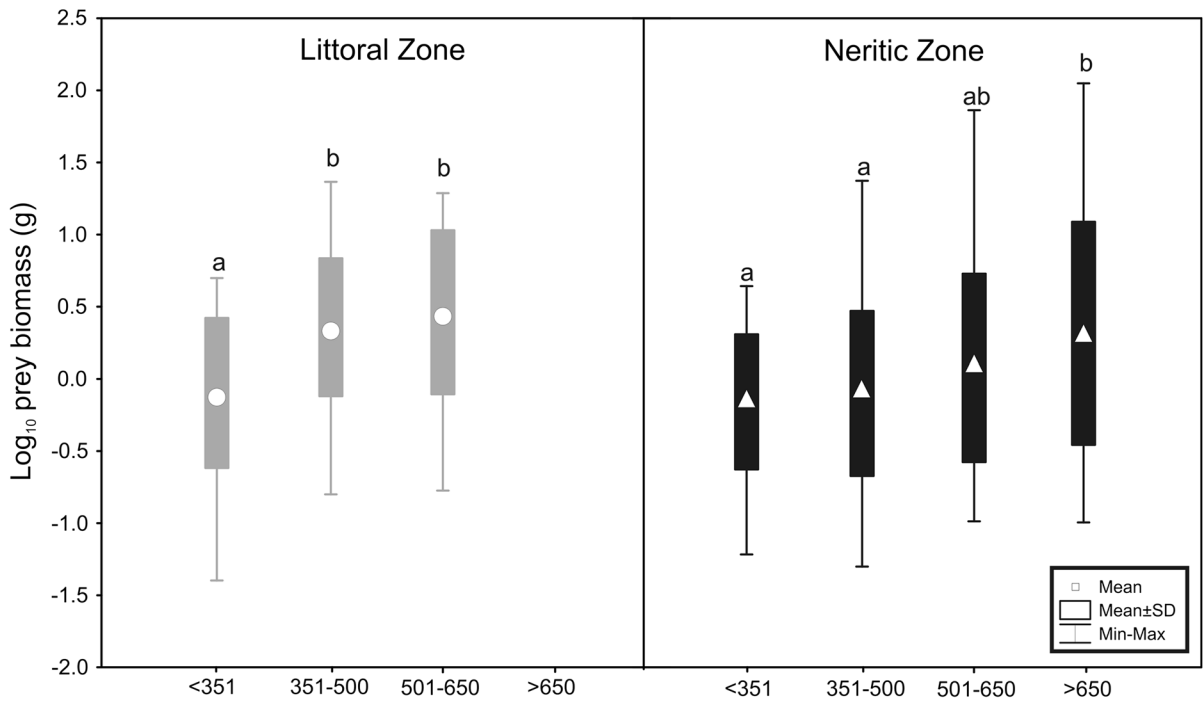


and subsequently among size classes (larger individuals from the neritic zone tend to have higher values on NMDS axis 2, whereas larger individuals from the littoral zone had higher values on axis 1, though there is much overlap of size classes for both locations; Fig. 4). PERMANOVA results were significant for both location ( $F_1 = 6.5173$ ,  $P = 0.001$ ) and size class ( $F_3 = 1.3091$ ,  $P = 0.030$ ), and marginally significant for the location by size interaction ( $F_3 = 1.1990$ ,  $P = 0.063$ ).

In contrast with the diet data, stable carbon and nitrogen isotope ratios of dusky grouper exhibited only minor differences between sites and among size classes (Figs. 5, 6). Mean  $\delta^{13}\text{C}$  differed between sites ( $P < 0.01$ ), though arguably not on an ecologically

relevant level (difference of approximately 0.2‰ between sites), and there was no difference between sites in  $\delta^{15}\text{N}$  ( $P > 0.90$ ). Weak trends were observed among size classes for dusky grouper at both sites, with higher variability in  $\delta^{13}\text{C}$  and slightly increased  $\delta^{15}\text{N}$  with increasing body size (Fig. 6). Isotopic values of large males from the neritic zone were similar to those of large females from the same region (Fig. 6).

