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Original Article

Trophic niches and diet shifts of juvenile mullet species coexisting in marine and estuarine habitats

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Abstract

Food partitioning among coexisting species is often considered advantageous to minimize niche overlap and avoid inter-specific competition. Congeneric fish species such as the mullets *Mugil curema* and *Mugil liza*, which co-occur across marine and estuarine habitats, are good models to evaluate resource use and niche overlap or partitioning. We used stomach contents (SCA) and stable isotope analysis (SIA) to assess potential trophic shifts and changes in niche overlap associated with the mullets transitioning from marine to estuarine habitats. SIA included different fractions of organic matter in suspension and in the sediment to estimate the contribution of micro, nano and pico-organisms to the mullets' diets. We hypothesized higher resource partitioning in the less resource-diverse system (marine surf-zone) than in the more diverse one (estuary). SCA showed diet differences between *M. curema* and *M. liza* according to the habitat. They showed distinct diets in the marine area ($P < 0.001$), but similar diets in the estuary ($P = 0.226$). A lower niche breadth was observed for both species in the marine area (*M. curema* = 0.03, *M. liza* = 0.06) compared with the estuary (*M. curema* = 0.14, *M. liza* = 0.16). Isotopic niches of both species were higher in the estuary (64.7%) compared with the marine area (0.7%). These findings corroborated our hypothesis of higher food partitioning in the marine surf-zone. We also demonstrated using SIA the shift from planktonic to benthic feeding following the recruitment of the mullets from the surf-zone into the estuary.

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Introduction

Understanding the processes and mechanisms controlling a species' trophic niche and potential consequences for coexistence of phylogenetically, morphologically or functionally similar species is a long-standing problem in ecology (Schoener, 1974; Layman & Winemiller, 2005; Andrade *et al.*, 2019). Niche overlap occurs when two or more species share, to some extent, the same realized niche (Hutchinson, 1957). If niche overlap is low or resources (e.g. food) are abundant, species will coexist without competition, but high niche overlap and limited resource availability will promote competition (Pianka, 1974; Giller, 1984). Food resources used by a species (i.e. the trophic niche) represent one of the most studied dimensions of the niche (Bearhop *et al.*, 2004). However, the myriad of factors affecting the trophic niche are complex due the spatiotemporal variations in food availability, prey–predator interactions and consumer trophic plasticity (Giller, 1984; Park *et al.*, 2016; Silva *et al.*, 2017).

Food partitioning among species with inter-specific differences in morphological traits or through space and time is often considered an adaptation to minimize competition (e.g. for fishes: Alexandrou *et al.*, 2011; Cardona, 2015; Rohan & Buckley, 2018). Several studies in distinct ecosystems revealed marked variation in food partitioning patterns among fishes coexisting in the same habitat (e.g. Correa & Winemiller, 2014; Juncos *et al.*, 2015; Andrade *et al.*, 2019; Malinowski *et al.*, 2019). For instance, prior work on food partitioning in iliophagous mullet species (i.e. feeding mostly on microorganisms associated with fine sediment and debris; Dualiby, 1988; Vieira, 1991; Cardona, 2015) has shown trophic niche segregation in some estuaries (Le Loc'h *et al.*, 2015; Garcia *et al.*, 2018), but trophic niche overlap in others (Cardona, 2001). Such discrepancies may be associated with differences in habitat characteristics, between-site variations in food availability, as well as technical differences employed to describe diets.

An interesting model to investigate use of food resources by sympatric species in coastal ecosystems is mullet species inhabiting estuaries. For example, the mullets *Mugil curema* Valenciennes, 1836 and *Mugil liza* Valenciennes, 1836 are highly euryhaline fishes that occur in marine, estuarine and freshwater ecosystems along tropical, subtropical and temperate systems (Crosetti & Blaber, 2015; Nelson *et al.*, 2016). These species are abundant in temperate and subtropical estuaries, comprising a major part of the fish assemblage in shallow areas

(Garcia *et al.*, 2004; Possamai *et al.*, 2018; Vieira *et al.*, 2019). They are marine estuarine-dependent (Elliott *et al.*, 2007) species that usually spawn in the sea whereas juveniles use shallow estuarine areas as nursery and feeding areas (Vieira, 1991; Lemos *et al.*, 2014; Garcia *et al.*, 2018; Mai *et al.*, 2019). Juveniles of mullets typically change feeding habits from planktonic to iliophagous (Blaber & Whitfield, 1977; Dualiby, 1988), and this planktonic-benthic diet shift usually occurs between 20 and 30 mm total length (TL) depending on the species (Cardona, 2015). In Patos Lagoon estuary in southern Brazil, juveniles of *M. curema* and *M. liza* consume microalgae, foraminifers and microcrustaceans and both recruit into Patos Lagoon estuary at less than 35 mm TL, suggesting they may shift feeding habits from planktivore in the marine adjacent surf-zone to iliophagous in the estuary benthic habitats (Vieira, 1991).

Studies of food partitioning among iliophagous species are relatively scarce, probably due to technical difficulties in describing detailed diet composition and food assimilation patterns (Cardona, 2015). A study carried out at Tramandaí-Armazém estuary (South Brazil) compared the diet of two mullet species using stable isotopes analysis and stomach contents techniques and observed different niche overlaps depending on the habitat occupied by both species (Garcia *et al.*, 2018). However, although their work provided relevant evidence on the trophic niche of these iliophagous species, technical aspects hinder their conclusion. For example, the study had a low number of stomach content samples and lacked temporal replicates (i.e. it was only a diet snapshot based on a single field campaign). Their isotopic analysis was restricted to consumers' variability and did not include food sources and isotope mixing model analysis, which are essential for a comprehensive investigation of the trophic niche (Phillips *et al.*, 2014). This last caveat was probably due to the difficulty to obtain pure samples of food items (microorganisms, especially microalgae) in an adequate amount to allow the determination of isotopic composition. Instead of analysing the isotopic composition of particular food items, prior studies had analysed particulate organic matter in suspension (POM) or in the sediment (SOM) as proxies for phytoplankton and phytobenthos, respectively, considering only single large (1.2–300 µm) pools of organic matter (Faye *et al.*, 2011; Le Loc'h *et al.*, 2015; Carassou *et al.*, 2017; Cicala *et al.*, 2019). This approach, however, does not allow differentiation among particle size classes such as picoplankton, nanoplankton and microplankton (Sieburth *et al.*, 1978). The aggregation of a broad range of particle sizes into a single source pool prevents detection of possible resource partitioning based on particle sizes, which are potentially composed by distinct microalgae communities.

Aiming to advance the knowledge of iliophagous food habits and evaluate diet shifts associated with estuarine recruitment and trophic niche overlap between *M. curema* and *M. liza*, we used a technique to separate the particles size fractions of organic matter and estimated their isotopic values. We also compared the use of food resources between juveniles of the mullets *M. curema* and *M. liza* in marine and estuarine habitats of a subtropical coastal system. Based on the fact that mullet species can exhibit differences in gill rakers morphology (Konan *et al.*, 2014; Cardona, 2015) and these may lead to trophic niche partition by selection of food sizes (Rohan & Buckley, 2018), we investigated which size fractions (1.2–20, 20–68 and 68–250 µm) of particulate organic matter were most assimilated in each habitat.

