



# Dietary and habitat niche partitioning in congeneric cryptobenthic reef fish species

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**Abstract** Ecological niches hold critical information concerning the eco-evolutionary dynamics that govern biodiversity and abundance patterns. Cryptobenthic reef fishes account for approximately half of all reef fish species and are an abundant and important group on coral reefs worldwide. Yet, due to their small size and inconspicuous lifestyles, relatively little is known about the ecological niches of most cryptobenthic species. Here, we use gut content DNA metabarcoding to determine dietary niche overlap and prey richness in four sympatric species of cryptobenthic reef fishes in two genera (*Acanthemblemaria aspera*, *A. spinosa*, *Enneanectes altivelis*, and *E. matador*). Furthermore, we test whether dietary differentiation corresponds with differences in species distribution patterns across twelve sites on the Mesoamerican Barrier Reef in Belize. Our approach reveals dietary partitioning among

the four species, which is further supported by low edge density and high modularity in the resulting trophic network. *A. spinosa* and *E. matador* consume a significantly higher richness of prey items than their congeners. This result corresponds with non-random distributions and co-occurrence patterns in both species pairs: the two high prey richness species (*A. spinosa* and *E. matador*) co-occur more frequently than predicted by chance, but they are exclusive to exposed forereef sites with high wave action. In contrast, their congeners occur across exposed forereef and sheltered backreef sites, but they do not increase in numbers at sheltered sites. Our findings suggest that *A. spinosa* and *E. matador* monopolize a wide variety of prey in exposed habitats, but they are unable to meet the energetic demands of their adaptation to high-flow habitats in sheltered areas, possibly due to lower prey availability. This, in turn, indicates strong ecological differentiation among closely related species of cryptobenthic fishes, driven by links between diet, physiology, prey availability, and wave exposure.

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## Introduction

Species-specific adaptations integrate with biotic and abiotic factors to shape organismal parameters such as growth, survival, and reproductive success. These parameters ultimately govern a species' existence and resource use at a given location: the ecological niche (Hutchinson 1957; Chesson et al. 2001). Niches and their differences among taxa, in turn, determine the coexistence of sympatric

species and shape the contribution of each species to ecological processes (i.e., fluxes of energy and nutrients). Therefore, quantifying species' niches, for example by proxy of species occurrence across environmental gradients (i.e., the Grinnellian niche) or dietary preferences (i.e., the Eltonian niche), is a cornerstone of ecology across taxa and biomes (e.g., Herder and Freyhof 2006; Soberón 2007; Kartzinel et al. 2015).

With more than 6000 species, teleost fishes are a dominant group of consumers on coral reefs that are involved in a wide range of ecological processes (Brandl et al. 2019a). Thus, much work has been performed to determine the ecological niches of reef fishes. Reef fish distribution patterns, for example, have been examined at local, regional, and global scales, and there is considerable evidence for strong effects of environmental conditions on the presence and abundance of reef fish species at all scales (Robertson 1995; Dornelas et al. 2006; Eurich et al. 2018). Wave exposure, in particular, appears to have a strong bearing on species' existence and fitness at a given site (Gust et al. 2001; Bejarano et al. 2017; Taylor et al. 2018). Similarly, several studies of nominally herbivorous (Robertson and Gaines 1986; Brandl and Bellwood 2014; Eurich et al. 2019) and carnivorous coral reef fishes (Leray et al. 2015; Matley et al. 2016; Casey et al. 2019) have identified strong differences in resource use, including both marked trophic differentiation among functional groups and fine-scale partitioning within such groups (Choat et al. 2002; Clements et al. 2016; Leray et al. 2019). In contrast, several other studies on dietary overlap have yielded little segregation across taxa in similar functional groups (Pratchett 2005; Barnett et al. 2006; Bellwood et al. 2006; Frédérick et al. 2009). To gauge whether dietary niche differentiation is a general pattern among sympatric reef fish species in similar taxa or trophic groups, further work is required. Yet, one challenge for detecting dietary partitioning among small reef fish species or those that feed on microscopic prey lies in the taxonomic resolution of prey species that we can obtain from visual analyses (Longenecker 2007).

Cryptobenthic reef fishes are the smallest of all reef fishes, but they include a vast number of species that are often highly abundant on coral reefs and in some temperate regions (Brandl et al. 2018). Due to their unusual life-history strategy, which includes extremely short life cycles, fast generational turnover, and rapid growth (Depczynski and Bellwood 2006), cryptobenthic reef fishes play an important role in coral reef trophic dynamics by providing readily available and swiftly replenished fish tissue for consumption (Brandl et al. 2019b). Central to the functional role of cryptobenthic fishes is their feeding on a variety of microscopic prey items that are often inaccessible for larger species (Brandl et al. 2018, 2019b). Thus, knowledge concerning the dietary niches of cryptobenthic

fishes and how biotic and abiotic factors integrate with organismal adaptations to shape the distribution patterns of these small vertebrates is relevant for our understanding of the biodiversity of cryptobenthic fish species, their coexistence in highly diverse assemblages, and their involvement in secondary biomass production on coral reefs.

Distribution patterns of cryptobenthic reef fishes can be difficult to establish since their small size and cryptic lifestyle make visual detection unreliable (Ackerman and Bellwood 2000). Nevertheless, available evidence from tropical and temperate ecosystems suggests that species' distributions are not random. Indeed, habitat specificity can include dependence on single coral species (Munday et al. 1997) or precisely sized shelter holes (Wilson et al. 2013), depth stratification (Clarke 1992; Patzner 1999; Wilson 2001; Feary and Clements 2006; Wellenreuther et al. 2007) or specificity to certain substrates (Depczynski and Bellwood 2004; Feary and Clements 2006; La Mesa et al. 2006; Santin and Willis 2007; Harborne et al. 2012; Ahmadi et al. 2018), reef zones (Depczynski and Bellwood 2005), or larger geographical features (Syms 1995; Goatley et al. 2016; Coker et al. 2017).

Concurrently, several attempts have been made to quantify the diets of cryptobenthic fishes, but the reported levels of overlap among species have varied extensively. While most studies found separation among cryptobenthic fishes into distinct trophic guilds based on clearly identifiable, broad prey items such as detritus, algae, and copepods (Kotrschal and Thomson 1986; Muñoz and Ojeda 1997; Depczynski and Bellwood 2003; Hernaman et al. 2009), less evidence exists for fine-scale dietary differences among closely related species (Lindquist and Kotrschal 1987; Clarke 1999; Feary et al. 2009). While it is possible that there is little dietary diversification among closely related species, reliable visual identification of prey items from a few milligrams of partially digested, poorly known prey taxa such as micro-invertebrates is difficult and may mask fine-scale differences (Longenecker 2007).