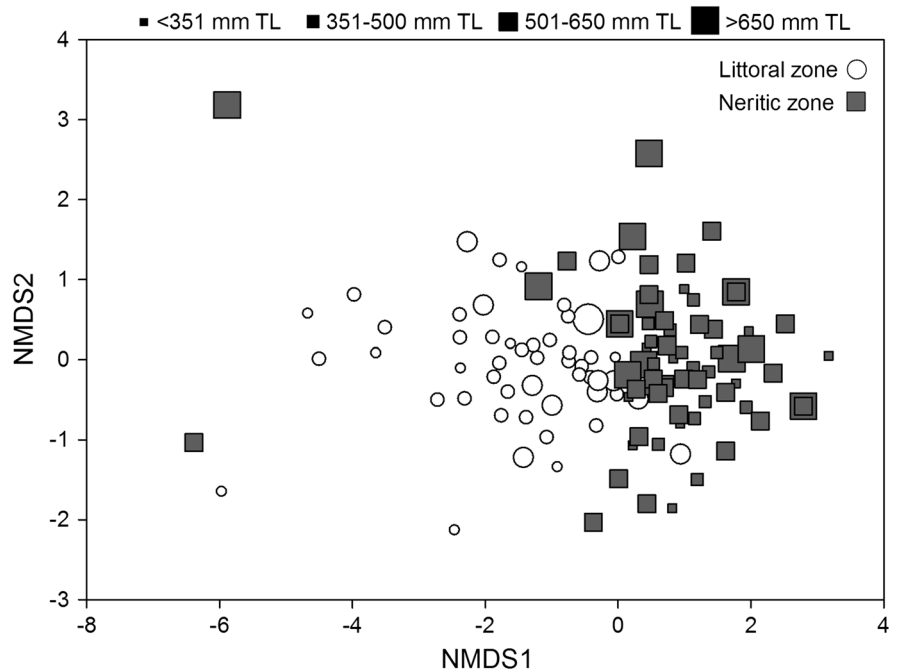
At both sites, dusky grouper had carbon isotope values intermediate compared with  $\delta^{13}\text{C}$  of other consumers in the food webs and nitrogen isotope values more enriched than all other sampled species, including other piscivorous fishes such as cutlassfish *Trichiurus lepturus* and bluefish *Pomatomus saltatrix*



**Fig. 3** Average values ( $\pm$ SD) of prey biomass (g) in the stomach contents of dusky grouper *Epinephelus marginatus* in the littoral zone (A) and neritic zone (B). Data are summarized

for four size classes (<351 mm TL, 351–500 mm TL, 501–650 mm TL, and >650 mm TL), and shared letters above each box indicate non-significant differences among size classes

**Fig. 4** Non-metric multidimensional scaling ordination (stress = 0.053) of dusky grouper diets based on prey relative biomass in littoral and neritic habitats along the southern Brazilian coast. Each point is an individual dusky grouper; symbol and shading denote location (=littoral zone; =neritic zone), and symbol size denotes size class (<351 mm TL, 351–500 mm TL, 501–650 mm TL and >650 mm TL)

