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REGULAR PAPER

Discerning the dietary habits of the smooth butterfly ray *Gymnura lessae* using two distinct methods, otolith identification and metagenetics

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Abstract

Two different methods, metagenetics and free-otolith identification, were used to identify prey in the stomach contents of 531 *Gymnura lessae* captured by trawling in Mobile Bay, Alabama 2016–2018. Both methods were found to produce analogous results and were therefore combined into a single complete dataset. All prey were teleosts; the families Sciaenidae and Engraulidae were the most important prey (prey specie index of relative importance 89.3% I_{RIps}). Multivariate analyses indicated that the diet of *G. lessae* varied with sex and seasonality. Specifically, variability was probably due to morphologically larger females consuming larger teleost prey species compared with males, whereas seasonal variability was probably due to changes in the available prey community composition. The findings indicate that both metagenetics and free otolith identification, used independently or complementarily, offer robust means of

characterising dietary habits for teleost-specialised species such as *G. lessae*, which may play an important role in the structure and maintenance of coastal food webs such as those in Mobile Bay.

KEYWORDS

Gymnura lessae, metagenetics, otoliths, prey identification, smooth butterfly ray, stomach contents

1 | INTRODUCTION

Understanding the diet of a species is vital for understanding trophic interactions and appropriately implementing ecosystem-based fisheries management (Bizzarro *et al*., 2017; Brown *et al*., 2012; Chipps and Garvey, 2007). Without dietary information, changes in predator–prey interactions and food web dynamics can go undetected, resulting in poor management decisions due to erroneous assumptions (Kemper *et al*., 2017). Despite the clear need for dietary data, studies describing these interactions are often lacking (Bizzarro *et al*., 2007; Grüss *et al*., 2018). The most common method used to interpret a species' diet is stomach-content analysis, a straightforward means for obtaining a snapshot of the prey an individual has recently consumed (Hyslop, 1980). However, in many cases, the separation of gut contents into

unambiguous prey categories is impossible (Baker *et al*., 2014), potentially limiting further quantification and ecological inference.

Additional techniques can complement traditional gut-content analyses. For example, the examination of free otoliths (loose otoliths not affixed to an intact prey item) is a useful tool for identifying otherwise unknown teleost prey, as prey free otoliths are often one of the last structures to leave the stomach (Jobling & Breiby, 1986). However, the use of free otoliths can bias results by over-representing teleost species with larger and slower-digesting otoliths and underrepresenting invertebrate prey (Granadeiro & Silva, 2000). As such, free otoliths are sometimes excluded from the dietary analyses of piscivorous elasmobranchs and teleosts (Albaina *et al*., 2012; Joyce *et al*., 2002). Unfortunately, it is difficult to quantify the degree to which free otoliths are used in the diet studies of piscivorous elasmobranchs and teleosts because their application (or lack thereof) is generally undescribed. Another increasingly common method used to identify unknown prey items is DNA analysis by sequencing species' delimiting genetic markers such as cytochrome oxidase subunit 1 (*coI*), commonly called DNA barcoding (Carreon-Martinez *et al*., 2011; Jakubavičiute *et al*., 2017; Leray *et al*., 2015; Pompanon *et al*., 2012; Smith *et al*., 2005). However, traditional DNA barcoding can be problematic when prey items are heavily degraded as the prey sample is often overwhelmed by the more abundant and less degraded predator DNA present in the stomach. Alternatively, a metagenetics approach using universal primers and massively parallel sequencing (hereafter referred to as metabarcoding) allows for the amplification and sequencing of DNA from multiple organisms in

a single sample in a cost-effective manner without the use of cloning libraries (Taberlet *et al*., 2012). Metabarcoding allows for the identification of a prey item even when the sample is overwhelmed by predator DNA and when the prey has been completely digested by the predator such that prey DNA remains in only minute amounts.

The smooth butterfly ray *Gymnura micrura* (Bloch and Schneider 1801) was recently identified as a species complex comprising *G. micrura* and two newly described species: *Gymnura sereti* Yokota and Carvalho 2017 and *Gymnura lessae* Yokota and Carvalho 2017. The species that is now described as *G. lessae* is a common coastal ray ranging from the northern Caribbean Sea to the northeast Atlantic Ocean (Yokota & Carvalho, 2017). Diet studies of other Gymnurids have found these species to be teleost-specialised feeders that feed intermittently on relatively large prey (Jacobsen *et al*., 2009; Yokota *et al*., 2013). Therefore, quantifying the diets of Gymnurids can be difficult because of high frequencies of empty stomachs and extended periods of digestion resulting in poor prey identification (Bizzarro, 2005; Jacobsen *et al*., 2009; Yokota *et al*., 2013). In addition, the only *G. micrura* diet study was conducted in north-eastern Brazil and thus does not portray the dietary habits of the North American *G. lessae* (Yokota *et al*., 2013). Given the lack of dietary information for *G. lessae*, a species with implications for fisheries management due to potentially being a high trophic level predator, the aim of this study was to examine the diet of the species in a northern Gulf of Mexico estuary using a combination of free-otolith identification and genetic techniques.

2 | MATERIALS AND METHODS

2.1 | Study Area

Mobile Bay is one of the largest estuaries in the United States (Kindinger *et al*., 1994; Figure 1). It is relatively shallow with an average depth of 3 m, with the exception of the shipping channel where the average depth is 12 m (Schroeder & Wiseman, 1988). The estuary receives the sixth greatest annual freshwater discharge in North America from the Mobile River system while simultaneously receiving saltwater inputs from the Gulf of Mexico (Park *et al*., 2007). These freshwater and saltwater inputs cause the salinity throughout the estuary to range from 0 to 35 throughout the year, which leads to extreme seasonal stratification (*i.e.*, hypoxic and anoxic events; Cowan *et al*., 1996; Schroeder & Wiseman, 1988).