Only few studies on cryptobenthic fish species have integrated both distribution and diet-based information to examine the potential interplay between species' niches and environmental gradients (Clarke 1992; Hilton et al. 2008). Yet, this integration offers an intriguing line of inference concerning eco-evolutionary dynamics that underpin the diversity and abundance of cryptobenthic fishes (Brandl et al. 2018). Herein, we examine dietary partitioning and prey richness of four sympatric cryptobenthic fish species in two genera using gut content DNA metabarcoding, and we link these results to local distribution patterns of the four species across twelve reefs in Belize.

## Materials and methods

The present study is centered on two pairs of congeneric, cryptobenthic reef fish species in the suborder Blennioidei. The focal species are two-tube blennies *Acanthemblemaria aspera* and *A. spinosa* (family Chaenopsidae), and two triplefins *Enneanectes altivelis* and *E. matador* (family Tripterygiidae). All four species co-occur on reefs of the Mesoamerican Barrier Reef and are considered part of a core group of cryptobenthic fish families (Brandl et al. 2018). Adult tube blennies are small, large-mouthed, and virtually stationary, as they occupy invertebrate tubes or tests for shelter from which they dart out of to feed on benthic or planktonic prey that is accessible from their perch (Kotrschal and Thomson 1986). Triplefins are equally small, but slightly more mobile fishes that employ a roaming foraging strategy that involves picking on a wide variety of benthic prey (Kotrschal and Thomson 1986; Longenecker and Langston 2005). We chose the two pairs to determine whether we are able to detect fine-scale differences in the niches of closely related, co-occurring species and whether the obtained results are consistent across two distinct families.

### Fish collections

All fishes in the present study were collected in March and April 2016 from reef outcrops on twelve distinct reefs in the vicinity of the Smithsonian field station on Carrie Bow Cay, Belize (cf. Brandl et al. 2017). Reefs around Carrie Bow Cay can be roughly divided into two habitats: exposed fore reefs and sheltered back reefs (Fig. 1; Rützler and Macintyre, 1982). To sample cryptobenthic fishes, we used enclosed clove-oil stations (Ackerman and Bellwood 2002; Brandl et al. 2017). Specifically, we covered a small, elevated area of reef (mean estimated surface area =  $5.856 \text{ m}^2 \pm 0.414 \text{ SE}$ ) with a fine mesh net and an impermeable tarpaulin and subsequently inundated the area under the tarpaulin with an anesthetic (1:5 clove oil/ethanol solution). Following application of the anesthetic, a team of two SCUBA divers removed the tarpaulin and collected all fishes using forceps. Upon completion of the collection (i.e., when more than five minutes were spent searching by both divers without collecting an additional fish), fishes were brought to the surface, euthanized with an overdose of anesthetic, and placed in an ice-water slurry (Brandl et al. 2017). All samples were photographed, measured, identified, and placed in 95% percent ethanol immediately upon return to the field station on Carrie Bow Cay. All sampling was performed under ethics approval SERC-IACUC-10-05-15 and collection permit 000005-16.

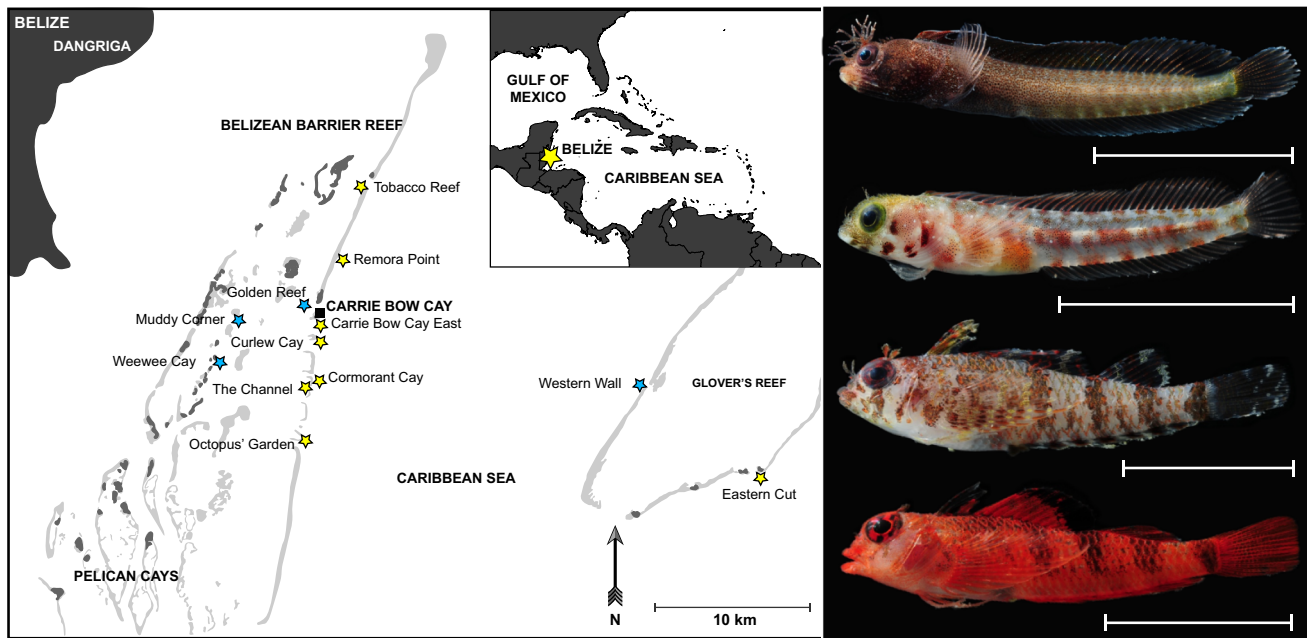
## Sample preparation and metabarcoding

To ensure that dietary patterns were not influenced by local prey availability, we performed gut content metabarcoding only on individuals from sites where all four species co-occurred (three sites: Cormorant Cay, Curlew Reef, and Remora Point; all exposed fore reefs). Across these sites, we randomly subsampled seven (*A. aspera*, *A. spinosa*, *E. altivelis*) and nine (*E. matador*) individuals, respectively, for gut content analyses. Samples were stored in 95% ethanol (10:1 ethanol to tissue ratio) for approximately one year, but flushed with fresh ethanol after the first 3 d of preservation. We performed all laboratory work on a clean, sterilized laboratory bench at the Laboratories of Analytical Biology (LAB) at the National History Natural History (NMNH) in Washington DC, USA. We dissected out the alimentary tract of each individual, removed the liver, gonads, and other organs, and then placed each entire alimentary tract (from the esophagus to the anus) into separate vials for DNA extraction. All dissecting tools were sterilized between individuals by rinsing and incubating them in a series of falcon tubes containing soap, 1:10 bleach and sterile water, and sterile water. We extracted DNA with a PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) and used a DNEasy PowerClean Cleanup Kit (Qiagen, Hilden, Germany) to clean DNA from PCR inhibitors before library preparation (Casey et al. 2019).