The marine surf-zone is dominated by dense blooms of microalgae (especially diatoms) comprising most of the *in situ* primary production (Odebrecht *et al.*, 2010), whereas the estuary harbours more diverse phytoplanktonic (Haraguchi *et al.*, 2015; Mendes *et al.*, 2016) and benthic microalgae assemblages (Coutinho & Seeliger, 1984; da Silva *et al.*, 2010). In this sense, we expected that diet and food partitioning between juvenile mullet species would change across these coastal habitats with contrasting food availability. We hypothesized that with higher availability of diverse size food-resources (estuary), there will be no food-size partitioning between the species, while in the lower availability of diverse size food-resources (marine surf-zone) the species will partition the particle sizes. Moreover, this proposed methodology will allow us for the first time to evaluate isotopically the occurrence of feeding habit shifts from planktonic to iliophagous on mullets.

Materials and methods

Study area

This study was carried at Patos Lagoon estuary and the adjacent marine surf-zone in southern Brazil (Figure 1). Patos Lagoon constitutes the largest choked lagoon (10,360 km^2) in the world (Kjerfve, 1986) and its estuarine area comprises about 10% of total lagoon area, connected with the ocean through a single inlet about 4 km long and 740 m wide at the mouth (Seeliger & Odebrecht, 2010) (Figure 1). Tidal range influence in the estuary is minimal (~0.47 m), and the hydrodynamic of the estuary is driven mainly for the winds, which are predominantly from NE to SW in the region (Möller *et al.*, 2001). The shallow waters bottom (<1.5 m) is composed of sand and the channel substrate by sand, silt and clay (Calliari *et al.*, 1977; Ortega *et al.*, 2020). Patos Lagoon estuary harbours more than 90 species of benthic macroalgae, including colonial and filamentous cyanobacteria, chlorophytes, phaeophytes, xanthophytes and rhodophytes. The main microalgae are diatoms, cryptophyta, cyanobacteria and dinoflagellates, and their temporal distributions may vary seasonally and also due to changes in abiotic factors such as salinity (Seeliger *et al.*, 1997).

The adjacent marine surf-zone is characterized by an extensive sandy coastline of ~220 km with dissipative beaches directly exposed to waves with medium to high energy. The main autotrophs in this ecosystem are microalgae that are responsible for high *in situ* phytoplankton production and blooms dominated by frequent and dense diatoms, mainly *Asterionellopsis guyunusae* (Odebrecht *et al.*, 2010, 2013; Franco *et al.*, 2016). Centric diatoms and dinoflagellates are important components of the phytoplankton particularly during summer (Seeliger *et al.*, 1997). Therefore, in contrast with the estuary, this marine surf-zone is characterized by the dominance of phytoplankton and the absence of other autotrophs such as aquatic macrophytes, macroalgae beds and seagrasses (Seeliger *et al.*, 1997).

Field collections and sample processing

Juvenile mullets (*M. curema* and *M. liza*) were sampled monthly during the austral summer (January to March) of 2018 in four sampling stations with depth <1.5 m: two inside the estuary (Estu1, Estu2) and two in the adjacent marine surf-zone (Mar1, Mar2) (Figure 1). This season was chosen based on prior work showing that both juvenile mullet species co-occur in this estuary and its adjacent marine shallow area only during summer (Vieira, 1991; Rodrigues *et al.*, 2015). Mai *et al.* (2018) demonstrated through chemical analysis of otoliths that both species spawn in marine waters. However, some specimens of *M. curema* can spend their entire life cycle in seawater, others have sporadic entries in brackish or fresh water and finally there are some individuals who remain in brackish water for a longer period of time (Mai *et al.*, 2018). The species *M. liza*, on the other hand, seems to be more dependent on the estuary, as they spend most of their life cycle in fresh and/or brackish water (Mai *et al.*, 2018). The mullet species reach the first maturity for both sexes with an average size of 408.3 mm for *Mugil liza* and 248.6 mm for *Mugil curema* (Fernandez & Dias, 2013; Lemos *et al.*, 2014). Fishes were sampled

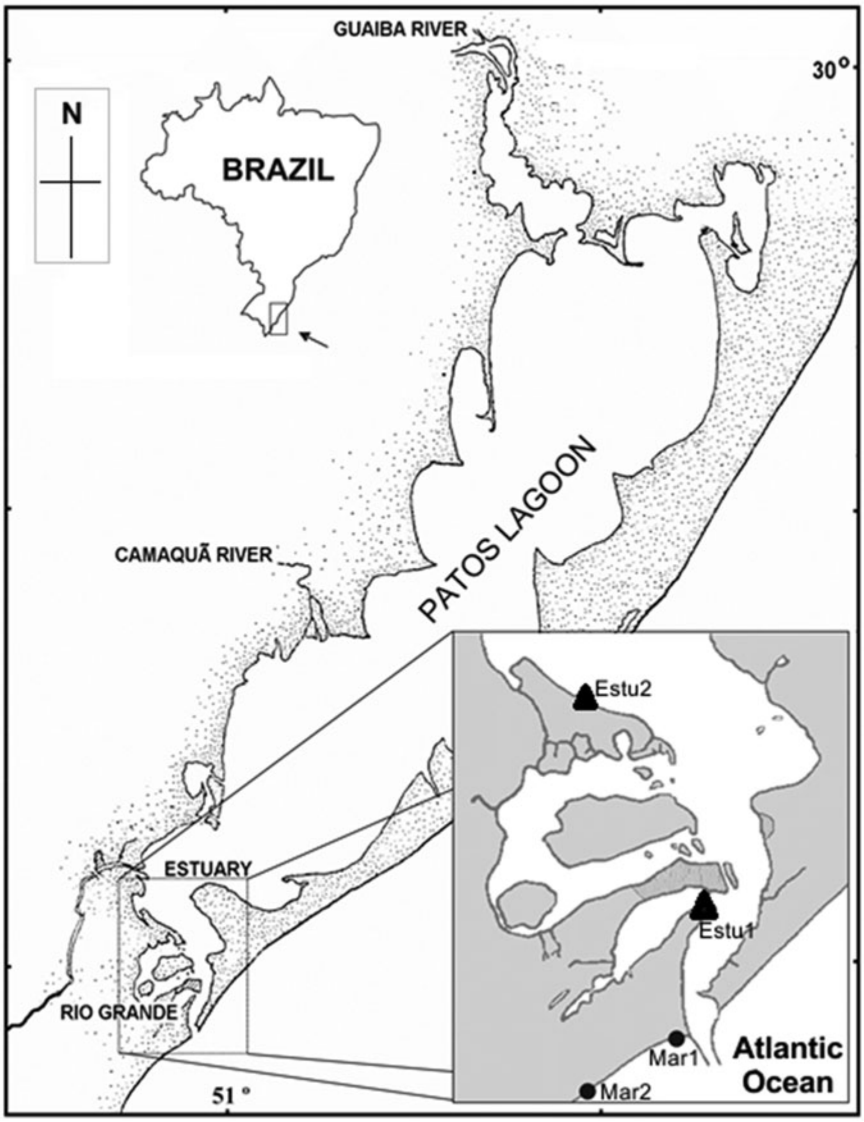

Fig. 1. Patos Lagoon and its estuarine zone in southern Brazil. Black triangles in the inset figure denote sampling sites in the estuary (Estu1, Estu2) and black dots denote the adjacent marine surf-zone (Mar1, Mar2).

using a 9 m beach seine (13 mm bar mesh in the wings and 0.5 mm mesh in the 3 m centre section) that was pulled to cover an area of about 60 m^2 during each haul (Garcia *et al.*, 2001).