2.2 | Sampling Methods

From February 2016 to May 2018, *G. lessae* were sampled opportunistically from non-targeted bottom trawls performed in and around Mobile Bay, Alabama. All trawls were conducted off the 19.8 m RV *Alabama Discovery* using a 7.6 m otter trawl between 08:00 and 1700 hours (n = 1–5 trawls per day). Tows were performed in 5 to 10 m of water at *c.* 4.6 km h^{-1} for *c.* 30 min. Water

temperature data were collected from the nearest Mobile Bay National Estuarine Program environmental monitoring station (www.mymobilebay.com; Figure 1).

Each individual was weighed (M_T) to the nearest gram and disc width (W_D) measured to the nearest mm). When possible, individuals were dissected onboard the vessel and stomachs were frozen at –29˚C for further analysis. Otherwise, each individual was frozen until it could be adequately assessed under laboratory conditions. Each individual was also assigned a maturity status according to Burgos-Vázquez *et al*. (2019).

2.3 | Laboratory Methods

All stomach contents were removed and examined using instruments that were cleaned with a 10% bleach solution for sterilisation. If the stomach showed signs of regurgitation (*e.g*., the stomach was partially retracted into the oesophagus), it was excluded from further analysis. All prey items were separated, identified to lowest possible taxon, counted, blotted dry and weighed to the nearest 0.01 g. All free otoliths in the stomach that were not associated with an intact prey item were counted and identified to the lowest possible taxon using an in-house reference set and an otolith key specific to fishes from the Gulf of Mexico (Baremore & Bethea, 2010). After free otoliths were separated by prey group, each count was divided by two and then rounded up to a whole number as a conservative estimate of the original number of prey that generated the otoliths.

Prey items that could not be visually identified to species, including free muscle tissue, were stored in 200% proof ethanol for future genetic analysis. To test the applicability of traditional barcoding, well digested (n =4) and partially digested (n = 1) teleost prey items were extracted and the *coI* locus was amplified using the *Vf-2*, *Fish-F2*, *Vr-1* and *Fish-R2* primers and methods of Ward *et al*. (2005). The resulting PCR products were Sanger sequenced bidirectionally on an ABI 3730 capillary sequencer (www.appliedbiosystems.com) at the Genomics Core Laboratory at Texas A&M University-Corpus Christi (TAMU-CC). Three out of the five sequences came back as *G. lessae*, one did not successfully amplify and the less digested prey item, a flatfish, came back a reasonable match.

As these traditional DNA barcoding methods failed to identify prey, a metagenetics approach was warranted. DNA extraction from muscle samples, PCR amplification and post-PCR processing and pooling were performed at the Genomics Core Laboratory at TAMU-CC. The marker used in this study is a 313 bp section of the *coI* locus sequenced *via* a paired end fashion on an Illumina MiSeq (www.illumina.com) at the New York University School of Medicine's Genome Technology Center. The specific protocols followed by the Genomics Core Laboratory for DNA extraction, PCR amplification and quality control, pooling and library preparation before Illumina sequencing and bioinformatics related to demultiplexing, read clustering and separation and identification into putative operational taxonomic units (OTU) can be found in the methods of Drymon *et al*. (2019). The universal metazoan primers *MlcoIint-F* (primer sequence: 5′- GGWACWGGWTGAACWGTWTAYCCYCC-3′; Leray *et al*., 2013) and

Jghc-02198 (5′- TAIACYTCIGGRTGICCRAARAAYCA-3′; Geller *et al*., 2013) were used in PCR amplification and a *G. lessae* annealing blocking primer (*SmbrblkcoI-f*; 5′-TACCCCCCATTAGCTGGT AACCTGG-C3-3′) was used to reduce the amplification of any predator DNA. Following bioinformatics processing, each prey sample was assigned a single, final OTU, which was assumed to represent the prey. To be assigned a final OTU, the sample was required to have at least ten reads matching the putative prey species and at least twice as many reads for that species as any other potential prey species in the sample. To be discriminated at the species level, the final OTU sequence was required to have a > 98% sequence match with that of a species in the reference libraries (Leray *et al*., 2013).

The goal of this study was to characterise the diet of *G. lessae* as thoroughly as possible; therefore, the free-otolith data and the metabarcoding data were combined into one single dataset for further analysis. Before this step, it was critical to ensure that the inclusion of free otoliths did not overrepresent prey with large otoliths and bias the results. To address this, before combining the results of both methods, two independent datasets were created and examined separately to investigate if incorporating the free-otolith identification would alter the dietary characterisation. Both datasets included the base visual prey identification results, but one included the results of the free-otolith analysis (the otolith data) and the other included the metabarcoding results (the metabarcoding data). If this bias was determined to be inconsequential, then the two methods would be used in tandem to create a single combined dataset.

2.4 | Data Analysis

All data analysis was performed in R 3.5.2 (www.r-project.org). Cumulative prey curves were created for species richness using the Mao tau estimate to determine if a sufficient number of stomachs had been sampled to adequately describe the diet of *G. lessae* (Colwell *et al*., 2012; Ferry & Cailliet, 1996). Prey curves were generated using prey identified to the lowest taxonomic category possible in the vegan community ecology package (Oksanen *et al*., 2019). Diet was considered to be adequately sampled once a prey curve approached an asymptote, defined by whether the slope of a linear regression (*b*), fit to the final five randomly sampled stomachs, was < 0.05 (Bizzarro *et al*., 2009).

Prey groups were quantified using single and compound indices, including average per cent number (%N_p), average per cent mass (%M_p), prey-specific number (%N_{ps}), prey-specific weight (%M_{ps}) and frequency of occurrence (%O) (Brown *et al*., 2012; Chipps & Garvey, 2007; Hyslop, 1980). The prey-specific index of relative importance (%I_{RIps}) was used to create an unbiased metric to determine the relative importance of each prey group in the diet of *G. lessae*, as well as to make comparisons to other studies (Brown *et al*., 2012). The formulas for %N_p, %M_p, N_{ps}, %M_{ps}, %O and % I_{RIps} are as follows, where %A_{ij} is the per cent abundance (by number or mass) of prey category i in stomach sample j, n_i is the number of stomachs containing prey i and n is the total number of stomachs containing prey (Brown *et al*., 2012).