We targeted the 313 bp mitochondrial cytochrome c oxidase subunit I (COI) region with seven tailed primer pairs of m1COLintF and jgHCO2198 (Geller et al. 2013; Leray et al. 2013). Detailed descriptions of the PCR reactions, touchdown PCR protocol, verification of successful amplification, pooling of PCR product, quantification of PCR product, pooling of primer pairs, bead cleaning, library preparation, sample normalization, and sequencing protocol on an Illumina MiSeq are provided in Casey et al. (2019) and the Electronic Supplemental Material (ESM).

### Bioinformatic processing

We used BFC (Li 2015) to correct Illumina sequencing errors and recover short reads and USEARCH (Edgar 2010) to assemble pair-end reads and initial quality filtering. All further sequence processing (filtering, dereplicating sequences, sequence alignment, trimming ends, denoising, chimera removal, clustering, and the generation of an OTU table) was conducted with Mothur (Schloss et al. 2009). We removed all reads with ambiguous base calls, mismatches in primer sequences, homopolymer regions longer than 8 bp, and those shorter than 300 bp. After dereplication, sequences were aligned to a reference dataset curated from the Moorea BICODE barcode library (Meyer 2016). Finally, we trimmed sequence ends,



**Fig. 1** Map of the study area around the Smithsonian Field Station on Carrie Bow Cay, Belize, and the four focal species. Sample sites relevant for the present study are indicated with stars, with blue stars indicating sheltered backreef sites and yellow stars indicating exposed

forereef sites. The inset on the upper right shows the location of Belize in the Caribbean. The four species on the right are (from top to bottom): *Acanthemblemaria aspera*, *A. spinosa*, *Enneanectes altivelis*, and *E. matador*. Scale bars represent 10 mm

merged sequences within two nucleotides, and used VSEARCH to remove chimeras (Rognes et al. 2016). We clustered sequences into operational taxonomic units (OTUs) and assigned taxonomy with the basic local alignment search tool (BLASTn) through a local BIOCODE database (Meyer 2016) and GenBank (<https://www.ncbi.nlm.nih.gov/genbank>). We obtained proportional values of identification success and query coverage for all matches. To obtain broad taxonomic assignments of OTUs, we assembled a phylogenetic tree that included all BIOCODE sequences and OTUs from the present dataset. If OTUs without matches were nested within a broad taxonomic clade (e.g., Platyhelminthes, Polychaeta), we assigned the OTU to this clade. We labeled OTUs without matches (< 85% identity match) on NCBI or BIOCODE and for which no clear phylogenetic nestedness was evident as “Unidentified.”

### Data analyses and visualization

First, we removed all OTUs with a single occurrence across all individuals (i.e., singletons) from the data. Then, we removed “self-hits” from the dataset (i.e., all OTUs identified as *A. aspera*, *A. spinosa*, *E. altivelis*, and *E. matador*). While this precludes detection of cannibalism or trophic linkages among the four species, this step was necessary to exclude host tissue as well as safeguard our results from false inference due to the collection and preservation of the

four species in the same containers. Then, we calculated relative abundances of all OTUs based on the total number of sequences from each individual.

To illustrate patterns of prey use among the four species, we performed a non-metric multidimensional scaling ordination (nMDS) in two dimensions based on the Bray–Curtis dissimilarity of individuals. To test for similarity among species and sites from which individuals were sampled, we performed a permutational analysis of variance (PERMANOVA) on the same distance matrix, using *Species* and *Site* as the two predictor variables with 999 permutations. We tested homogeneity of group dispersion using the PERMDISP routine. We also calculated overlap among the four species using the Morisita-Horn index, which ranges between 0 (no overlap) to 1 (complete overlap).

Furthermore, we visualized associations between higher-level prey taxa and the four fish species in a bipartite network based on the relative abundances of prey OTUs. We simplified the network by discarding all OTUs with < 0.5% relative abundance across all species (after deleting self-hits). We assigned all OTUs that were identified with at least a 95% identity match to the level of order (subject to sufficient taxonomic resolution). We assigned OTUs to the level of phylum when they had a 75% identity match and/or were nested within phyla on the phylogenetic tree. For the largest group of prey taxa (Crustacea, > 85% identity matches), we visualized links

between OTUs and the four fish species in a bipartite network tree. We calculated both edge density (i.e., the ratio of realized connections vs. potential connections; a measure of randomness of linkages) and modularity (i.e., the grouping structure) of the resulting network. The former can take on values between 0 and 1, with low values indicating sparse, highly selective linkages and high values indicating near complete linkages among all nodes. Modularity values range between  $-1$  and  $1$  and take on positive values if more edges occur within the network's identified groups of nodes than expected at random.

We also compared the richness of prey (i.e., OTUs) ingested by the four species using sample-based rarefaction curves, extrapolated to 15 individuals for each species (Hsieh et al. 2016) using Chao's diversity estimator (Chao et al. 2014). We divided species into "low-diversity feeders" and "high-diversity feeders" based on these results. Finally, using the data on all four species' abundances across different sample sites, we examined co-occurrence patterns among the four species using a probabilistic model of species co-occurrence with a combinatorics approach (Griffith et al. 2016) to establish whether any of the species pairs co-occur more or less frequently than expected by chance. We then compared the abundance (response variable) of high- and low-diversity feeders (*PreyDiversity*) across sites with varying exposure regimes (sheltered vs. exposed; *Exposure*) using a generalized linear model (GLM) with a negative binomial error distribution and a log-link function, with an interaction term between the predictor variables *PreyDiversity* and *Exposure*. We visually assessed the model assumptions and calculated the pseudo- $R^2$  of the model.