Stable isotopic analysis

Ten individuals measuring between 23 and 45 mm TL of each species (*M. curema*: 33.9 ± 3.0 mm and *M. liza*: 30.4 ± 5.8 mm TL) at each sampling station were collected in March 2018. The exemplars were euthanized with eugenol (0.4 ml/l of eugenol solution 1:10 in ethanol 70%) for subsequent collection of muscle tissue for analysis of carbon and nitrogen stable isotope ratios (δ^{13}C and δ^{15}N, respectively). This anaesthetic was used because it does not significantly alter stable isotopic composition (Nahon *et al.*, 2017). Only the individuals collected in March were used in the SIA because their isotopic turnover reflects food assimilation in the prior two months of sampling (Oliveira *et al.*, 2017) and thus the time that both species co-occur in the study system.

In order to quantify potential diet shifts, different size classes of particulate organic matter in suspension (POM) and in the sediment (SOM) were sampled and used as proxies for basal food sources (planktonic and benthonic microalgae) consumed by juvenile mullets. One sub-sample of each SOM size fraction was analysed under the microscope to ensure that the samples contained microphytobenthos. Three samples of each size fraction of POM and SOM were obtained monthly between January and March 2018 at each sampling station. The POM fractions were obtained by filtering water through a sequence of filters with decreasing porosity. Initially, water was passed through a 250 µm pore sieve to retain and subsequently discard larger zooplankton and coarse detritus (e.g. sediment, leaves). The remaining water was filtered in sequence into 68 µm and 20 µm pore sieves and finally a pre-combusted (450 °C for 4 h) 1.2 µm Whatman glass-fibre filter. The material obtained in the 68 µm and 20 µm pore sieves and the 1.2 µm filter was placed in Petri dishes, sealed and stored on ice until their transport to the laboratory. These procedures resulted in three fractions of POM with different size classes: 1.2–20 µm, 20–68 µm and 68–250 µm. A similar procedure was applied to obtain size fractions of SOM (i.e. microphytobenthos). Initially, the upper 2 cm from the sediment was collected using a plastic core (10 cm diameter) at each sampling station and stored on ice until processing. In the laboratory, the sediment was moved to a clean plastic bottle, partially filled with distilled water and manually mixed. After sedimentation of sand grains, the supernatant was collected and filtered into the sieves and glass-fibre filter following the same procedures described for POM. The samples were not acidified because the sediments of Patos Lagoon estuary are poor in carbonates and previous studies did not find differences in the carbon isotopic composition of acidified samples compared with non-acidified (Claudino *et al.*, 2013).

All samples for SIA were processed in the laboratory following standard procedures (Garcia *et al.*, 2007; Hoeinghaus *et al.*, 2011). Briefly, muscle tissue was dissected from the anterior-dorsal region of each individual fish, rinsed with distilled water, and dried in sterile Petri dishes in an oven at 60 °C to constant weight (minimum of 48 h). Similarly, POM and SOM size fractions, including the filter for the smallest size fraction, were dried in sterile Petri dishes to constant weight. Dried samples with the exception of filters were ground to a fine powder with a mortar and pestle and stored in clean Eppendorf tubes. Subsamples were weighed (~1 mg for animal tissues and 25–30 mg for SOM and POM) and pressed into ultra-pure tin capsules (Costech Analytical Technologies, Valencia, CA, USA). Isotopic analyses were conducted at the Stable Isotope Ecology Laboratory at the University of North Texas using continuous flow elemental analyser isotope ratio mass spectrometry (EA-IRMS) in a system comprised by a Thermo Flash 2000 EA, ConFlo IV interface and Delta V Advantage IRMS. Modified single-point normalization (equivalent to two-point and multi-point normalization methods; Paul *et al.*, 2007; Carter & Barwick, 2011) was used to normalize preliminary data, based on quantified and known values of two certified reference materials (USGS 40 and USGS 62) analysed with each sequence. Reference materials were chosen such that their known values approximately bracket the estimated ranges of C and N isotope values of samples being analysed. Stable isotope values are reported as parts per thousand (‰) differences from corresponding international standards VPDB (Vienna PeeDee Belemnite) and air for carbon and nitrogen, respectively: $\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$, where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$. Standard deviations for $\delta^{13}C$ and $\delta^{15}N$ of replicate analyses of lab standards analysed with each sequence were 0.13‰ and 0.07‰ for animal, and 0.08‰ and 0.24‰ for plant, respectively.

Stomach content analysis

For the stomach content analyses (SCA), five individuals ranging from 25–50 mm of total length of each species (*M. curema*: 41.4 ± 5.7 mm and *M. liza*: 32.8 ± 8.5 mm TL) at each sampling station in January and February 2018 were randomly selected from the individuals collected. In the laboratory, stomachs were removed and fixed in 10% formaldehyde for four days then stored in 70% alcohol until analysis. Determination of stomach contents was based on subsamples. For each individual, the contents of the stomach were removed, mixed with ethanol and stirred. Following procedures in Cardona (2015) and Garcia *et al.* (2018), one aliquot was sampled with a micropipette and put into a Fuchs Rosenthal chamber (0.2 mm deep with a grid of 16 quadrats of 1.0 mm^2 each) where microalgae and zooplankton were identified and counted at 400× magnification using a light microscope. Items were counted until reaching at least 200 individuals (cells, colonies or filaments) in each sample. In case of not reaching 200 individuals in the first aliquot, an additional aliquot was analysed up to the maximum of three aliquots or until reaching the established limit of counted individuals. Additional subsamples were acid cleaned and mounted on glass slides using Naphrax™ (Brunel Microscopes Ltd, Chippenham, UK) in toluene and examined at 1000× under a light microscope (Biggs & Kilroy, 2000) to identify diatoms. Microalgae and zooplankton were identified to the lowest practical taxonomic level based on specialized literature.

Data analyses

SIA – sources contribution to the mullets

Initially, a two-way ANOVA was used to test for differences in average $\delta^{13}C$ and $\delta^{15}N$ among species (*M. curema* and *M. liza*) and habitats (Mar1, Mar2, Estu1, Estu2), followed by a Tukey (HSD) test for post-hoc comparisons. Biological data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Cochran test). This analysis did not reveal differences in $\delta^{13}C$ or $\delta^{15}N$ for either species between the two sampling stations within the marine surf-zone or within the estuary (*P*-value >0.05). Therefore, subsequent SIA were carried out considering only marine *vs* estuary habitats without specific locations within each.