Average percent abundance (%N_p and %M_p), $\%A_i = (\sum_{j=1}^{n} \%A_{ij})(n)^{-1}$; prey-specific abundance, (%N_{ps} and %M_{ps}): $\%A_{pi} = (\sum_{j=1}^{n} \%A_{ij})(n_i)^{-1}$; frequency of occurrence, $\%O_i = (n_i)(n)^{-1}$; prey-specific index of relative importance, % $I_{Rips} = (\%O_i \%N_{pi} + \%M_{pi})$. An index of vacuity (%$I_V$) was calculated by dividing the total number of stomachs without prey items by the total number of stomachs sampled (Hyslop, 1980).

The Bray-Curtis index was used to create a dissimilarity matrix for the dependent variables %N_p and %M_p, with each individual ray stomach treated as an individual sampling event and prey species treated as the response variables (Clarke *et al*., 2014). A permutational multivariate analysis of variance (PERMANOVA) was executed on the dissimilarity matrix to test whether the measured independent variables [sex, W_D, life-history stage (immature *v.* mature), season (meteorological spring, summer and fall), day length and water temperature (^oC)] showed significant explanatory value to the primary dietary variables. The variables sex, life-history stage and season were treated as factors and the variables disc width, day length and water temperature were treated as covariates. All variables were initially tested independently and then a final model was then created using forward, stepwise model selection to determine which combination of response variables best explained the variability in the data (Anderson & Burnham, 2002; Bizzarro *et al*., 2017). Permutation tests for heterogeneity of multivariate group dispersions were run to test all response variables, as PERMANOVA is known to be sensitive to sample dispersion (Anderson & Walsh, 2013). A PERMANOVA was also run on the results of the independent metabarcoding and otolith data pooled together, with sampling method treated

as an independent variable, to test if the two sampling methods produced significantly different results. All PERMANOVAs were permutated 9999 times using the vegan community ecology package (Oksanen *et al*., 2019). Differences were considered significant if *P*-values were < 0.05. A canonical correspondence analysis (CCA) was used to complement the final model of the PERMANOVA analysis and biplots were created to visualise the association of prey items and the response variables (Braak & Verdonschot, 1995). Results from CCA are strongly influenced by the inclusion of rare species; therefore, to help maximise the explanatory power of the model, individual prey categories were only included in the model if they occurred in at least five stomachs (Kemper *et al*., 2017). The significance of the overall models and single constraining axes and variables were tested using permutational tests.

3 | RESULTS

3.1 | Sample collection

Five hundred and thirty-one *G. lessae* were sampled for stomach-content analysis from February 2016 to May 2018. Of these, 316 were male (19–50 cm W_D) and 215 were female (19–89 cm W_D; Figure 2). The majority of *G. lessae*, 58.2% of males and 54.0% of females, were sexually mature. Of the *G. lessae* used in this study, 99.2% were captured within a 16 km wide sampling

region (Figure 1) and 100% were captured within 8 km of that sampling region's boundary, so differences in the available prey community between locations were presumed to be minimal.

3.2 | Stomach-content analysis

One hundred and seventy-eight prey items were analysed genetically, including 12 prey items that were previously morphologically identified to species (as a procedural control). Final OTUs were assigned to 96 of the 178 total prey items (53.9%), with all final OTUs representing teleost taxa. Final OTUs were assigned for 13 different teleost species; one additional prey item was only identified to genus because a reference for that species was not available. For those items that were not assigned final OTUs, 31 failed to amplify, 18 failed due to poor amplification of prey DNA and 29 did not meet the OTU assignment criteria. All prey items for which identities were previously known through morphologic identification and with DNA amplified, were correctly identified with metabarcoding. When analysed independently, the genus richness was 12 for both the otolith and metabarcoding data, but species richness was greater for the metabarcoding data (11 and 14, respectively; Table 1).

Prey in the families Sciaenidae and Engraulidae were the two most important prey families for both the otolith data and metabarcoding data, with $\%I_{\mathrm{RIps}}$ values of 92.2% and 86.9%, respectively, among identified prey. Prey in the family Sciaenidae, which have relatively large otoliths, had a greater $\%I_{\mathrm{RIps}}$ in the otolith data (60.9%) than in the metabarcoding data

(50.1%). Prey in the family Engraulidae, which have relatively small otoliths, had a slightly lower $\%I_{RIps}$ in the otolith data (31.3%) compared with the metabarcoding data (36.8%). Independent PERMANOVA analyses for both datasets included the variables season and sex in the final models, with the otolith dataset also including their interaction. A PERMANOVA analysis on the pooled metabarcoding–otolith data with each dataset treated as a factor found no difference between the datasets ($F = 0.784$, $R^2 = 0.004$, $P > 0.05$. Given these results, both datasets were combined into a single consolidated dataset that was then used to characterise the diet of *G. lessae*.

3.3 |Diet characterisation

Of the 531 stomachs examined, 204 stomachs contained prey items, resulting in $\%I_V = 61.6\%$. A total of 242 prey items were found and 169 (69.8%) of those prey items were identified to species. The maximum number of prey items in a single stomach was four but most stomachs (85.8%) contained only a single prey item. The use of a combined dataset increased the number of prey identified to species nearly tenfold when compared with a dataset that used neither free otoliths nor metabarcoding (*i.e.*, base visual stomach content analysis alone; Table 1). Cumulative prey curves indicated that the sample size of the study was sufficient to adequately describe the diet of *G. lessae* at the species level ($b = 0.036$), but not for males ($b = 0.077$) or females ($b = 0.067$) alone (Figure 3).

All prey were teleosts, with unidentified teleost prey having the greatest $\%I_{RIps}$ (31% ; Table 2). Eight prey families were identified, with the majority of prey coming from the families Sciaenidae and Clupeidae, with a combined $\%I_{RIps}$ = 89.3% when compared with other identified prey. Sixteen species were identified; Atlantic croaker *Micropogonias undulatus* (L. 1766) was the most important prey species, with a $\%I_{RIps}$ = 19.3% , followed by bay anchovy *Anchoa mitchilli* (Valenciennes 1848) and broad-striped anchovy *Anchoa hepsetus* (L. 1758) with $\%I_{RIps}$ values of 12.9% and 9.7% , respectively (Table 2).