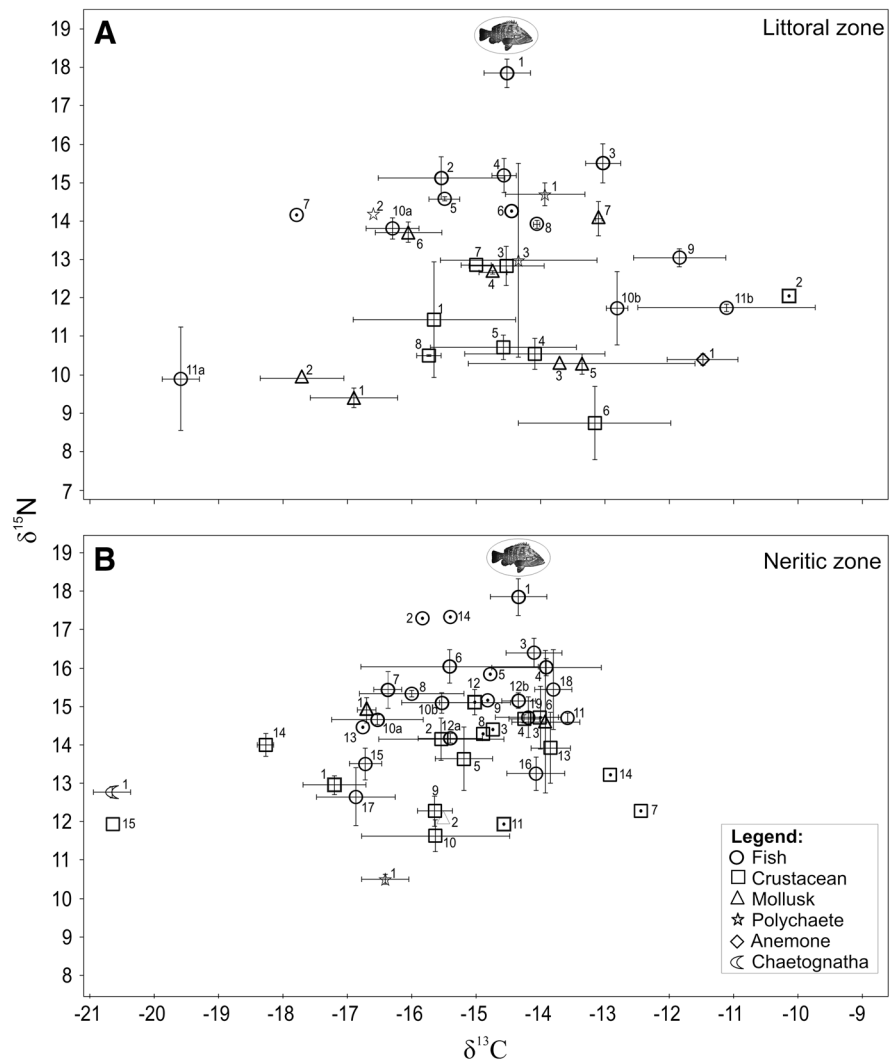


collected from the neritic site (Tables S2 and S3; Fig. 5). At both sites, sciaenids (e.g., whitemouth croaker *Micropogonias furnieri*, Argentine croaker

*Umbrina canosai*, southern kingcroaker *Menticirrhus americanus*, barbel drum *Ctenosciaena gracilicirrhus*, and stripped weakfish *Cynoscion guatucupa*) and



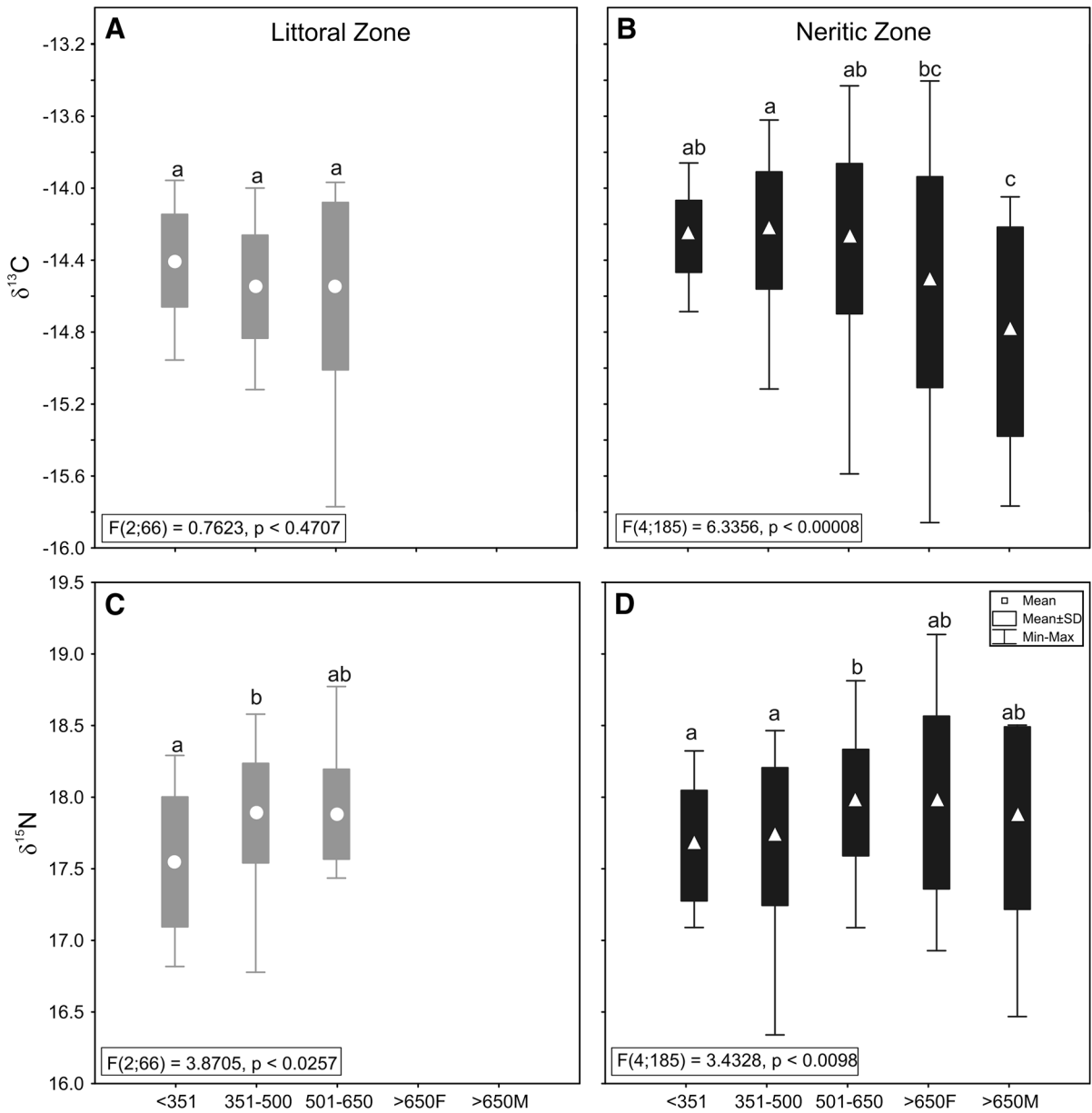
**Fig. 5** Carbon and nitrogen stable isotope bi-plots (mean  $\pm$  SD) of all individuals collected in the littoral (A) and neritic (B) zones. Symbols identify major taxonomic groups, as indicated in the inset legend in B. Numbers identify species listed in Table S2 (littoral) and Table S3 (neritic); dusky grouper are fish species 1 in both bi-plots