Biplots of $\delta^{13}C$ and $\delta^{15}N$ were used to first examine between-habitat patterns in isotopic composition of consumers (*M. curema* and *M. liza*) and basal food sources (Peterson & Fry, 1987). A Bayesian isotope mixing model (SIMMR; Stable Isotope Mixing Models in R; Parnell, 2016) was used to estimate relative contributions of different size fractions of POM and SOM (1.2–20 µm, 20–68 µm and 68–250 µm) assimilated by each species in each habitat. This method employs Gaussian likelihood and fits the model to the data via Markov chain Monte Carlo (MCMC). For each mixing model, 500,000 iterations were run, followed by a burn-in and thinning of 50,000 and 5000, respectively. Models were run with uninformative priors. The trophic enrichment factor (TEF) values used were 2.1 ± 0.7 for carbon and 3.8 ± 0.4 for nitrogen, estimated specifically for juvenile mullet *M. liza* through controlled diet experiments (Oliveira *et al.*, 2017). In the absence of similar experimental data for its congeneric species, the same TEF values for *M. curema* were applied in the Bayesian mixing models. The nitrogen content in the POM and SOM samples was relatively low and close to the detection limit of the Isotope Ratio Mass Spectrometry (IRMS), therefore one additional standard deviation was added to the instrumental precision of $\delta^{15}N$ values obtained for each fraction of POM and SOM to cope with this potential analytical variability.

A fundamental assumption of isotope mixing models is that the isotopic variability of consumers, after accounting for fractionation corrections, is contained within the variability of assimilated food sources (Phillips *et al.*, 2014). This assumption was evaluated using isotope mixing polygon simulations, which quantitatively establish boundaries of possible source values in the $\delta^{13}C$-$\delta^{15}N$ biplot space that can explain the isotopic variability of consumers (Smith *et al.*, 2013). These simulations were run with the packages sp (Pebesma & Bivand, 2005; Bivand *et al.*, 2013) and splancs (Rowlingson & Diggle, 2017) in R (R Core Team, 2019). Simulations were conducted for each habitat separately and those individual consumers located outside the 95% mixing polygon region (Supplementary Figure S1), which indicate they could not be confidently explained by the food sources (Phillips *et al.*, 2014), were omitted from the subsequent Bayesian isotope mixing model analyses. For the surf-zone, the majority of individuals could not be explained by local basal food sources in the polygon simulation. In this sense, we added the POM offshore as an additional source in the Bayesian Mixing Model once it meets the marine surf-zone polygons. These samples (N = 6) were obtained during spring 2014 between 5 and 150 nautical miles off the coast (E. Secchi & S. Botta, unpublished data; Possamai *et al.*, 2020). Although offshore food sources were not collected in the same season and year of the fish samplings, the use of these data is justified by the lower variation in isotopic values of marine POM compared with marine surf-zone POM (Bouillon *et al.*, 2011; Rosli *et al.*, 2017; Garcia *et al.*, 2019).

SIA – isotopic niche overlap

Isotopic niche overlap was evaluated by the area and overlap among standardized isotopic ellipses (SEAc) in the bivariate $\delta^{13}C$–$\delta^{15}N$ space for mullet species at each habitat using the SIBER (Stable Isotope Bayesian Ellipses, Jackson *et al.*, 2011) package in R. Overlap in SEA_C between juvenile mullet species in each habitat was expressed as a proportion of the sum of the non-overlapping areas of the ellipses, where values range from

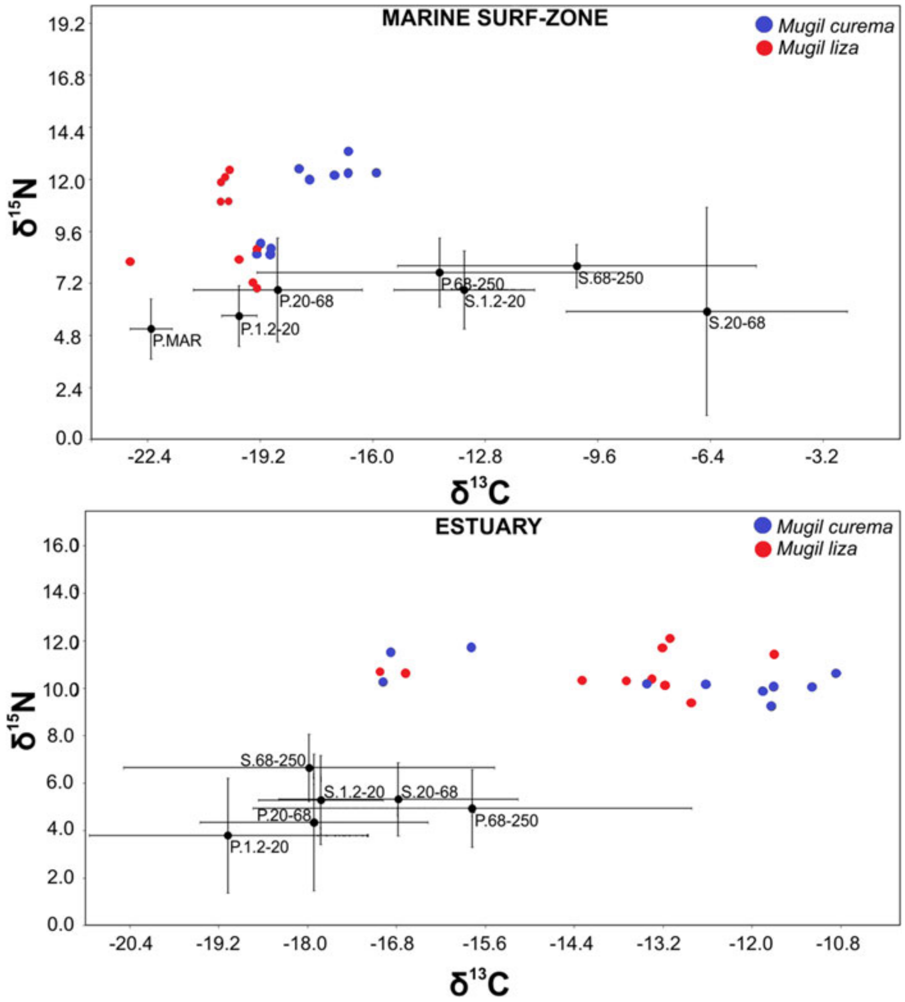

Fig. 2. Carbon (δ^{13}C) and nitrogen (δ^{15}N) isotopic values of the mullets *Mugil curema* (open/blue circles) and *M. liza* (grey/red circles) and average (± SD) values of the different size fractions (1.2–20, 20–68, 68–250 µm) of particulate organic matter in suspension (POM: P) and in the sediment (SOM: S) in the marine surf-zone (upper panel) and in the estuary (lower panel).

zero (ellipses are completely distinct) to 1 (ellipses are identical) (Jackson, 2019). Although not directly equivalent to trophic niche metrics based on stomach contents analyses (Bearhop *et al.*, 2004; Newsome *et al.*, 2007; Hoeinghaus & Zeug, 2008; Hette-Tronquart, 2019), isotopic ellipses can be useful as a proxy to compare amplitude and overlap among species' trophic niches (Jackson *et al.*, 2011).