3.4 | Dietary variation

The final models for the PERMANOVA analysis for $\%N_p$ and $\%M_p$ included the variables season and sex with no interaction. The final models for $\%N_p$ and $\%M_p$ explained 7.5% and 6.6% of the dietary variability, respectively (Table 3). None of the six variables (sex, maturity, W_D, temperature, season and day length) had heterogeneity of multivariate group dispersion.
The CCA models for both $\%N_p$ and $\%M_p$ using the variables from the final models of the PERMANOVA analysis (variables season and sex) were significant overall and for both axes (CCA1 and CCA2; Figure 4). The models explained 9.9% and 8.5% of the overall dietary variability for $\%N_p$ and $\%M_p$, respectively. The models for $\%N_p$ and $\%M_p$ were similar, both having the prey species spot croaker *Leiostomus xanthurus* Lacépède 1802 correlated with both females and fall. Both models also displayed correlations with spring and the species sand

weakfish *Cynoscion arenarius* Ginsburg 1930, with males and the species *A. mitchilli* and with summer and the species *A. hepsetus* and bay whiff *Citharichthys spilopterus* Günther 1862.

4 | DISCUSSION

This study documents that *G. lessae* in Mobile Bay are teleost-specialised predators, with most prey belonging to just two families (Engraulidae and Sciaenidae). This is consistent with trends described in long-tailed butterfly ray *Gymnura poecilura* (Shaw 1804) in Mumbai, India and spiny butterfly ray *Gymnura altavela* (L. 1758) in Brazil (Raje, 2003; Silva and Vianna, 2018). While studies investigating the diets of other Gymnurids often found non-teleost prey, these prey generally accounted for an insignificant portion of their diets (Bizzarro, 2005; Jacobsen *et al*., 2009; James, 1966; Rastgoo *et al*., 2018; Yemışken *et al*., 2018; Yokota *et al*., 2013). As seen with *G. micrura* and Australian butterfly ray *Gymnura australis* (Ramsay & Ogilby 1886), there was typically only one, often large, prey item oriented head first in each *G. lessae* stomach (Jacobsen *et al*., 2009; Yokota *et al*., 2013). This consumption of large prey, combined with the ambush feeding style observed in captive *G. lessae* and other Gymnurids, suggests that *G. lessae* probably bury themselves in the substrate to ambush passing prey and then strike and stun the prey using their pectoral fins, before consuming it whole (Henningsen, 1996; Schreiber, 1997; Smale *et al*., 2001).

Intermittent feeding on a small number of relatively large prey is common with ambush predators and is frequently seen in batoids that have the ability to stun their prey (Jacobsen & Bennett, 2013; Wetherbee *et al*., 2012). However, it should be noted that *G. lessae* frequently regurgitated prey items during capture, as was evident from the large, partially digested teleosts often found in the trawl net with *G. lessae*. Thus, it is possible that the proportion of empty stomachs seen in this and other Gymnurid diet studies is artificially high due to this stress response, making feeding rates difficult to quantify. However, this stress response could be less effective with large undigested prey as, due to their orientation in the stomach, fin spines would probably flare out, resulting in the fish getting lodged in the oesophagus.

Final models from the multivariate analyses contained the variables season and sex. The seasonal shift in diet was not surprising as the diets of batoids frequently vary seasonally due to changes in the available prey community (Platell *et al*., 1998; Szczepanski & Bengtson, 2014; White *et al*., 2004). In this instance, the seasonal shift appears to be driven by increased consumption of *A. hepsetus* in the summer, which parallels a significant increase in seasonal availability of *A. hepsetus* in Mobile Bay during that time (Sean Powers, unpubl. data). This increase in *A. hepsetus* consumption in the summer corresponds to a decrease in the consumption of other prey species. *Gymnura lessae* typically only consume a single prey item at a time; therefore, as long as feeding rates do not change, an increase in the consumption of one prey species will result in a decrease in the consumption of others. Consequently, *A. hepsetus* may act

as a temporary prey buffer for other prey species, such as *M. undulatus*, during the summer months (Saunders *et al*., 2006).

Sex-specific differences in diet seem to be driven by greater consumption of *L. xanthurus* by female *G. lessae*. One possible explanation for the observed dietary differences is that *L. xanthurus* are on average larger and deeper bodied than the four other most commonly consumed teleosts in our study area; thus, despite its relative abundance, *L. xanthurus* may be too large for most males to swallow whole. While the deeper body size of *L. xanthurus* may protect them from consumption by male *G. lessae*, this attribute might also make them a preferred prey of larger females, which are probably selecting for the largest, most calorific-rich teleost prey they can swallow. This hypothesis though will require further investigation and analyses.

Sex-specific differences in diet are sometimes seen in elasmobranchs, but these dissimilarities are frequently attributed to changes in habitat use due to sexual segregation (O'Shea *et al*., 2013; Springer, 1967), which does not appear to be the case here. While differences between male and female mouth width relative to W_D have not been reported, mouth widths of the largest *G. lessae* can be over three times wider than those of smaller individuals (Yokota and Carvalho, 2017). Interestingly, the larger females were not the only individuals that consumed *L. xanthurus*. Of the seven females in this study between the W_D of 40 and 50 cm, three of them were found to have consumed *L. xanthurus*, while none of the 37 males in that W_D range did. However, the veracity of this inference requires further investigation, since confounding factors may have contributed to the observed sex-specific difference in diet for animals of this size range.