All analyses and visualizations were performed in R (R Core Team 2018), using the *tidyverse* and the packages *vegan*, *colorRamps*, *bipartite*, *GGally*, *cooccur*, *iNEXT*, *MASS*, and *igraph*. All data and analyses used for the present paper are provided in the Supporting Information.

## Results

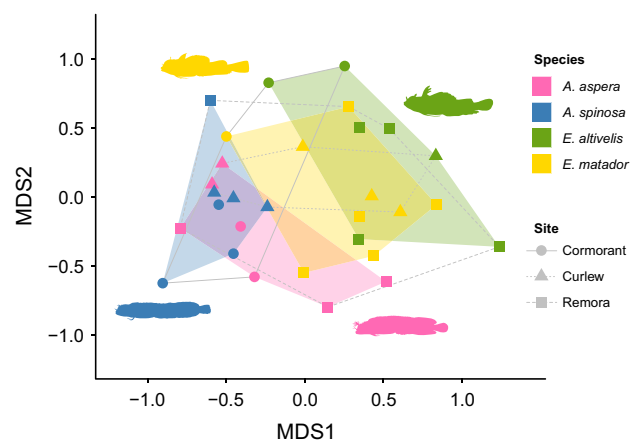
The prevalence of self-hits varied substantially among the four species, with the two triplefins having lower percentages (*E. matador* = 55.0% and *E. altivelis* = 58.7%) than the two-tube blennies (*A. aspera* = 80.8% and *A. spinosa* = 89.7%). This is likely a consequence of foraging intensity, with triplefins showing more active foraging and fuller stomachs compared to tube blennies (Kotrschal & Thompson 1986). Forty-three percent of specimens contained sequences from one of the four study species other than itself (most likely due to preservation in the same container), but these hits were restricted to a single sequence in  $> 80\%$  of the cases and accounted for an

average of only 1.4% of sequences across individuals (excluding all species-specific self-hits). After removing all self-hits from the dataset, gut content analyses resulted in 367 OTUs from 30 individuals. We were able to assign 341 OTUs to at least the phylum level (35 based on their position within clades of the phylogenetic tree). Twenty-six OTUs (7%) had no matches in either database and did not cluster unambiguously into a specific phylum on the tree.

Convex hulls calculated for each species in the nMDS ordination (stress = 0.226) showed clustering of the four species, but no obvious separation among the three sites (Fig. 2). Morisita-Horn indices showed limited overlap among congeners in both pairs (Table 1) as well as across genera. The mean overlap across species was  $0.197 \pm 0.031$  (SE).

The respective distinctness of species and site groupings was supported by the PERMANOVA, which showed higher explanatory power for species as a grouping variable (PERMANOVA:  $df = 3$ ,  $F = 1.507$ ,  $R^2 = 0.146$ ,  $P = 0.0003$ ) than for site ( $df = 2$ ,  $F = 1.269$ ,  $R^2 = 0.082$ ,  $P = 0.039$ ). All species and sites showed homogeneous dispersion in ordination space based on their centroids (PERMDISP<sub>species</sub>:  $df = 3$ ,  $F = 2.375$ ,  $P = 0.093$ ; PERMDISP<sub>site</sub>:  $df = 2$ ,  $F = 2.002$ ,  $P = 0.155$ ).

The bipartite network of all prey items showed distinct relative prey contributions across the four species at taxonomic resolution ranging from phyla to orders (Fig. 3). The two-tube blennies ingested predominantly arthropods. While *A. aspera* fed to a greater extent on taxa that were unidentifiable beyond the phylum Arthropoda, *A. spinosa* contained mainly calanoid, cyclopoid, and harpacticoid copepods. *A. aspera* contained more annelid and molluscan sequences than *A. spinosa*, while *A. spinosa* contained



**Fig. 2** Non-metric multidimensional scaling ordination (nMDS) of prey items used by the four fish species, based on Bray–Curtis dissimilarity. Convex hull polygons delineate the four species (symbol and polygon colors) and the three sites (line type and symbol shapes in gray)

**Table 1** Morisita-Horn index values of overlap among the four species and their lower (LCI) and upper (UCI) 95% confidence intervals

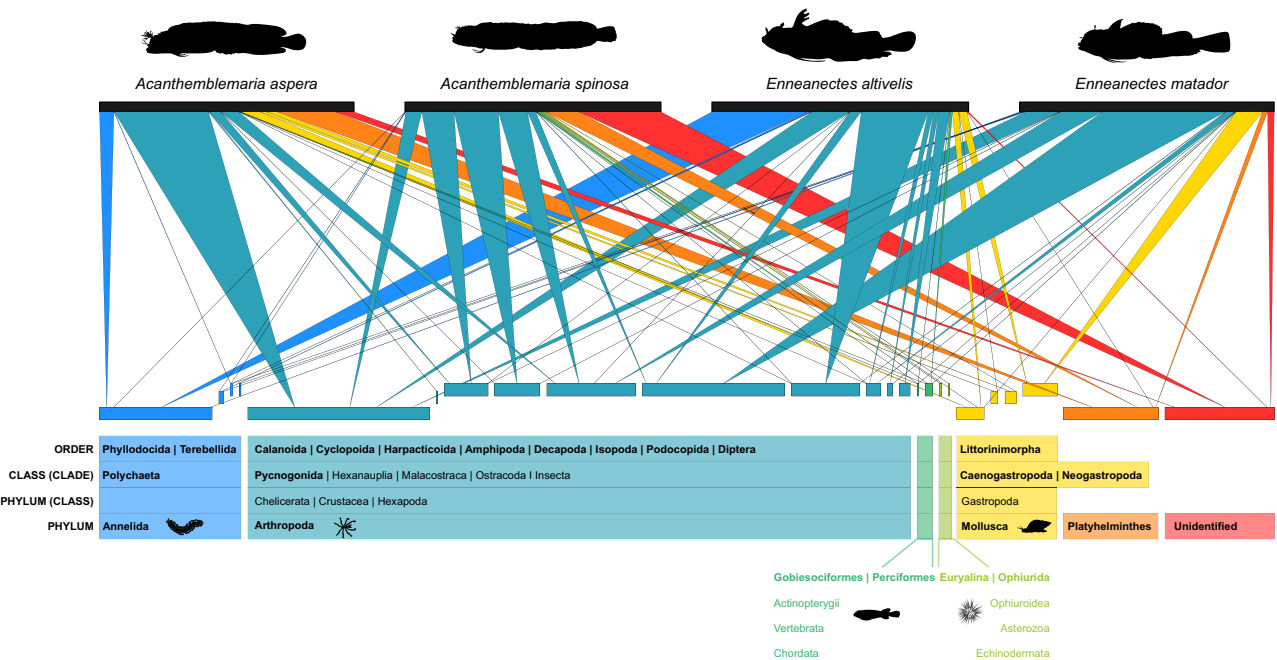
Species 1	Species 2	Overlap	LCI	UCI
<i>A. aspera</i>	<i>A. spinosa</i>	0.331	0.287	0.394
<i>A. aspera</i>	<i>E. altivelis</i>	0.134	0.072	0.216
<i>A. aspera</i>	<i>E. matador</i>	0.219	0.170	0.229
<i>A. spinosa</i>	<i>E. altivelis</i>	0.114	0.077	0.198
<i>A. spinosa</i>	<i>E. matador</i>	0.168	0.115	0.202
<i>E. altivelis</i>	<i>E. matador</i>	0.221	0.209	0.264