other benthic predators (e.g., gobies, skates, predatory polychaetes) had nitrogen isotope values approximately 2–2.5‰ depleted compared with dusky grouper, whereas nitrogen isotope values of crabs (both non-blue crabs and blue crabs) and shrimp were approximately 6‰ more depleted than grouper.

Stable isotope mixing models yielded slightly different patterns of relative importance of primary diet categories than observed in stomach contents. For grouper from the littoral zone, models identified fishes as the dominant prey category across all size classes, with 95% credibility intervals of 57–84, 77–88, and 74–90% contribution in size classes <351 mm, 351–500 mm, and 501–650 mm, respectively (Fig. 7A–C). Blue crabs and non-blue crabs, in that order, completed

most of the rest of the estimated percent contribution to littoral grouper diets, and 95% credibility intervals for both decreased with increasing grouper size, concomitant with increasing contribution of fishes. In contrast, shrimp and fishes were the dominant sources identified by mixing models for grouper from the neritic zone, with shrimp decreasing in importance with increasing grouper size (95% credibility intervals for grouper <351 mm = 30–58%, and 18–46% for grouper >650 mm) and fishes becoming the most important diet category in the largest grouper size class (95% credibility interval 0.42–0.68; Fig. 7E–H). When separating the ‘fishes’ diet category into benthic omnivores and piscivores, 95% credibility intervals for benthic omnivores were 36–48% compared with 12–22% for



**Fig. 6** Mean values ( $\pm$ SD) for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of the dusky grouper *Epinephelus marginatus* in the littoral zone (A and C) and neritic zone (B and D). Data are summarized for four size classes (<351 mm TL, 351–500 mm TL, 501–650 mm TL, and