The PERMANOVA and CCA analyses only described a small portion of the observed dietary variability for *G. lessae*. Much of the unexplained variability probably stems from the variability in the prey communities themselves. Many studies address this by sampling the prey communities concurrently, because without a measure of the relative abundance of prey, determining whether a species is selecting for certain prey is difficult (Ajemian & Powers, 2012; O'Shea *et al*., 2017). Although the available prey communities were not concurrently quantified in this study, the prey consumed by *G. lessae* did appear to reflect the demersal fish communities that were also captured in the trawls. One notable exception was the lack of prey from the sea catfishes family, Ariidae, that were commonly caught in the trawls. This is unsurprising given the large serrated venomous spines of these species, which make them hazardous to consume (Ronje *et al*., 2017); however, it is an additional example of teleost-prey selectivity displayed by *G. lessae*.

Both the metabarcoding and free-otolith analyses were integral in describing the diet of *G. lessae* in this study. While metabarcoding better explained the total diversity of prey species in the diet of *G. lessae* (n = 14 *v.* 11), both datasets independently drew similar conclusions regarding prey consumption and the factors that best explain dietary variability. In addition, when both datasets were pooled together, PERMANOVA analysis found that the two sampling methods did not produce significantly different results. While free-otolith analysis was valuable in this study, the utility of otoliths in other dietary studies would be diminished if common teleost prey had indistinguishable otoliths or if the species of interest fed on more than just

teleosts, potentially leading to under or overestimation of teleost *v.* non-teleost prey. Additionally, the free otolith analysis exhibited a slight bias towards prey in the family Sciaenidae, which have large otoliths, but this bias appeared to have a negligible influence on the dietary characterisation and variation. These results suggest that free-otolith analysis should generally be used in primary diet analysis, even when genetic techniques are used, because free otoliths can provide valuable information undetected by genetic techniques. Regardless of a dietary study's methods, dietary studies on predators that are at least partially piscivorous should note the degree to which free otoliths were analysed for reproducibility purposes.

Metabarcoding was successful in identifying prey remains from samples containing DNA that was probably too degraded to be amplified using traditional barcoding methods. The lack of invertebrate DNA in the metabarcoding data further confirmed that *G. lessae* are teleost-specialised feeders. However, the power of metabarcoding can introduce potential bias due to secondary consumption and amplification of DNA that was introduced to the stomach *via* the water column (Taberlet *et al*., 2012; Jakubavičiute *et al*., 2017). While the lack of invertebrate DNA in the samples implies that little DNA was added to the stomach during the trawl itself, DNA of other batoid species that were often placed in a temporary holding tank with *G. lessae* after capture were frequently found in the samples. Though *G. altavela* is known to occasionally consume elasmobranchs, there was no evidence to suggest *G. lessae* consumed elasmobranchs in this study, indicating that the DNA from other batoids is most probably environmental contamination (Daiber & Booth, 1960). Thus, appropriate protocols for prey determination

should be established before analysing metabarcoding data since DNA presence in a sample is not always the result of consumption (Leray *et al*., 2015).

Gymnura lessae are relatively large and abundant teleost-specialised predators; as such, they presumably play an important role in the structure and maintenance of coastal food webs like those in Mobile Bay through top-down effects. The results of this study suggest that *G. lessae* consume the most abundant forage fish in their region, which may affect nutrient transfer to other predators through exploitative competition (Pikitch *et al*., 2014). Although this study found neither commercially nor recreationally important teleost species in *G. lessae* diets, this may not be the case throughout other parts of their range. While simple visual stomach content analysis alone would have indicated the piscivorous nature of *G. lessae*, metagenetics and otolith identification each quantified more prey items to species and revealed several abiotic and intraspecific variables that influence the foraging habits of *G. lessae*.

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CONTRIBUTIONS

All authors listed on the title page have agreed to be listed and have approved the submitted version of the manuscript. M.B.J. is the primary author, he examined the diet contents, analysed the data and drafted the manuscript. M.J.A and M.E.C. helped with data analysis–interpretation and critically reviewed the manuscript at multiple stages of development. P.T.C. was of great assistance with the genetics aspect of the study and also critically reviewed the manuscript. Lastly, J.M.D. assisted with all aspects of the project including design, data analysis–interpretation and critical review of the manuscript at multiple stages.

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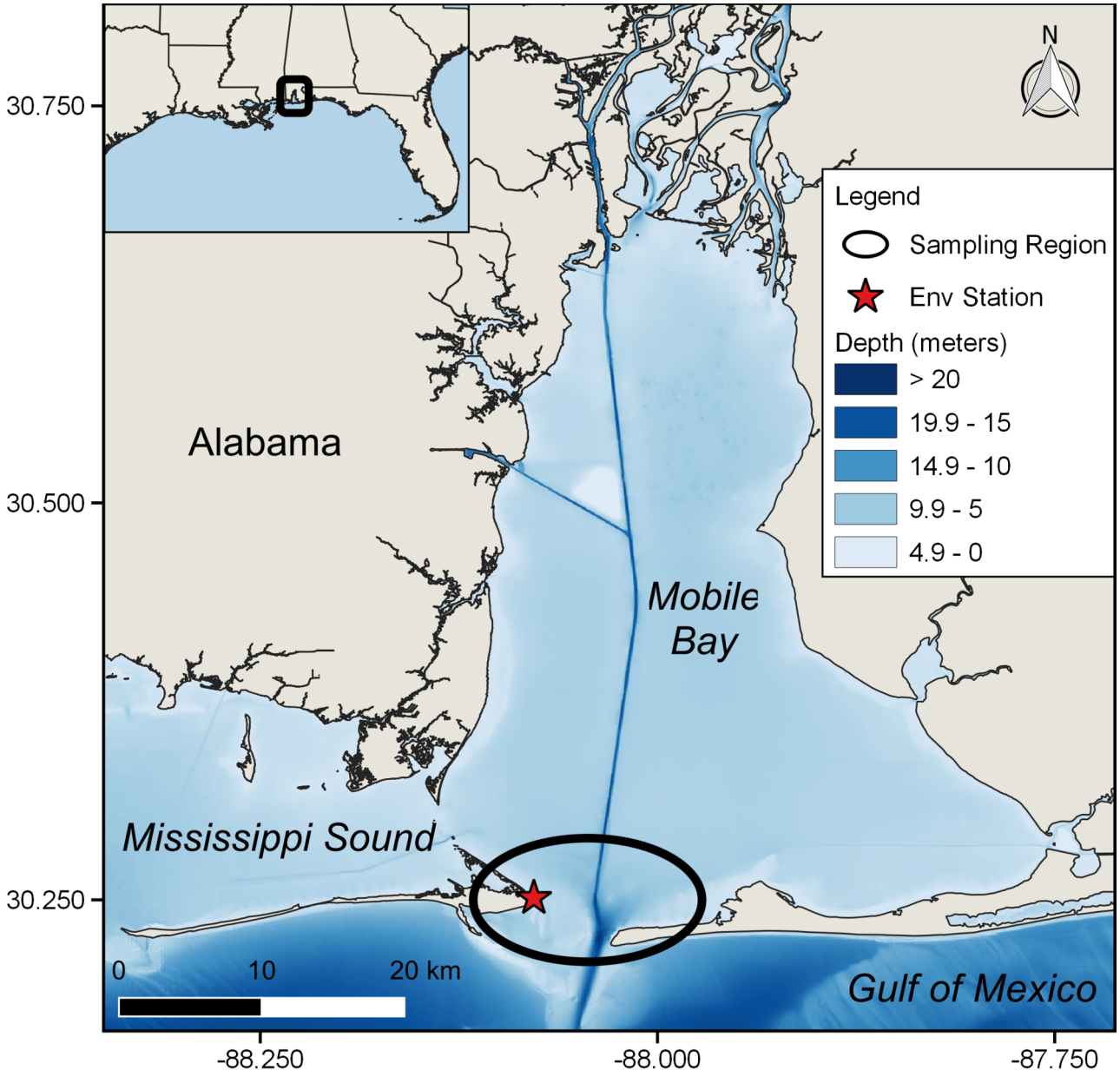