more teleost DNA (Gobiesociformes and Perciformes). Both *Acanthemblemaria* species also contained substantial numbers of platyhelminth sequences, which were unidentifiable beyond phylum. The two triplefins ingested mainly arthropods, but they differed in their dominant prey orders (amphipods for *E. matador*, decapods for *E. altivelis*). *E. altivelis* contained the highest proportional contribution of annelids, while *E. matador* contained the highest proportion of littorinimorph gastropods.

The bipartite network tree of crustacean prey OTUs and their links with the four host species also showed clear partitioning of prey taxa at a higher taxonomic resolution (Fig. 4). Edge density in the network was very low (0.007),

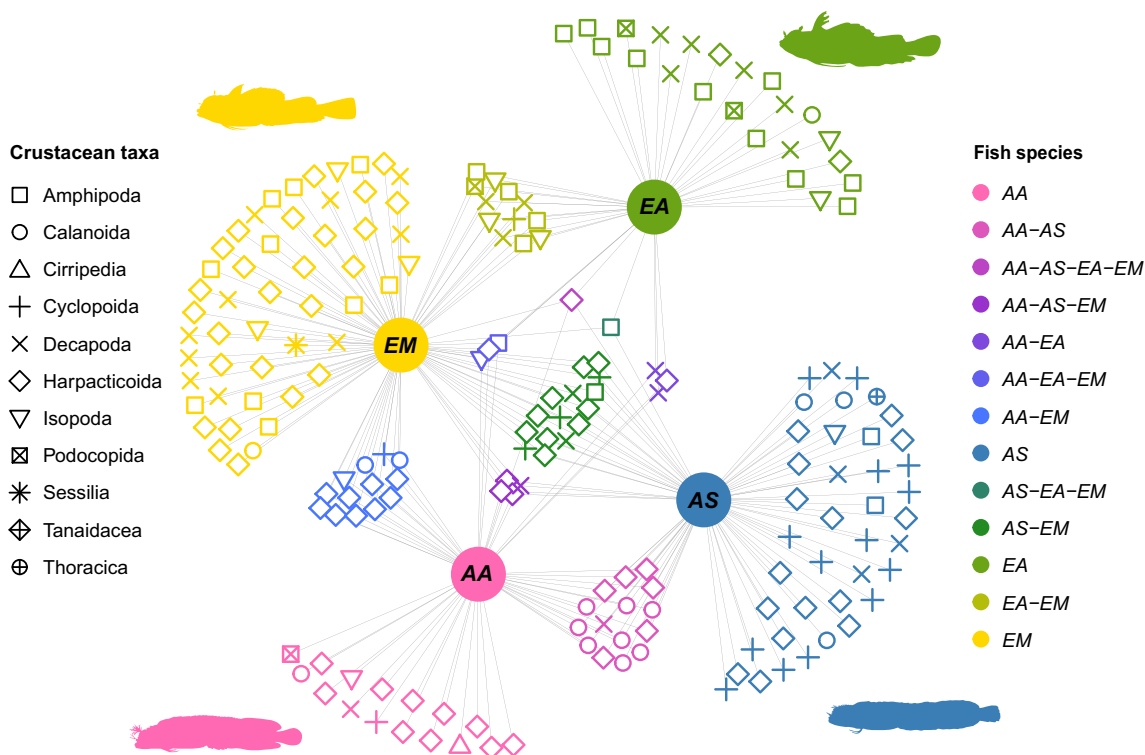
suggesting sparse, selective linkages among the nodes of the network. Thus, edges (i.e., links between focal fish species and their prey) appear to be selective rather than distributed at random. Network clustering revealed four distinct groups, which corresponded to the four host species’ nodes, and modularity for these groups was high (0.463). Only one OTU (a harpacticoid copepod) was shared among all four species. There were no pairwise linkages between *A. spinosa* and *E. altivelis* and no three-way linkages between *A. aspera*, *A. spinosa*, and *E. altivelis*.

In terms of prey richness, *E. matador* had the highest number of prey OTUs across individuals (161 OTUs), followed by *A. spinosa* (134), *A. aspera* (112), and *E. altivelis* (106), which was supported by the rarefaction curves (Fig. 5). However, sample-based rarefaction showed that none of the species were sampled exhaustively enough to obtain complete coverage of prey items. Nevertheless, both empirical values and extrapolated prey richness obtained from the rarefaction analysis with a sampling depth of up to 15 individuals per species also showed *E. matador* with the highest prey richness (248 OTUs; LCI = 217; upper 95% confidence interval UCI = 280), followed by *A. spinosa* (213; LCI = 184; UCI = 244). *A. aspera* exhibited lower prey richness (184; LCI = 159; UCI = 210) but was similar to *E. altivelis* (176;



**Fig. 3** Bipartite network plot of the four host species and their proportional use of prey items. Thickness of the downward facing arrows represents the relative abundance of each prey taxon in the species’ gut contents. The height of the prey rectangles and labels reflects the level of taxonomic resolution, from phylum (lowest) to