SCA – mullets' diet composition and trophic niche overlap

The comparison of species' diet was performed by a Permutational Analysis of Variance (PERMANOVA) for each habitat separately, using the vegan package in R software. The abundance matrix of diet items was used and the $\alpha = 0.05$. In order to express the main food items consumed by each species, the Numeric Frequency was calculated by $FN\% = (N_i/N_t) \times 100$, where N_i is the total abundance of item i and N_t is the total abundance of items.

Trophic niche overlap between juveniles of the two mullet species was based on stomach contents data for each habitat and was analysed using the Morisita–Horn index:

$$\hat{C}_H = \frac{2\sum_i^n p_{ij}p_{ik}}{\sum_i^n p_{ij}^2 + \sum_i^n p_{ik}^2},$$

where $\hat{C}_H$ is the diet niche overlap between species j and k, p_{ij} is the proportion of resource i from the total resources used by species j, p_{ik} is the proportion of resource i from the total resources used by species k, n is the total number of resources. This index varies from 0 to 1, with higher values showing higher dietary niche overlap (Krebs, 1998). The Morisita–Horn index was calculated using the divo package in R software, calculating 500 bootstrap values and confidence interval of 95% (Erazmus *et al.*, 2018).

The trophic niche breadth of each juvenile mullet at each habitat was estimated using Levins' standardized niche breadth index (Hurlbert, 1978) following the formula:

$$\mathrm{Ba} = \frac{\left(1/\sum p_j^2\right) - 1}{n - 1}$$

where Ba is the trophic niche breadth, p_j is the proportion of the food item j in the diet of species i and n is the number of food items. This index ranges from 0 to 1, where 0 is an extreme specialist and 1 an extreme generalist. The trophic niche breadth was calculated with 95% confidence intervals using the Ecological Methodology program (Krebs, 1998).

Results

Food assimilation and isotopic niche inferred by SIA

The range of δ^{13}C values in the estuary was from −16.9 to −10.9‰ for *M. curema* and from −17.0 to −11.7‰ for *M. liza*, and in the marine surf-zone was −19.2 to −15.9‰ and −22.9 to −19.3‰, respectively (Figure 2, Table 1). For δ^{15}N values, the ranges in the estuary were 9.2–11.7‰ for *M. curema* and 9.4–12.1‰ for *M. liza*, and in the marine surf-zone was 8.6–13.2‰ and 7.0–12.3‰, respectively. In relation to size fractions of POM and SOM, wider range and lower overlap among average δ^{13}C and

Table 1. Number of samples (*n*) and average values (±SD) of carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotope values of consumers (*Mugil curema* and *M. liza*) and the different size fractions (1.2–20, 20–68, 68–250 µm) of particulate organic matter in suspension (POM) and in the sediment (SOM) in the marine surf-zone and in Patos Lagoon estuary

	Marine surf-zone				Estuary			
Consumers/sources	*n*	δ^{13}C	*n*	δ^{15}N	*n*	δ^{13}C	*n*	δ^{15}N
Mugil curema	10	−17.9 ± 1.2	10	11.0 ± 1.9	10	−13.30 ± 2.4	10	10.06 ± 0.7
Mugil liza	10	−20.2 ± 1.0	10	9.8 ± 2.1	10	−13.91 ± 1.7	10	10.69 ± 0.8
POM 1.2–20 µm	5	−19.85 ± 0.5	5	5.65 ± 1.4	5	−19.08 ± 1.9	5	3.80 ± 2.4
POM 20–68 µm	17	−18.69 ± 2.4	17	6.91 ± 2.4	17	−17.91 ± 1.5	16	4.34 ± 2.9
POM 68–250 µm	18	−14.13 ± 5.2	14	7.75 ± 1.6	18	−15.78 ± 3.0	13	4.93 ± 1.6
POM-MAR	6	−22.3 ± 0.6	6	5.1 ± 1.4	–	–	–	–
SOM 1.2–20 µm	6	−13.36 ± 2.0	6	6.90 ± 1.8	6	−17.82 ± 0.8	6	5.28 ± 1.9
SOM 20–68 µm	18	−6.55 ± 4.0	2	5.93 ± 4.8	18	−16.78 ± 1.6	18	5.32 ± 1.5
SOM 68–250 µm	18	−10.19 ± 5.1	1	8.04 ± 1.0	18	−17.98 ± 2.5	15	6.64 ± 1.4

δ^{15}N values were observed in the marine surf-zone than in the estuary (Figure 2). Average δ^{13}C values were significantly different across habitats ($F = 45.359$, df = 3, $P < 0.001$) and species ($F = 9.347$, df = 1, $P = 0.004$), but the interaction was not significant ($F = 2.239$, df = 3, $P = 0.102$) (Figure 3). In contrast, average δ^{15}N did not vary across habitats ($F = 0.698$, df = 3, $P = 0.560$) or species ($F = 1.263$, df = 1, $P = 0.269$) (Figure 3).

Bayesian mixing models revealed that offshore POM was the most assimilated basal food source at the marine surf-zone by both *M. curema* (Median = 60%; 95% Credibility Interval = 7–77%) and *M. liza* (88%; 74–95%) (Figure 4). In contrast, the largest size fraction (68–250 µm) of SOM was the most assimilated in the estuary by *M. curema* (48%; 3–80%) and *M. liza* (43%; 2–77%) (Figure 4).

The size and overlap of isotopic ellipses (SEAc) of both species differed across habitats (Table 2). In the marine surf-zone, *M. curema* presented lower SEAc than *M. liza* (4.11 and 7.62, respectively), whereas in the estuary both species showed similar values (4.73 and 4.85, respectively). The proportional overlap between isotopic ellipses of both species was higher in the estuary (64.7%) compared with the marine surf-zone (0.7%) (Table 2).

Diet composition and trophic niche inferred by SCA

Stomach content analyses (SCA) of both mullet species revealed diets composed of microalgae such as Bacillariophyceae, Coscinodiscophyceae, Mediophyceae, Chlorophyceae, Cyanophyceae, Euglenophyceae, Zygnematophyceae, and also fragments of zooplankton (Supplementary Table S1).

The comparison of the diet between *M. curema* and *M. liza* showed different results depending on the habitat. In the marine area, the species presented distinct diets ($F = 4.829$, df = 1, $P < 0.001$); however, in the estuary, the diet was similar ($F = 1.308$, df = 1, $P = 0.226$). In the marine surf-zone, *M. curema* feed mainly on *Asterionellopsis guyunusae* (Bacillariophyceae), while *M. liza* consumed mainly Coscinodiscophyceae (fragments) (Supplementary Figure S2). Nevertheless, although in the estuary both species fed on a greater diversity of algae, the four more consumed algae were the same for these two species: Bacillariophyceae spp., *Cylindrotheca closterium*, *Nitzschia* sp. 4 and *Skeletonema* spp., together comprising 40.8% of the *M. curema* diet and 39.9% of the *M. liza* diet (Supplementary Figure S2). Moreover, these four algae species were also consumed in similar proportions.