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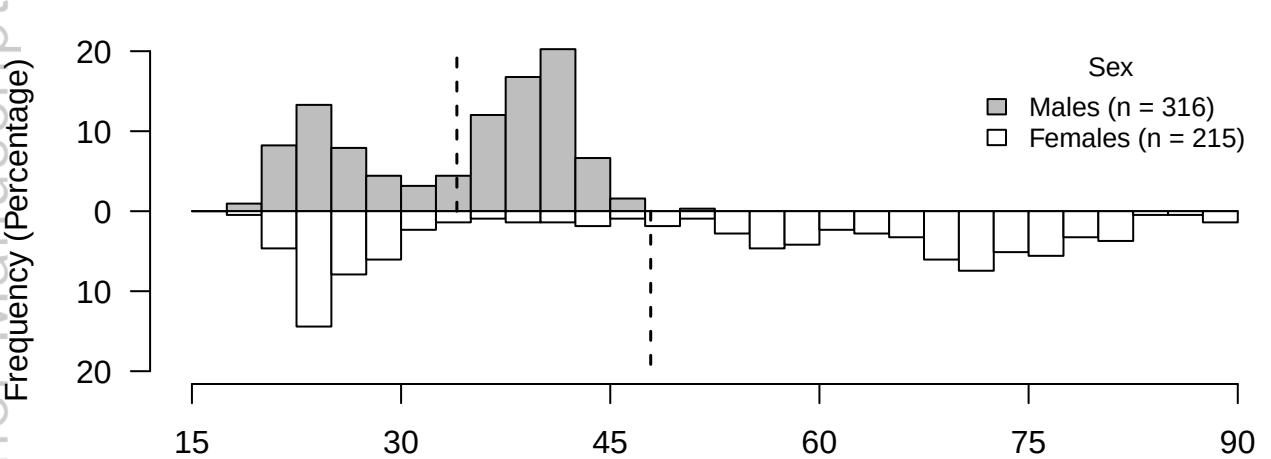

