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Trophic ecology of three estuarine predatory fishes revealed through a multi-method approach

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ABSTRACT: Estuaries are characterized by wide ranges of environmental conditions that support diverse communities and complex food webs. When trophic structure is assessed at a broad scale, large predators are often regarded as redundant, fulfilling comparable ecological functions. We used stomach content analysis (SCA), fecal DNA metabarcoding (fDNA), and stable isotope analysis (SIA) of carbon (δ^{13}C), nitrogen (δ^{15}N), and sulfur (δ^{34}S) to investigate the trophic ecology of 3 co-occurring predatory fishes: alligator gar *Atractosteus spatula*, bull shark *Carcharhinus leucas*, and Atlantic stingray *Hypanus sabinus* in a sub-embayment of a large estuarine complex in the western Gulf of Mexico. Stomach contents showed that alligator gar and bull shark preyed almost exclusively on teleost fishes, but on different species, while Atlantic stingray fed mostly on crustaceans. fDNA confirmed these dietary patterns and provided higher resolution on prey composition and diversity. Alligator gar displayed lower δ^{34}S values than bull shark and Atlantic stingray, indicating a diet from low-salinity environments with freshwater-derived organic matter. Atlantic stingray exhibited the broadest trophic niche, while alligator gar and bull shark had more restricted, overlapping niches despite feeding on different species. Alligator gar and bull shark were classified as top predators, and Atlantic stingray as a mesopredator. Seasonal, life stage, and sex-based trophic patterns showed no clear distinctions, likely due to limited sample sizes. This study presents new insights into the trophic ecology of these species and provides the first comprehensive characterization of trophic dynamics using an integrative approach combining SCA, fDNA, and SIA.

KEY WORDS: Estuarine predatory fishes · Fecal DNA · Stable isotope analysis · Stomach content analysis · Trophic ecology

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1. INTRODUCTION

The dynamic nature of estuaries makes them highly productive, but also challenging for many wildlife species to thrive or even persist in. Large inputs of organic matter and nutrients from land and oceans support primary and secondary production in estuaries (McLusky & Elliott 2004), and the combination of freshwater flow from rivers and tidal currents from the ocean creates environmental gradients that shape ecological communities in these systems (Whitfield et al. 2024). Within estuaries, physiologically flexible euryhaline species exhibit broader geographical distributions compared to stenohaline species that are largely confined to either low- or high-salinity habitats (Martino & Able 2003). In turn, physiological tolerance to variability in salinity affects the ecological roles many species have within these ecosystems, with euryhaline species more likely to have wider niches (Park et al. 2023). For example, spotted seatrout *Cynoscion nebulosus* actively retreat from low-salinity regions after the release of

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fresh water into estuaries and thus top-down effects linked to this predator may shift to more saline lower estuarine habitats (Callihan et al. 2015, TinHan et al. 2018). As a result, species that display flexible patterns in habitat use and trophic interactions often fulfill important ecological roles, especially highly mobile euryhaline fishes (Stevens et al. 2018).

Given the productivity of estuarine ecosystems, multiple predator species frequently co-occur, sharing habitats and food resources. Large predators are often viewed as redundant generalists fulfilling similar functional roles in food webs, but a growing body of literature indicates greater ecological complexity, with seemingly similar species exhibiting different roles (Hussey et al. 2015, Shipley et al. 2019b). The behavior of predators reflects morphological traits, prey availability, and prey preferences that have evolved over evolutionary timescales and therefore varies among co-existing species (Heithaus & Vaudo 2004). While some co-occurring predators compete for prey, most partition food resources and/or use habitats differently, reducing interspecific competition and promoting coexistence (Malinowski et al. 2019, Maitra et al. 2020). For example, bonnetheads *Sphyrna tiburo* and red drum *Sciaenops ocellatus* coexist in coastal ecosystems, but spatiotemporal differences in distributions coupled with varying foraging tactics lead to distinct ecological roles (Kroetz et al. 2017). As such, most species are functionally unique within a given predator guild, highlighting their individual ecological function.