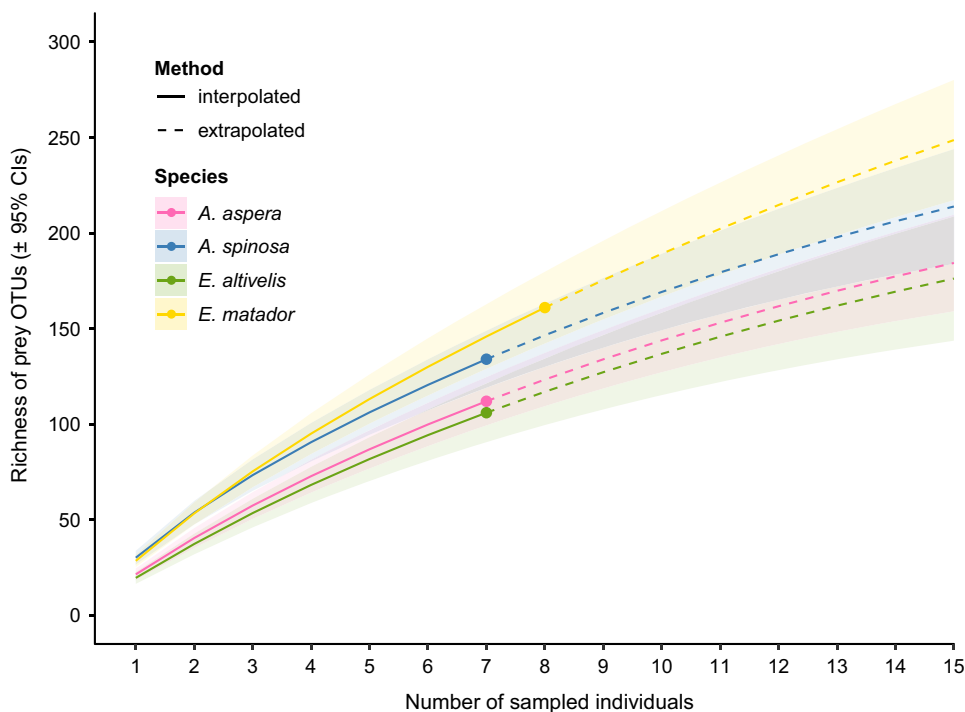
subphylum, class, and order (highest). Bold letters mark taxa that have unique rectangles in the network. All labels and rectangles are ordered from left to right. Colors reflect prey phyla. Taxonomic levels are provided on the left, with levels in parenthesis applying to molluscan prey items



**Fig. 4** Bipartite network tree of the linkages between the four fish species and their crustacean prey items based on presence/absence. Colors represent the four fish species and their various combinatory linkages, while symbol shapes correspond to crustacean taxa. Each

species ingested a large portion of unique taxa (peripheral symbols clouds), while smaller numbers of OTUs were shared among different combinations of the four fishes

**Fig. 5** Sample-based rarefaction curves of prey richness in the four species of cryptobenthic fishes, denoted by the four colors. Circles mark the empirical sampling depth, while line types indicate whether estimates are interpolated (solid) or extrapolated (dashed). Ribbons mark 95% confidence intervals (CIs). Based on extrapolations to 15 individuals and their 95% CIs, *E. matador* had the highest richness of OTUs, followed by *A. spinosa*, which had higher prey richness than *A. aspera* and *E. altivelis*



LCI = 143; UCI = 209), which had the lowest richness of prey items.

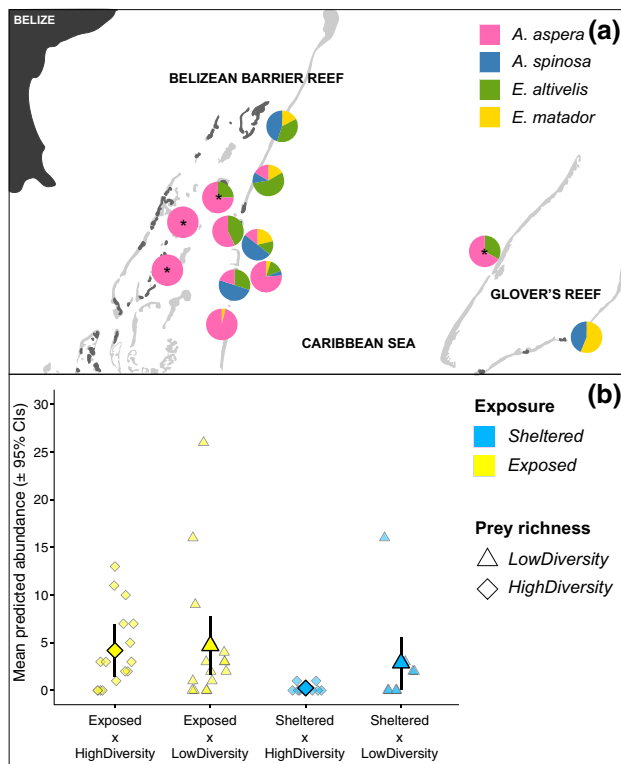
Finally, the distribution patterns of the four species among the twelve sampled reefs (Fig. 6a) showed that *A. aspera* was the most consistently and abundantly present species (ten out of twelve sites), followed by *E. altivelis* (eight sites). *A. spinosa* and *E. matador* occurred only at six sites each, but they co-occurred at all but one of the sites. The probabilistic model revealed that this level of co-occurrence was higher than expected by chance (Table 2), while all other pairwise co-occurrences were not different from random. In addition, the GLM showed a significant effect of the interaction term (*Sheltered* × *LowDiversity*:  $\beta = 2.330$ ,  $SE = 1.076$ ,  $z = 2.165$ ,  $P = 0.030$ ) and a significant negative effect of *Sheltered* sites ( $\beta = -2.818$ ,  $SE = 0.899$ ,  $z = -3.136$ ,  $P = 0.002$ ) on fish abundance (Fig. 6b) (pseudo- $R^2 = 0.320$ ). The size structure of each species' population provided no indication for recruitment

**Table 2** Observed and expected probabilities of co-occurrence between species pairs based on a probabilistic model

Species 1	Species 2	$C_{obs}$	$C_{exp}$	$P_{co}$	$P_{low}$	$P_{high}$
<i>A. aspera</i>	<i>A. spinosa</i>	4	5.0	0.417	0.227	1
<i>A. aspera</i>	<i>E. altivelis</i>	7	6.7	0.556	0.909	0.576
<i>A. aspera</i>	<i>E. matador</i>	4	5.0	0.417	0.227	1
<i>A. spinosa</i>	<i>E. altivelis</i>	5	4.0	0.333	0.970	0.272
<b><i>A. spinosa</i></b>	<b><i>E. matador</i></b>	<b>5</b>	<b>3.0</b>	<b>0.25</b>	<b>0.999</b>	<b>0.040</b>
<i>E. altivelis</i>	<i>E. matador</i>	4	4.0	0.333	0.727	0.727