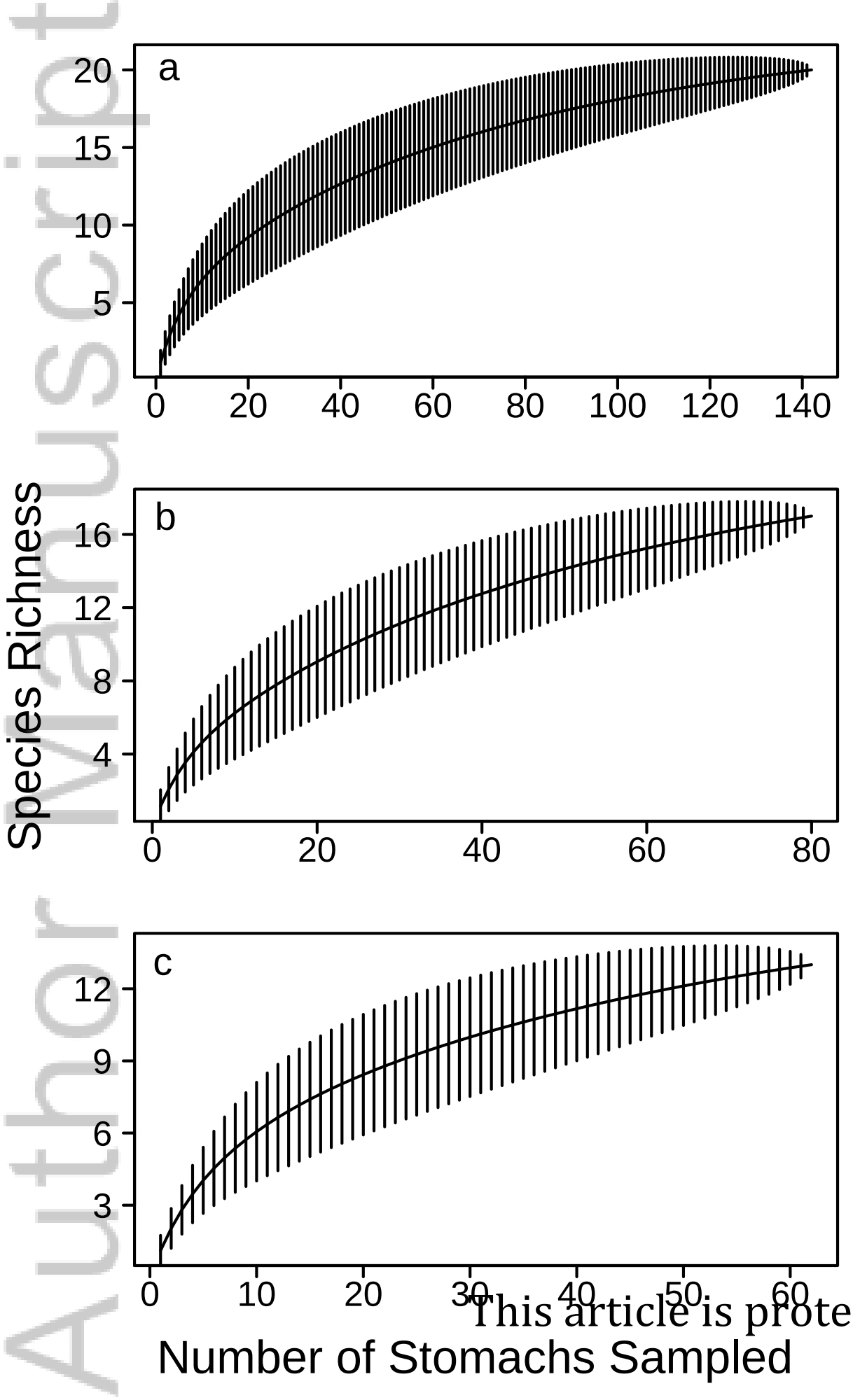

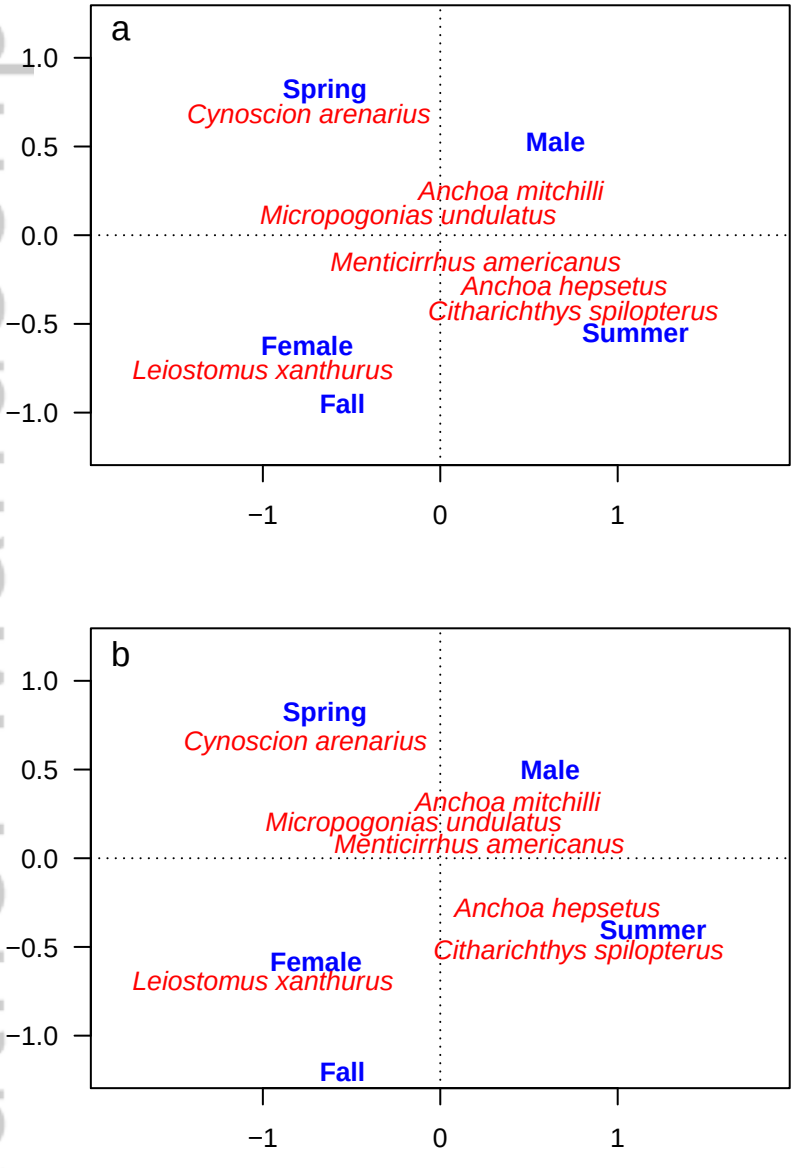

Figures

FIGURE 1 Map of Mobile Bay, Alabama, showing the location of 8 km sampling region (O) from which 99.2% of *Gymnura lessae* were collected and the environmental monitoring station (★) used for water temperature data

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1 All roman script

2 Remove minus sign from longitude values

3 Replace compass arrow with simple latin cross

4 Delete legend, Sampling Region (and symbol) and Env Station (and red star)

FIGURE 2 Disc width (W_D)-frequency distributions for male (, n = 316) and female (□, n = 215) *Gymnura lessae* from Mobile Bay, Alabama. ¦, Disc width at 50% maturity

Typesetter

1 Sex and the information shown beneath it.

2 Change Disc Width to W_D

3 Change (percentage) to (%)

FIGURE 3 Cumulative prey curves (± SD) for *Gymnura lessae* from Mobile Bay, Alabama, showing species richness (number of prey species) for (a) all stomach contents, (b) male stomach contents and (c) female stomach contents

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1 Change a, b, c to (a), (b), (c)

2 Change x-axis to sentence case

3 Change y-axis label to Species richness (*n*)

FIGURE 4 Canonical correspondence analysis (CCA) biplots for (a) per cent number of prey (%N_p) and (b) per cent mass of prey (%M_p) showing the relationships between the response variables (blue) from the final model in the PERMANOVA analysis and prey species (red)

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2 Delete numerical values from x-axis of (a)

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Tables

TABLE 1 Comparisons of the *Gymnura lessae* diet metrics examined using a variety of methods: lacking free-otolith identification and metabarcoding (base analysis), using free-otolith identification (free-otolith analysis), using metabarcoding (metabarcoding analysis), and using free otolith and metabarcoding methods combined (complete analysis)

Sampling method	Stomachs with prey	Total prey	Prey identified to genus	Genus richness	Prey identified to species	Species richness
Base analysis	170	187	30	6	17	6
Free-otolith analysis	204	240	126	12	89	11
Metabarcoding analysis	170	187	122	12	122	14
Complete analysis	204	242	175	15	169	16

TABLE 2 Diet composition of *Gymnura lessae* collected in Mobile Bay from February 2016 to May 2018.