Trophic niche overlap (CH) of *M. curema* and *M. liza* showed similar results between marine surf-zone (mean = 0.86; 95% Credibility Interval = 0.69–0.97) and estuary (mean = 0.83; 95% Credibility Interval = 0.76–0.90) (Table 2). Trophic niche breadth (Ba) did not vary between species within the same habitat, but lower niche breadth was observed for both species in the marine surf-zone (*M. curema* = 0.03 and *M. liza* = 0.06) compared with the estuarine zone (*M. curema* = 0.14 and *M. liza* = 0.16) (Table 2).

Discussion

Assimilated diet and isotopic niche overlap inferred by SIA

Stable isotope analyses based on different size fractions (1.2–20, 20–68 and 68–250 µm) of organic matter revealed the preference of both species for larger particles in the estuary. Compared with previous studies (e.g. Faye *et al.*, 2011; Le Loc'h *et al.*, 2015; Carassou *et al.*, 2017; Cicala *et al.*, 2019) that analysed bulk organic matter that included all size fractions, our analyses provide new isotopic evidence supporting the hypothesis that juvenile mullets exhibit particle preferences during feeding. Higher assimilation of SOM with size particles between 68 and 250 µm suggests a feeding preference for microphytobenthos (20–200 µm), compared with smaller cell sizes such as nanophytobenthos (2–20 µm) and picophytobenthos (0.2–2 µm).

SIA also revealed a diet shift between juvenile mullets caught in the marine surf-zone and inside the estuary. Particulate organic matter in suspension (POM) was the most assimilated dietary item by juvenile mullets in the marine surf-zone, suggesting planktonic feeding in the water column. In contrast, the most assimilated item inside the estuary was SOM, which suggests iliophagous feeding behaviour (i.e. consuming microorganisms associated with fine sediment and detritus). This diet shift has already been described for other Mugilidae species (Cardona, 2015) and has also been suggested for *M. liza* based on inspection of stomach contents (Vieira, 1991). The present work is the first to provide isotopic evidence of the planktonic–benthic diet shift for *M. curema* and *M. liza* during their recruitment from the marine surf-zone into the estuary. Finally, it is worth noting that comparison of food niche partitioning between congeneric species in different types of habitat could be partially confounded by potential differences in feeding preference simply related to ontogeny. In order to minimize this potential effect, we analysed only juvenile forms with similar body size ranges (25–50 TL mm) in both habitats. Nevertheless, future experimental studies could investigate if diet changes in juvenile mullets may occur between

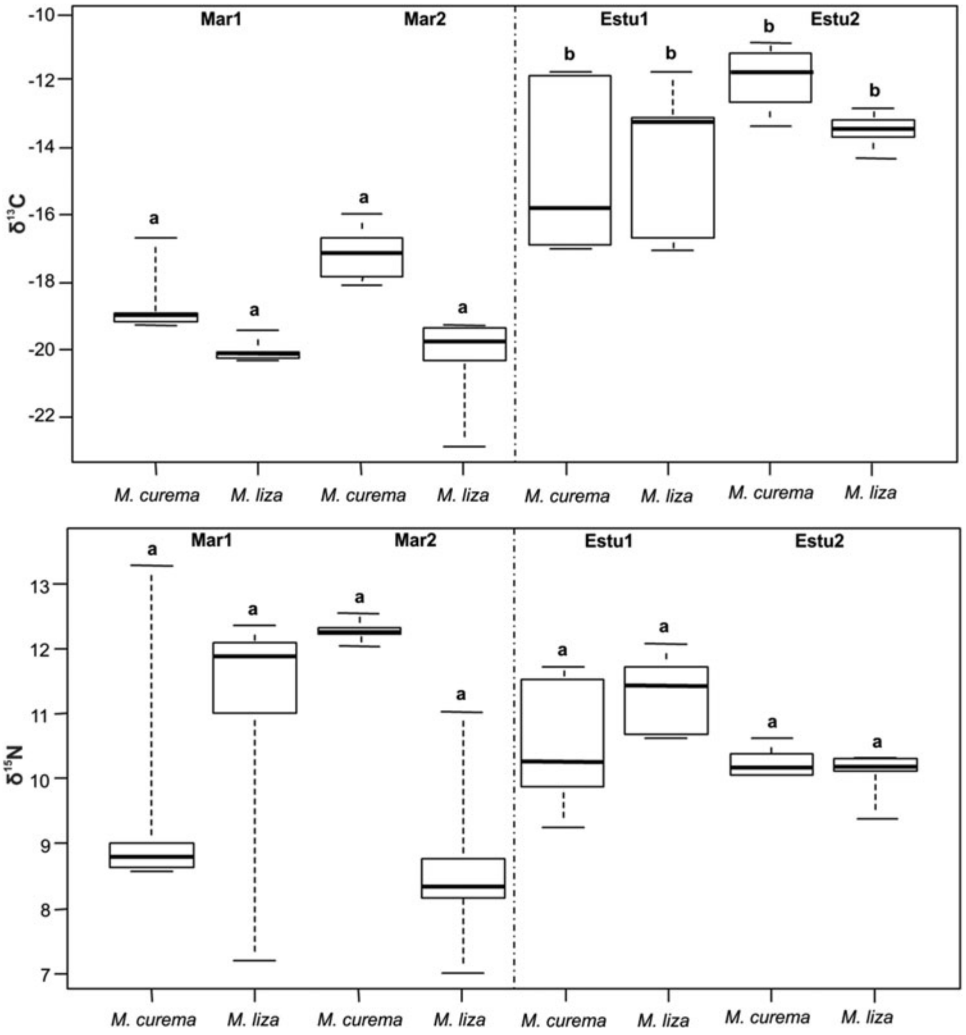

Fig. 3. Comparison between carbon ($\delta^{13}C$) (upper panel) and nitrogen ($\delta^{15}N$) (lower panel) isotope values of the mullets *M. curema* and *M. liza* in the marine surf-zone (Mar1, Mar2) and in the estuary (Estu1, Estu2). Different letters (a, b) denote statistically significant differences (Tukey post-hoc test, $P > 0.05$) between average values.

early developmental stages compared with later ones regardless of the resource diversity available.