Species 1 and Species 2 denote the focal pair

$C_{obs}$ , observed co-occurrence,  $C_{exp}$ , expected co-occurrence,  $P_{co}$ , probability of co-occurrence,  $P_{low}$ , probability that a lower frequency than observed is obtained by chance,  $P_{high}$ , probability that a higher frequency than observed is obtained by chance. Values indicate that only one pair (*A. spinosa* and *E. matador*, bold letters) co-occurs at a frequency not likely to be obtained at random (i.e.,  $P_{low}$  and  $P_{high}$  in the 95th percentile)



**Fig. 6** **a** Relative abundance of species across the twelve sampled reefs. Colors in pie charts reflect the different species. *A. spinosa* and *E. matador* co-occurred more often than expected by chance alone, while all other co-occurrence patterns did not differ from random. Asterisks indicate sheltered sites. **b** Mean predicted abundances ( $\pm$  95% CIs) of the four fish species, divided by the interaction of exposure regime (*Exposed* vs. *Sheltered*) and the richness of ingested prey items (*HighDiversity* vs. *LowDiversity*, based on rarefaction curves). Transparent, superimposed points show the raw data, while caterpillar plots indicate the model fit ( $\pm$  95% CIs) obtained from a GLM with a negative binomial error structure

pulses or bottlenecks that could drive the obtained distribution patterns (ESM Fig. 1).

## Discussion

Dietary and distributional differences in congeneric cryptobenthic fish species may hold valuable information concerning selective pressures and adaptive responses in these short-lived, abundant vertebrates. This, in turn, can increase our understanding of the factors that govern diversification and species coexistence in highly diverse assemblages. Here, we use a molecular approach to show dietary partitioning among four blennioid cryptobenthic reef fish species in two genera (*Acanthemblemaria aspera*, *A. spinosa*, *Enneanectes altivelis*, and *E. matador*) at both coarse and fine-scale taxonomic resolution of prey taxa (i.e., across different phyla and within the most heavily consumed prey subphylum Crustacea). Furthermore, we find that the four species differ in the diversity of prey ingested. Notably, the two species with higher prey species richness than their congeners show positive, non-random co-occurrence at exposed forereefs while being completely absent from back reef sites. Reconciling these findings with existing literature suggests that adaptive differences in resource use and metabolic demands interact with site-specific conditions pertaining to wave action and prey availability to shape the distribution of the four species.

### Prey use and dietary partitioning

Overlap in resource use can determine the likelihood of competitive interactions among species. As one of the most



intuitive niche axes, investigations of species' diets are particularly frequent, and new methods, such as gut content DNA metabarcoding, compound-specific isotope analysis, or biochemical diet analyses, are steadily improving the resolution with which we can examine species' prey ingestion (Leray et al. 2013, 2019; Kartzinel et al. 2015; Bradley et al. 2016; Clements et al. 2016; Casey et al. 2019).

All four species contained large amounts of arthropod prey, but there were several differences both within and across the two genera. Triplefins fed on a wide range of benthic and sessile prey taxa (e.g., annelids, molluscs; Kotrschal and Thomson 1986; Longenecker and Langston 2005; Brandl et al. 2018), and differed from tube blennies in the dominant arthropod taxa: while *A. aspera* and *A. spinosa* predominantly consumed unidentified arthropods and copepod taxa, respectively, *E. altivelis* and *E. matador* mainly ingested decapods and amphipods, respectively. Furthermore, *E. altivelis* consumed more annelid prey than its congener. The prevalence of copepod prey in *A. spinosa* is in accordance with previous examinations (Kotrschal and Thomson 1986; Clarke 1999), but *A. aspera* also ingested a large proportion of annelids. The distinction between the two-tube blenny species mirrors potential reliance on prey from pelagic (copepods) and benthic (annelids) origins (Clarke 1999).

Notably, both *A. aspera* and *A. spinosa* ingested large amounts of platyhelminthes (flatworms), and their guts contained a substantial number of sequences that could not be confidently assigned to any phylum, even in one of the world's best studied marine bioregions. Platyhelminthes accounted for 22.5% of all sequences (including 22 OTUs) in *A. aspera* and 12.4% (including 11 OTUs) in *A. spinosa*'s guts. Similarly, unidentified OTUs accounted for 7.2% of sequences (across 8 OTUs) in *A. aspera* and 32.1% (across 24 OTUs) in *A. spinosa*'s guts. Poorly known taxa such as flatworms are highly diverse but dramatically understudied (Rawlinson 2008), and they have not been identified as important prey taxa in previous investigations of Caribbean blennioids (Lindquist and Kotrschal 1987; Clarke 1992, 1999).

We are unable to exclude the possibility that some platyhelminthes are parasites, living within the intestinal system, which highlights the limitations of gut content analysis, especially in small-bodied organisms. Alongside the considerably large relative abundances of unidentified OTUs, this issue also highlights the limitations of metabarcoding from a naturalistic perspective. We still lack basic taxonomic information for a wide range of coral reef organisms that may play important roles in fishes' diets and energy fluxes on coral reefs (Fisher et al. 2015; Korzhavina et al. 2019), and well-curated DNA barcode inventories are not available for most regions. Clearly,

there is still an urgent need for basic naturalism and taxonomy of poorly known organisms such as micro-invertebrates (Rocha et al. 2014).

Yet, despite the lack of naturalistic information on prey taxa and the relatively low sample size in the present study, our results highlight advantages of the level of taxonomic detail that metabarcoding can provide for understanding eco-evolutionary dynamics in small-bodied species. Previous examinations of the diets of small consumers like cryptobenthic fishes have been largely limited to broad taxonomic categories for prey items, such as the crustacean taxa Amphipoda, Decapoda, and Copepoda (e.g., Castellanos-Galindo and Giraldo 2008; Depczynski and Bellwood 2003; Hernaman et al. 2009), with few more detailed prey assignments (e.g., (Clarke 1999; Feary et al. 2009; Kotrschal and Thomson 1986; Longenecker 2007). In these studies, dietary overlaps among species (scored between 0 and 1) occupy a broad range of values, but are often reported as higher than 0.6, a common threshold for "dietary similarity" (Castellanos-Galindo and Giraldo 2008; Hernaman et al. 2009; Hundt et al. 2014). The average overlap of 0.197 among the four blennioid species in our study is considerably lower. There are three non-mutually exclusive explanations for this difference: (1) previous studies examined fish species with broader diets and, thus, higher overlap, (2) the low sample sizes in the present study decreased overlap among species, or (3) the higher resolution of identifying prey taxa with DNA metabarcoding can unmask niche differentiation among species that are not detected using visual methods (cf. Kartzinel et al. 2015), especially due to the small size of cryptobenthic fishes.