Order	**Family**	**Species**	**%*O***	**%N_p**	**%N_{ps}**	**%M_p**	**%M_{ps}**	**%I_{RIps}**
Unidentified Teleostei			32.4	31.5	97.2	30.5	94.1	31.0
Clupeiformes			27.9	25.3	90.5	25.8	92.4	25.5
	Clupeidae	*Dorosoma petenense*	1.0	1.0	100.0	1.0	100.0	1.0
	Engraulidae		27.0	24.3	90.2	24.8	92.1	24.6
		Anchoa hepsetus	10.8	9.2	85.6	10.1	93.5	9.7
		Anchoa mitchilli	14.7	13.1	89.2	12.8	86.9	12.9
		Anchoa spp.	2.0	1.7	87.5	1.5	78.5	1.6
		Engraulis sp.	0.5	0.2	50.0	0.4	88.0	0.3
Gadiformes	Phycidae	*Urophycis* sp.	0.5	0.1	25.0	0.0	1.8	0.1
Gobiiformes	Gobiidae		2.0	1.6	81.3	1.5	62.9	1.5
		Ctenogobius boleosoma	1.5	1.5	100.0	1.5	100.0	1.5
		Gobiidae	0.5	0.1	25.0	0.0	1.6	0.1
Carangiformes	Carangidae	*Chloroscombrus chrysurus*	1.0	1.0	100.0	1.0	100.0	1.0
Pleuronectiformes			4.4	4.2	94.4	4.4	99.2	4.3
	Achiridae	*Trinectes maculatus*	1.5	1.2	83.3	1.4	97.6	1.3
	Paralichthyidae		2.9	2.9	1.0	2.9	1.0	2.9
		Citharichthys spilopterus	2.5	2.5	100.0	2.5	100.0	2.5
		Etropus crossotus	0.5	0.5	100.0	0.5	100.0	0.5
Sciaeniformes	Sciaenidae		36.8	36.4	92.7	36.9	92.5	36.6
		Bairdiella chrysoura	1.0	0.5	50.0	0.1	5.8	0.3
		Cynoscion arenarius	5.9	4.8	81.3	4.6	78.0	4.7
		Larimus fasciatus	1.0	0.7	75.0	0.5	51.4	0.6
		Leiostomus xanthurus	8.3	7.4	88.7	7.3	88.0	7.4
		Menticirrhus americanus	2.9	2.3	79.2	2.9	99.0	2.6
		Menticirrhus spp.	1.5	1.2	83.3	1.3	87.7	1.3
		Micropogonias undulatus	21.1	19.0	89.9	19.7	93.6	19.3
		Stellifer lanceolatus	0.5	0.5	100.0	0.5	100.0	0.5

%*O*, Frequency of occurrence; %N_p, average per cent number of prey; %M_p, average per cent mass of prey; %N_{ps}, prey-specific number; %M_{ps}, prey-specific mass; %I_{RIps}, prey-specific index of relative importance

TABLE 3 PERMANOVA models for the diet composition of *Gymnura lessae*.

Model(s)	Variable(s)		%N_p			%M_p		
		df	*F*	R^2	*P*	*F*	R^2	*P*
Independent variables	Sex	1	4.284	0.030	< 0.001	3.396	0.024	< 0.01
	Maturity	1	1.660	0.012	> 0.05	1.350	0.010	0.209
	Disc width	1	2.911	0.020	< 0.01	2.223	0.016	< 0.05
	Temperature	1	3.176	0.022	< 0.01	3.039	0.021	< 0.05
	Season	2	3.538	0.048	< 0.001	3.275	0.045	<0.001
	Day length	1	2.635	0.018	< 0.05	2.818	0.020	< 0.01
Interactions	Sex x maturity	1	0.772	0.005	> 0.05	0.652	0.005	> 0.05
	Sex x temperature	1	0.958	0.007	> 0.05	1.073	0.007	> 0.05
	Sex x season	2	1.493	0.020	> 0.05	1.596	0.021	> 0.05
	Sex x day length	1	2.214	0.015	< 0.05	2.343	0.016	< 0.05
	Sex x disc width	1	0.903	0.006	> 0.05	0.795	0.006	> 0.05
	Disc width x temperature	1	2.281	0.015	< 0.05	2.577	0.018	< 0.05
	Disc width x season	2	1.114	0.015	> 0.05	1.244	0.017	> 0.05
	Disc width x day length	1	1.234	0.009	> 0.05	1.559	0.011	> 0.05
Final model	Season	2	3.613	0.048	< 0.001	3.324	0.045	< 0.001
	Sex	1	3.971	0.027	< 0.001	3.107	0.021	< 0.01
	Residuals	96		0.925			0.934	

%N_p, average per cent number of prey; %M_p, average per cent mass of prey

SIGNIFICANCE STATEMENT

We analyzed stomach contents of smooth butterfly rays, *Gymnura lessae*, using metagenetics and free otolith identification. We describe the diet of an abundant high trophic level predator that likely plays a significant ecological role in many coastal marine ecosystems of the United States and Mexico. We also demonstrate that both methods can be powerful tools for examining the diets of marine piscivorous fish, particularly in studies with an abundance of unidentified teleost prey.