Analysis of isotopic niche overlap of mullet species in the marine (0.07%) and estuarine (64.7%) habitats support our initial hypothesis of higher resource partitioning in the habitat with lower resource diversity. Interestingly, a preliminary study carried out in another subtropical system (Tramandaí-Armazém estuary) comparing the same species, found an opposite pattern, with isotopic niche overlap in the marine habitat, and resource partitioning in the estuary (Garcia *et al.*, 2018). This discrepancy could be related to differences between estuaries in food composition and availability and time of arrival of the juvenile mullets in the marine habitat. For example, individuals recently arrived in the marine habitat had less time to achieve isotopic equilibrium with local food sources in the current work compared with Garcia *et al.* (2018), which may have influenced the isotopic niche overlap. In future studies, it would be interesting to perform isotopic analysis of tissues with a faster turnover rate than muscle tissue (e.g. blood, plasma, liver), because they reflect what was consumed in a shorter period of time (Thomas & Crowther, 2015; Barton *et al.*, 2019). This would increase the likelihood of juvenile mullets reflecting food items eaten in the habitat in which they were captured. However, the comparison with this prior work is limited because they did not analyse isotopic composition of food sources. In contrast, the differentiation of the organic matter size fractions used in the present study revealed differences in food habits between *M. curema* and *M. liza*, which were not possible in other works that analysed the organic matter pool as a single larger (1.2–300 µm) fraction (Faye *et al.*, 2011; Le Loc'h *et al.*, 2015; Carassou *et al.*, 2017; Cicala *et al.*, 2019).

In the current study, the isotopic composition of juvenile mullets did not isotopically match the autochthonous organic matter pools in the marine surf-zone. Rather, Bayesian mixing models indicated that the juvenile mullets sampled in the marine surf-zone reflected assimilation of ocean-derived organic matter. This trophic connection between the marine surf-zone and the offshore ocean is plausible considering the life cycle of these mullet species. These marine estuarine-dependent species spawn in offshore ocean waters and their larvae and recruits migrate towards shallow coastal zones, such as marine beaches and estuaries (Vieira, 1991; Lemos *et al.*, 2014). Therefore, it is reasonable to suggest that the juvenile mullets found in the surf-zone with isotopic composition distinct from local autotrophic sources are oceanic-derived individuals who recently recruited into the surf-zone. As newly arrived individuals, they would not have enough time (∼2 months according to Oliveira *et al.*, 2017) to achieve isotopic equilibrium with local food sources, and still reflect isotopically their original oceanic habitat.

The high isotopic niche overlap found in the estuary for the juvenile mullets does not necessarily imply competition. High biomass and diversity of microalgae in the studied estuary suggest a lack of food limitation and, therefore, absence of intra- and

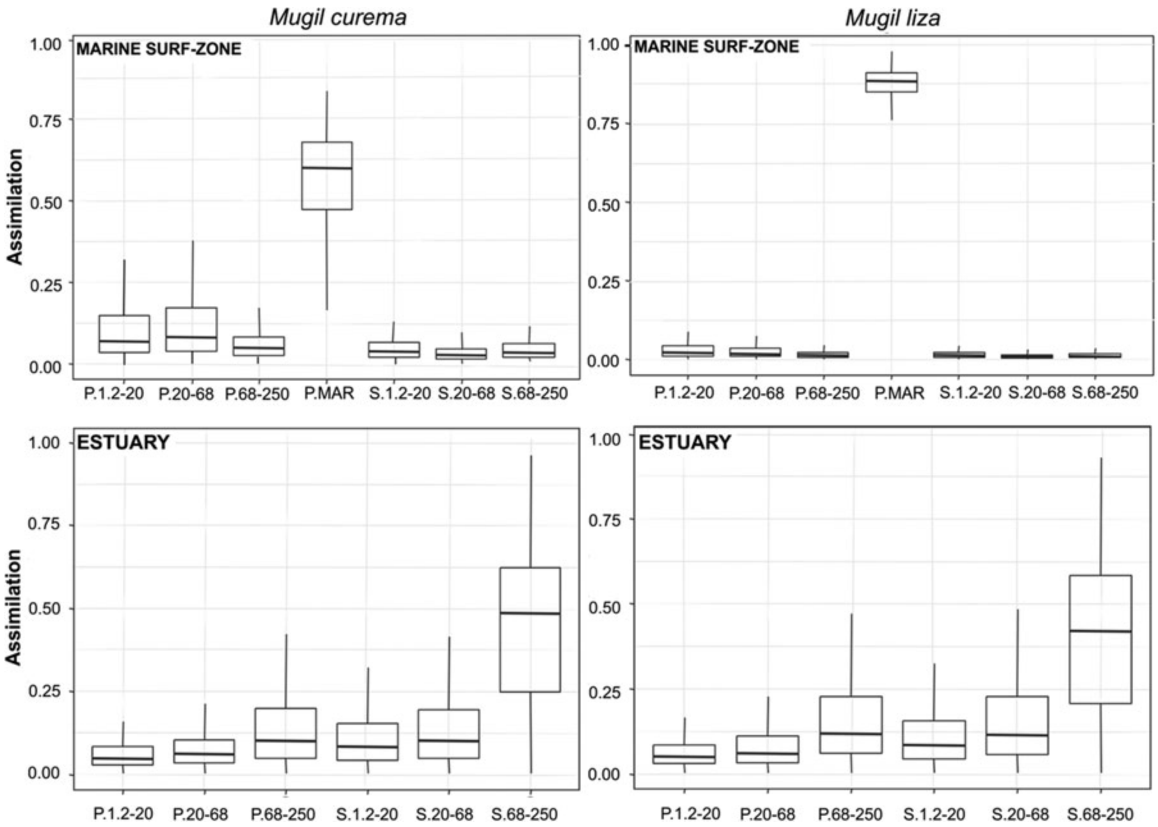

Fig. 4. Relative assimilation (Median: 50%; Credibility interval: 95%) of the different size fractions (1.2–20, 20–68, 68–250 µm) of particulate organic matter in suspension (POM: P) and in the sediment (SOM: S) by juvenile mullets *Mugil curema* and *M. liza* of the marine surf-zone (upper panel) and the estuary (lower panel). An additional food source (POM offshore) was included in the mixing models in the marine surf-zone (see Materials and methods for details).

Table 2. Niche width and niche overlap of *M. curema* and *M. liza* in Patos Lagoon estuary and adjacent marine surf-zone, South Brazil, estimated by both stable isotopes and stomach contents techniques

	Mugil curema		*Mugil liza*			
Habitat	SEAc (‰^{2})	Ba	SEAc (‰^{2})	Ba	INO (%)	CH
Marine surf-zone	4.11	0.03	7.62	0.06	0.7	0.86
Estuary	4.73	0.14	4.85	0.16	64.7	0.83

Isotopic niche width (SEAc: ‰^{2}), trophic niche breadth (Ba), isotopic niche overlap (INO) and trophic niche overlap (CH).

inter-specific competition for food resources in juvenile mullets. Other factors that minimize strength of competition and promote coexistence between these mullet species include high spatio-temporal variability of microalgae assemblages in this estuary (Haraguchi *et al.*, 2015; Mendes *et al.*, 2016) and trophic plasticity frequently observed in fishes, including mullets (Gerking, 1994; Cardona, 2015; Garcia *et al.*, 2018). Prior research suggested that these factors can suppress competition even in conditions of resource limitation (Cardona, 2001; Park *et al.*, 2016; Silva *et al.*, 2017). For instance, Garcia *et al.* (2018) attributed the trophic niche segregation between juveniles of *M. curema* and *M. liza* in the Tramandaí-Armazém estuary (southern Brazil) not to resource limitation and competition, but rather, to exploitation of microhabitats with different depths and/or particle size profiles harbouring distinct microalgae assemblages. Several studies of feeding apparatus anatomy in mullets have shown differences among species in the gap size between gill rakers that could lead to differential retention of particles (e.g. microalgae cells) (Guinea & Fernandez, 1992; Konan *et al.*, 2014; Cardona, 2015; Menezes *et al.*, 2015).