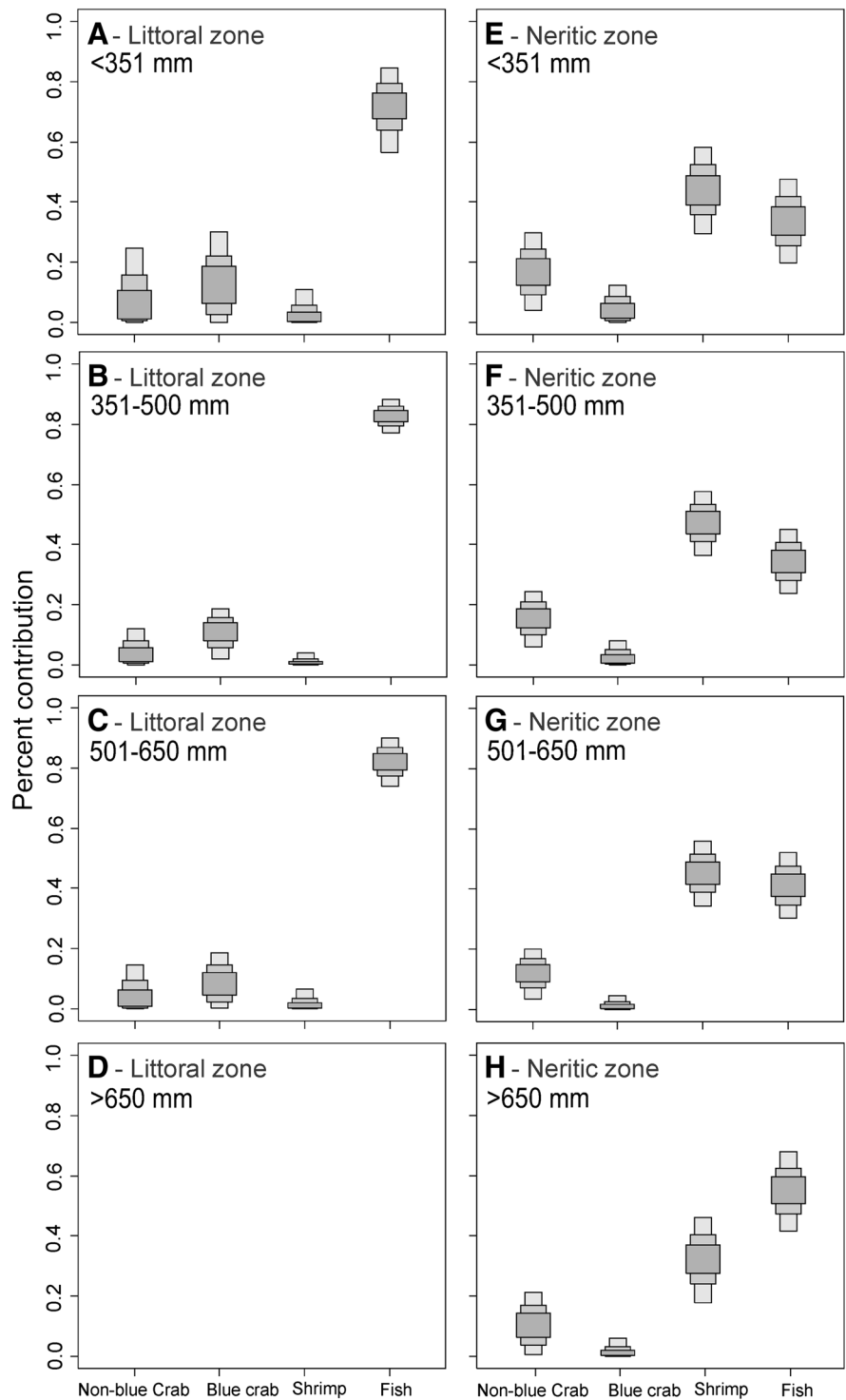
>650 mm TL), with males and females presented separately for the largest size class in the neritic zone. Shared letters above each box indicate non-significant differences among size classes

piscivores. Non-blue crabs contributed between 6 and 30% (lower and upper limits of the 95% credibility intervals across all grouper size classes), slightly decreasing in importance with increasing grouper size (Fig. 7E–H).

**Discussion**

Stomach contents analyses of dusky grouper from the littoral and neritic zones of southern Brazil indicated generalist predatory behavior with diets dominated by

**Fig. 7** Results of SIAR mixing models showing estimated percent contribution (with 95, 75, and 50% credibility intervals) of the sources to the dusky grouper collected in the littoral (A–D) and neritic (E–H) zones. Data are summarized for four size classes (<351 mm TL, 351–500 mm TL, 501–650 mm TL, and >650 mm TL)



fishes, crabs, and shrimp at both sites but with a large diversity of other prey types also consumed to a lesser degree (e.g., octopi, mussels, and snails). Ontogenetic

dietary shifts were observed for grouper at both sites, from diets dominated by comparatively small non-blue crabs to increased importance of larger prey such

as blue crabs and fishes for grouper from the littoral and neritic zones, respectively, with increasing body size. Stable isotope analyses also indicated grouper at these sites are generalist upper trophic-level predators, with intermediate values for  $\delta^{13}\text{C}$  and the highest observed values for  $\delta^{15}\text{N}$ . Stable isotope values for grouper did not differ appreciably between sites, but mixing models indicated differences in percent contribution of primary prey categories between sites, as well as among size classes for grouper from the neritic zone. Mixing models indicated a higher percent contribution of fishes to grouper growth than identified based on stomach contents analyses.