Although higher-order taxonomic assignments of prey items may be achievable (and comparable) between visual and genetic examinations of fish gut contents even for cryptobenthic fishes (e.g., dominance of copepods in *Acanthemblemaria spinosa*'s diet, importance of amphipods in cryptobenthic fish diets; Clarke 1999; Feary et al. 2009; Kotrschal and Thomson, 1986), there is value in obtaining precise taxonomic identities for prey items that are morphologically difficult to identify or not yet taxonomically described (e.g., copepods; Korzhavina et al. 2019). In this context, our results may support the notion that niche overlap among sympatric species can decrease as the resolution concerning a species' ecology is increased (Loreau 2004; Longenecker 2007). Yet, the significantly higher costs associated with a molecular approach may inhibit large sample sizes and subsequently mask important intraspecific variation. In addition, using multiple approaches to quantify species' dietary niches, such as isotope analysis or supplementary visual assessment alongside gut content metabarcoding, is desirable (Nielsen et al. 2018). Furthermore, due to the small size of cryptobenthic fishes,

it is necessary to process their entire alimentary tract. As a result, large proportions of sequences are often self-hits, which warrants caution when interpreting relative sequence abundances of prey items (Casey et al. 2019; Deagle et al. 2019).

Nevertheless, the lack of a strong signal of site-specific resource availability (as indicated by the explanatory power of *Site* in the PERMANOVA, which suggests present but minor site-specific prey use), along with weak ontogenetic or seasonal differences reported in the literature (Castellanos-Galindo and Giraldo 2008; Hernaman et al. 2009; but see Andrades et al. 2019) suggest that the diet of cryptobenthic fishes is bound by eco-evolutionary constraints rather than being solely shaped by opportunistic foraging, omnivory, and prey versatility (Depczynski and Bellwood 2003; Bellwood et al. 2006). The sparse nature of the trophic network (indicated by the low value of edge density) and the high values of modularity (delineated by the four fish species) further support this hypothesis.

### Prey richness and non-random co-occurrence

Besides prey composition, the number of distinct resources used by different species can provide critical information on species niches, fitness, coexistence in a range of different environments, and their susceptibility to environmental change (Clavel et al. 2011). Our results show that *A. spinosa* and *E. matador* consume higher richness of prey items than their congeners, which contradicts previous reports that suggest strong phylogenetic conservatism in the degree of specialization/generalization (e.g., generalized feeding by triplefins compared to tube blennies Kotschal and Thomson 1986).

Notably, the two high-diversity feeding species co-occurred more frequently than predicted by chance and did not exist at sheltered back reef sites. The division into low- and high-diversity feeding species in the two genera and their non-random spatial distribution provide evidence for the interplay between diet, physiology, behavior, and fine-scale differences in environmental conditions (e.g., wave action) between exposed fore reefs and sheltered back reefs. *A. spinosa* (the tube blenny with high prey richness) outcompetes *A. aspera* in the occupation of exposed, high water flow microhabitats on Caribbean reefs due to a higher resting metabolic rate (Clarke 1992, 1999). In turn, *A. spinosa* is unable to exist in lower quality habitats and is vulnerable to population collapse after disturbance because of its purported reliance on calanoid and cyclopoid copepods, while *A. aspera* can persist on a diet of benthic prey items (Clarke 1996). Planktonic copepods may provide more efficient nutrition due to larger body size and thinner exoskeletons compared to benthic copepods (Clarke 1999). Our data corroborate prey partitioning between the two

species, with a higher proportion of planktonic copepod species in *A. spinosa* (Clarke 1992, 1999), but they also emphasize the importance of prey diversity through concordant patterns in the two *Enneanectes* species. Specifically, *A. spinosa* and *E. matador* both appear able to monopolize a diverse range of prey items in exposed microhabitats, leading to environmentally mediated, positive co-occurrence of the two species, possibly due to an adaptive physiological trait (i.e., a high metabolism) that confers competitive superiority in high-exposure environments (Clarke et al. 2009). Metabolic adaptations to high wave action have been documented in cryptobenthic fishes (Hickey and Clements 2003; Hilton et al. 2008), and these intuitively link with competitive abilities (i.e., higher fitness in exposed habitats) and the availability of a wide range of nutritious prey species to meet higher energetic demands (Clarke et al. 2009). Thus, dietary differentiation pertaining to both composition and richness of prey items may play an important part in the evolutionary history and coexistence of tripterygiid and chaenopsid lineages in the Caribbean. Morphological, behavioral, and physiological adaptations, which have been documented for the two *Acanthemblemaria* species (Clarke 1999; Clarke et al. 2009; Eytan et al. 2012), support this hypothesis and highlight the importance of considering multiple facets of organismal adaptations when disentangling the ecological niches of closely related species.

In summary, by using a high-resolution molecular technique, we demonstrate substantial prey partitioning and a separation between high and low-diversity feeding species in two congeneric species pairs of cryptobenthic reef fishes. Along with non-random spatial distribution patterns across a wave exposure gradient, our results emphasize the importance of organismal adaptation and species-specific niches in determining distribution patterns across environmental gradients. Synthesizing across multiple ecological dimensions (e.g., diet, morphology, physiology, behavior) and using complementary approaches to quantify species' niches promises to provide further insights into the exceptional biodiversity of cryptobenthic fishes and their role for coral reef ecosystems.

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**Authors' contributions** SJB conceived the study; SJB and JMC collected the data; JMC performed laboratory work and bioinformatic processing; SJB performed the statistical analysis; SJB wrote the first

draft of the manuscript; and JMC and CPM contributed by editing the manuscript.

**Data accessibility** All data and code necessary to reproduce the results of the present paper are available on Zenodo (10.5072/zenodo.472332).

#### Compliance with ethical standards

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

**Ethical approval** All work was performed under ethics approval SERC-IACUC-10-05-15 (SJB) and collection permit 000005-16 (SJB).

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