Diet composition and niche overlap inferred by SCA

Diet composition of juveniles of both mullet species was dominated by microalgae and showed higher diversity in the estuary than in the marine surf-zone, coinciding with the high diversity and spatiotemporal variability in the estuarine microalgae assemblage (Odebrecht *et al.*, 2010; Haraguchi *et al.*, 2015; Mendes *et al.*, 2016). According to the Morisita–Horn index, the diet of both species showed a high overlap in both habitats. However, the PERMANOVA model demonstrated that the diet of both species was different in the marine surf zone, but it was not in the estuary, agreeing with our initial hypothesis that partitioning may occur in less resource-diverse environments.

Diatoms are the predominant microalgae throughout the year in both the marine surf-zone and estuary (Haraguchi *et al.*, 2015; Mendes *et al.*, 2016) and distinct diatom groups dominated the stomach contents of both species. Although diatoms are ubiquitous in both habitats, the marine area is largely dominated by dense (10^9 cells l^{-1}) blooms of the surf-zone diatom *Asterionellopsis guyunusae*; it is a very difficult environment due to the waves'

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energy, and few species are adapted to these conditions (Odebrecht *et al*., 2010; Franco *et al*., 2016; Mendes *et al*., 2016). In contrast, microalgal diversity is much higher in the estuary; in addition to diatoms, cyanobacteria, green algae and cryptophytes are important components of planktonic and benthic habitats (Mendes *et al*., 2016). Estuarine microalgal assemblages are characterized by marked variation in species composition and abundance at both short (hours, weeks) and long (seasons, years) time scales (Haraguchi *et al*., 2015). That variation is largely attributed to environmental variability in salinity, temperature, nutrients, freshwater inflow and water transparency (Haraguchi *et al*., 2015; Mendes *et al*., 2016). This high variability in species composition and abundance of microalgal assemblages is likely the main driver of higher trophic niche width of *M. curema* and *M. liza* juveniles in the estuary (0.14 and 0.16, respectively) compared with the adjacent marine habitat (0.03 and 0.06, respectively).

Although the marine surf-zone is less diverse and dominated by one microalgae species (Odebrecht *et al*., 2010), the two mullet species did not prey mainly on the *Asterionellopsis guyunusae* microalgae; instead, *Mugil liza* fed mainly on Coscinodiscophyceae (fragments). *Asterionellopsis guyunusae* was also an important item in the *M. liza* diet, however, it was the second most consumed by this species, while for *M. curema* it was the dominant consumed algae. This difference in the predominance of each algae in the stomach contents can show a degree of the partitioning of the resources, in that each mullet feeds on a specific prey, avoiding trophic overlapping in this less resource-diverse habitat. However, the Morisita–Horn index demonstrated a high similarity in the diet of both species in the marine surf zone, this may have occurred due to the high sensitivity to more abundant species (Chao *et al*., 2006). Therefore, as seen above, the mullet species consume the microalgae *Asterionellopsis guyunusae* and Coscinodiscophyceae (fragments), and they represent about 80% of their diet, but each mullet has a preference for one of these food resources. And the Morisita–Horn index, in addition to being insensitive to rare species (Chao *et al*., 2006), also did not detect this preference for the abundant food items present in the diet of both mullets.

Concerning the diet of the mullets in the estuary, a factor that contributes to higher diet diversity and niche width in this environment is greater microhabitat heterogeneity in the estuary compared with the marine surf-zone habitat (Seeliger *et al*., 1997). Due to this heterogeneity of habitats, and the nutrients input provided by the continent, the abundance and diversity of the phytoplankton in the estuary is higher than the marine surf-zone. With a high offer of food resources, the consumers did not need to partition resources, and this may be what is happening with these mullets in the estuary, since their diets were very diverse, but did not differ.

Integrating SCA, SIA and other diet tracer approaches

The results from both SIA and SCA showed that in the less resource-diverse environment (marine zone) the trophic niche overlap was lower than in the most resource-diverse environment (estuary). The diet of both species was different in the marine zone, but was not in the estuary, agreeing with our initial hypothesis that partitioning may occur in a less resource-diverse environment compared with a more diverse one. This difference was possible to observe due to the SCA, as opposed to the SIA that did not show this pattern. Using SIA we were able to describe the shift in the mullets' diet when they changed habitats, but as this analysis doesn't have the refinement of identifying each species used as a resource, we could not distinguish the differences in the resources used. However, when we analysed the trophic niche overlap, the SIA based on isotopic ellipses was more informative that the trophic niche calculated by SCA. So both analyses showed the same pattern, although they showed a different time-lag of resources use.

The SCA is a higher resolution snapshot of what was recently (hours to days) consumed, while the SIA provides time-integrated (weeks to months; Oliveira *et al*., 2017) data reflecting dietary items assimilated in consumer tissues according to the isotopic turnover time of each particular tissue type and metabolic pathway (Hesslein *et al*., 1993; Mont'Alverne *et al*., 2016). The isotopic approach has an advantage over stomach content analysis in estimating the trophic niche overlap because it reflects assimilated food sources over a wider time window of feeding activity, which is particularly relevant considering the marked spatio-temporal variability in the availability of microalgae in the estuary (Haraguchi *et al*., 2015; Mendes *et al*., 2016). Stomach contents analysis is complementary in that it provides a detailed taxonomic description of food items consumed, which is not feasible using SIA due to the lack of substantial isotopic differences among sources and technical difficulties in obtaining samples of microalgae at the species level. Moreover, SIA requires an isotopic equilibrium between sources and consumers, which is not easy to address in migratory organisms, as the mullets are in the present work. Therefore, the use of both approaches together is recommended to better evaluate the intra-specific interactions and clarify the use of resources by the species.

Research on the trophic ecology of iliophagous species, such as mullets, comes with several technical challenges, which could be addressed in part by integrating multiple approaches such as analyses of compound-specific stable isotopes, fatty acid biomarkers and DNA tracers (Majdi *et al*., 2018). Given the costs of those approaches and their own assumptions and limitations, we suggest that combined SIA and SCA approaches, as in this study, are important to provide specific hypotheses for more narrowly targeted research using those approaches. Such further studies to advance our understanding of the trophic ecology of mullets are needed, especially considering their commercial value for fisheries, increasing anthropogenic pressures on their habitats, and their important (yet not fully understood) role in estuarine food web structure and dynamics (Reis & D'incao, 2000; Oliveira *et al*., 2014; Haimovici & Cardoso, 2017; Santana *et al*., 2017; Possamai *et al*., 2020).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315421000242

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