Similar to previous studies that integrated stomach contents and stable isotope analyses (e.g. Winemiller et al., 2011; Davis et al., 2012), the two approaches were complementary and the combination provided a more robust understanding of grouper diet and its role in the food webs of our study sites. Specifically, stable isotope data indicate that unidentifiable fish species in the stomach contents were likely mostly sciaenids such as whitemouth croaker, Argentine croaker, southern kingcroaker, and barbel drum. Consumption of these predatory taxa yields the observed grouper isotope values when combined with other known important diet items (i.e., crabs and shrimp) that had significantly more depleted nitrogen isotope values. Furthermore, isotope mixing models indicated that fishes are far more important to assimilated diets than suggested by stomach contents analyses alone. This difference is likely due to relatively greater amount of refractory material in crabs (e.g., carapace) versus shrimp and fishes, such that crabs may be more frequently encountered in stomach contents due to slower digestion rates but also contribute less (per unit ingested biomass) to assimilated energy. Identification of differences between ingestion and assimilation is arguably only feasible when combining stomach contents and stable isotope analyses, and is especially relevant when examining the trophic ecology of generalist/opportunistic predators that consume diverse prey types. On the other hand, stomach contents analyses provided resolution on ontogenetic diet shifts, particularly related to prey size and biomass, and differences in the relative frequency of consumption of non-blue crabs, blue crabs, and shrimp between sites and size classes that would not have been apparent from stable isotope analyses alone due to the relative similarity in carbon isotope values for

these prey types and limited assimilation. Although mixing models indicated that crabs were of only minor importance to material assimilated by grouper, the large numbers of species and individuals identified from stomach contents analyses suggest that grouper may have strong top-down effects on crab populations even if their consumption does not contribute significantly to grouper growth.

The high frequency of occurrence of crabs and fishes in diets of dusky grouper from both the littoral and neritic zones in this study is similar to findings by Machado et al. (2008) from other coastal zones in Brazil. Research in the Mediterranean (Reñones et al., 2002; Linde et al., 2004; López & Orvay, 2005) also detected high frequency of occurrence of these prey types, along with high frequency of cephalopods, principally octopuses of the genus *Octopus* and common cuttlefish *Sepia officinalis*. The importance of cephalopods in dietary studies of dusky grouper from the Mediterranean, in contrast with studies from the Atlantic (Machado et al., 2008; this study), is likely mediated by water depth. Specifically, the most common cephalopod species found in southeastern Brazil (e.g., *Illex argentinensis*, *Doryteuthis plei*, *D. sanpaulensis*, *Octopus vulgares*, and *O. tehuelchus*) are generally encountered in deeper waters (Haimovici & Álvarez-Perez, 1990; Andriugetto & Haimovici, 1996; Santos & Haimovici, 2002) than the grouper populations sampled in this study.

Previous studies on dusky grouper identified ontogenetic diet shifts, transitioning from consumption of crustaceans at smaller sizes to gastropods and fishes for larger size classes (e.g., Reñones et al., 2002; Linde et al., 2004; Machado et al., 2008). Kulbicki et al. (2005) studied the diets of ten *Epinephelus* species and found that, in general, smaller individuals consumed mostly crabs, whereas larger individuals preferentially consumed fishes and mollusks. Ontogenetic diet shifts were also observed in this study, but with an increased importance of blue crabs for larger grouper from the littoral zone and fishes in the neritic zone. The difference in the importance of blue crabs in grouper stomach contents between sampling locations is likely influenced by higher densities of blue crabs in the littoral zone, especially as large numbers of adult female blue crabs leaving Patos Lagoon Estuary to spawn in higher salinity offshore waters must pass through this location as it provides the only connection between the estuary and the ocean. Linde et al. (2004)

suggested that ontogenetic diet shifts in grouper are associated with a change in foraging strategies, switching from an active predator preferring smaller prey (e.g., crabs) to an ambush predator targeting larger prey (e.g., fishes and cephalopods). These previous studies also noted a tendency for prey to increase in length or biomass with increasing grouper size, a pattern observed for both the littoral and neritic zones in this study. Integration of the isotope data suggests that consumption of crabs, especially by larger grouper, may be of minor importance to grouper growth. Even as such, crabs likely still provide an important link in carbon flow as prey for fishes such as whitemouth croaker that are consumed by grouper and contribute significantly to assimilated carbon.

As cited above, previous research on *Epinephelus* compared diets among species and or size classes, but information is lacking for variation in diet along a depth gradient or between comparatively shallow and deep sites. Our findings suggest that feeding behavior of dusky grouper is similar between littoral and neritic zones (in terms of general prey types and sizes), but with clear differences in the taxonomic composition of prey species. This difference in prey composition is expected to be the result of different environmental conditions between sites, as affecting prey composition and abundance, rather than intrinsic differences in foraging behavior of grouper at these sites. Specifically, the littoral site is strongly influenced by the surf zone and continental discharge from Patos Lagoon, and these conditions favor a unique suite of species such as the crabs *Menippe nodifrons* and *Callinectes danae*, whereas other species inhabit deeper marine waters, such as *Octopus* spp. and the hermit crab at the neritic study location.

Even though carbon isotope values of grouper were similar between sites, the basal carbon sources supporting secondary production of dusky grouper are expected to differ. The littoral site is strongly influenced by discharge from Patos Lagoon Estuary, and important basal carbon sources likely include saltmarsh vegetation, seagrass, macroalgae, and phytoplankton (Garcia et al., 2007; Hoetinghaus et al., 2011; Claudino et al., 2013). The neritic zone is dominated by marine influences and important basal carbon sources at this site are expected to be macroalgae, primarily Rhodophyta (Horta et al., 2008), and marine phytoplankton. Phytoplankton typically vary in  $\delta^{13}\text{C}$  along salinity gradients, with values being

more depleted as the relative contribution of DIC of freshwater origin increases (Peterson et al., 1994; Canuel et al., 1995; Bouillon et al., 2000; Kaldy et al., 2005), and saltmarsh vegetation can include both comparatively depleted  $\text{C}_3$  plants and enriched  $\text{C}_4$  plants depending on hydrogeomorphology of the estuarine zone (Hoetinghaus et al., 2011). Due to differences in composition and relative abundance of carbon sources, and importantly carbon isotopic values of the sources, we expected that dusky grouper collected at the neritic site would have more depleted carbon isotopic values than their littoral counterparts and that carbon isotopes could thus be useful as a tracer of origin or site fidelity of dusky grouper along our coastal zone (e.g., as in Hobson, 1999; McCarthy & Waldron, 2000; Harrod et al., 2005). Contrary to expectations, sampled species at both sites were relatively enriched in  $\delta^{13}\text{C}$ , ranging between  $-20$  and  $-10\text{‰}$ . This range is consistent with marine algae providing the depleted end-member and more enriched values may include macroalgae for the neritic site, with the addition of seagrass and  $\text{C}_4$  saltmarsh vegetation such as *Spartina alterniflora* for the littoral site (both likely through detrital pathways; Garcia et al., 2007; Hoetinghaus et al., 2011). The intermediate isotopic values of dusky grouper suggest that they integrate both marine pelagic and benthic food web pathways, with benthic carbon sources and consumers often having more enriched carbon isotope values than pelagic sources and consumers (France, 1995; Bergamino et al., 2011; Claudino et al., 2013). Isotopic analyses of dusky grouper in the Mediterranean also provided evidence of strong linkages to the benthic food web (Reñones et al., 2002). An alternative hypothesis is that dusky grouper are moving extensively between sites resulting in similar carbon isotope values for individuals at both sites. However, dusky grouper, like other grouper species, are known to be territorial and make only very limited movement during the year (e.g. Lembo et al., 2002; Pastor et al., 2009), with the exception of reproductively active adults specifically during the reproductive period (Zabala et al., 1997). Thus, it is not likely that all size classes of grouper are moving between sites with sufficient frequency to result in isotopic values that are considered a mixture from feeding at both sites.

It is important to further consider the role of animal movement on carbon dynamics and sources supporting secondary production of dusky grouper, especially

for the littoral zone study site. The littoral zone site receives not only passive transport of organic materials due to discharge from Patos Lagoon, but also is situated at the only access point through which estuarine or coastal organisms may move between systems. Animal movements across these ecosystems may provide unique carbon subsidies when an organism grows in one location and is consumed in another (e.g., Connolly et al., 2005; Garcia et al., 2007; Oliveira et al., 2014). Due to the nursery function of the estuary for many consumer species, such movements among estuarine and coastal zones are often tied to reproductive behaviors necessary to complete an organism's life-cycle, and previous research on coastal systems in this region identified significant differences in carbon isotopic values among reproductive guilds based on utilization of coastal and inshore environments (Garcia et al., 2007; Hoeninghaus et al., 2011). As mentioned above, the movement of adult female blue crabs from estuarine to higher salinity coastal zones for spawning may represent a subsidy of carbon predominantly derived from detrital *Spartina alterniflora* to coastal food webs (Hoeninghaus & Davis III, 2007). Another example can be seen in the carbon isotope signatures of the mullet *Mugil liza*. This species is a catadromous estuarine dependent, utilizing the coastal and estuarine zone for growth and migrating offshore for reproduction (Vieira, 1991). Juvenile mullets less than 30 mm TL occur in the marine environment where they feed on phytoplankton and zooplankton prior to moving inland, where larger mullets occur in the estuarine zones and shift to a diet based more heavily on detritus (Acha, 1990; Vieira, 1991). Our data from the littoral zone reflect this difference, with juvenile mullets (25 mm TL) having a mean  $\delta^{13}\text{C}$  of  $-19.59\text{‰}$  (carbon derived from marine phytoplankton) and adults ( $>120$  mm TL) with a mean  $\delta^{13}\text{C}$  of  $-11.11\text{‰}$  (estuarine detritus). Both of these examples support interpretations of the enriched end-member of the littoral zone being at least in part comprised by estuarine-derived carbon sources such as detritus from  $\text{C}_4$  saltmarsh grasses.

Through their role as upper trophic-level predators, grouper can exhibit strong effects on food webs of the ecosystems where they occur (Heemstra & Randall, 1993; Sluka et al., 2001). Reñones et al. (2002), studying dusky grouper populations in the Mediterranean, observed a strong correlation between  $\delta^{15}\text{N}$  and

body size, corroborating the increase in trophic position with increasing body size identified from stomach contents analyses. The authors suggest that because of this correspondence between diet and isotopes, in particular the positive correlation between  $\delta^{15}\text{N}$  and body size, stable isotope analyses can be particularly useful for studies in marine protected areas where fishes cannot be collected for stomach contents analyses. Unfortunately, we observed only very minor shifts in  $\delta^{15}\text{N}$ , even though ontogenetic dietary shifts were detected using both stomach contents and stable isotope analyses. This is likely due to grouper feeding across trophic levels at all size classes and may also reflect different  $\delta^{15}\text{N}$  baselines for pelagic and benthic carbon sources. The complex nature of these combinations precludes strong shifts in  $\delta^{15}\text{N}$ , even though ontogenetic diet shifts were detected for prey composition and size. Our results suggest that stomach contents analyses and stable isotope analyses are highly complementary for elucidating the trophic ecology of grouper, but that it may be premature to rely solely on stable isotope analyses in the study of protected populations. When possible, it seems prudent to first validate that strong correspondence exists between ontogenetic diet shifts and stable isotope ratios (e.g., as observed in Reñones et al., 2002).

Previous research from our study system on dusky grouper age, growth, and reproduction (Seyboth et al., 2011; Condini et al., 2014) suggests that elevated beach rocks in the neritic zone are better quality habitat than the man-made rocky jetties that provide littoral habitat. Barreiros & Santos (1998) suggest that the small size of caves or crevices in the rocky jetties, rather than other factors such as prey availability, may limit the occurrence of adult dusky grouper at this site. Due in part to the lack of adults (especially males) in the littoral zone and the relative proximity of the two sites, these two rocky habitats may be part of a single metapopulation with source-sink dynamics, where the neritic population (source) exports juveniles to the littoral population (sink). Although carbon and nitrogen stable isotopes alone were not useful as tracers of habitat use between the littoral and neritic zones, inclusion of a third isotope such as sulfur could provide further resolution for quantifying connectivity between these two locations (Fry & Chumchal, 2011; Nelson et al., 2012). In addition to understanding the trophic bases of secondary production, as investigated in this study, clarification of spatial and temporal

dynamics of population connectivity is critical for protection of this endangered species, especially considering that it is targeted by both littoral and offshore fisheries. The populations in natural and man-made habitats examined here provide a relevant case study for applications to other threatened grouper populations around the world, for example, as declining populations and habitat loss result in fragmented populations or if conservation strategies are considering inclusion of artificial substrates as part of protected area or reserve designs.

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