The trophic ecology of co-occurring predators has been quantified with a suite of approaches that assess direct effects on prey populations and the degree of overlap among predator diets. Stomach content analysis (SCA) provides a 'snapshot' of an individual's diet (Hyslop 1980) and provides high taxonomic resolution. However, identifiable prey are limited due to digestion, which can lead to underestimates in the amount of soft-bodied prey in the diet of a predator (Buckland et al. 2017, Amundsen & Sánchez-Hernández 2019). Consequently, dietary DNA metabarcoding has emerged as an alternative to morphological dietary assessment (de Sousa et al. 2019). Dietary DNA metabarcoding of stomach contents typically requires lethal sampling, whereas metabarcoding fecal DNA (fDNA) is less invasive and provides high taxonomic resolution of diet compositions, overcoming sensitivity issues of SCA (van Zinnicq Bergmann et al. 2021). Another method is stable isotope analysis (SIA), which typically uses stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) to examine the trophic ecology of predators (Newsome et al. 2007). Values of $\delta^{13}C$ reveal basal carbon source (e.g. terrestrial vs. marine production; Vander Zanden & Rasmussen 1999, Bearhop et al. 2004, Fry 2006), whereas values of $\delta^{15}N$ are used to infer trophic position (Fry & Sherr 1986, Rounick & Winterbourn 1986, France & Peters 1997). Stable isotopes of sulfur ($\delta^{34}S$) have increased in use as a trophic marker in the study of aquatic ecosystems (Raoult et al. 2024). In estuaries, where $\delta^{13}C$ values overlap across primary producers along a salinity gradient, $\delta^{34}S$ can provide increased resolution into foraging patterns of an individual by distinguishing between marine and freshwater-derived dietary sources (Connolly et al. 2004, Hussey et al. 2012). Combining SCA, fDNA, and SIA provides a comprehensive approach that offers valuable insights into the trophic ecology of predators and overcomes the biases of employing any single approach, ultimately enhancing our understanding of their ecological roles. This multi-method approach not only refines the interpretation of trophic interactions among co-occurring estuarine predatory fishes but also extends its applicability across diverse species and ecosystems, offering substantial value to researchers.

This study examined the trophic ecology of 3 commonly co-occurring predatory fishes in a large estuarine complex in the western Gulf of Mexico: alligator gar *Atractosteus spatula*, bull shark *Carcharhinus leucas*, and Atlantic stingray *Hypanus sabinus*. The selection of these species was driven by their ecological significance and unique role within the estuarine ecosystem. Alligator gar are top predators, primarily piscivorous, playing a critical role in controlling fish populations in freshwater and brackish habitats (Daugherty et al. 2018, Marsaly et al. 2023). They primarily inhabit low-salinity areas and spawn in fresh water, with seasonal movement in estuaries tied to temperature and environmental conditions (Daugherty et al. 2018, Livernois et al. 2021). Juvenile bull sharks are also primarily piscivorous but exhibit size-specific affinities for certain salinities, using freshwater regions as nursery grounds and moving freely throughout estuaries (Heupel & Simpfendorfer 2008, Froeschke et al. 2010). Atlantic stingrays are benthic feeders that primarily consume invertebrates and small fish, exerting control over benthic prey populations (Cook 1994, Bradley 1996). They are found in estuarine and nearshore regions, and exhibit seasonal movement between shallow, warm waters and deeper areas for overwintering (Snelson et al. 1988). These 3 species are abundant in the study area and occupy distinct ecological roles across the salinity gradient, offering an ideal context for exploring trophic interactions among co-occurring predatory fishes in estuarine ecosystems. In this context, the objectives of this study were to employ SCA, fDNA, and SIA to (1) characterize the diets of alligator gar, bull shark, and Atlantic stingray, (2) assess the degree of trophic overlap among these species, and (3) evaluate differences in the trophic ecology of these species by season, life stage, and sex.

2. MATERIALS AND METHODS

2.1. Study area

The study was conducted in West Bay and the adjacent waterbodies of Bastrop Bay and Christmas Bay in the southwestern Galveston Bay Complex (GBC) in Texas, USA (Fig. 1). West Bay is utilized by all 3 study species and has representative habitats found throughout the GBC (i.e. salt marsh, oyster reef, non-vegetated substrate). West Bay is mostly hydrologically separated from the main sources of freshwater input to the GBC, and most tidal exchange to the Gulf of Mexico is through San Luis Pass (Villalon et al. 1998). Freshwater enters West Bay through Bastrop Bayou and Chocolate Bayou, but these inflows are relatively limited, which results in West Bay being the most saline sub-bay of the GBC (Villalon et al. 1998). Because mobile euryhaline fishes in West Bay experience both freshwater and marine conditions within a modest spatial extent, this area provides an ideal context for assessing trophic interactions of predatory fishes across a salinity gradient.

2.2. Data collection

Alligator gar, bull shark, and Atlantic stingray individuals were opportunistically obtained from gillnet and bag seine surveys conducted by the Texas Parks and Wildlife Department (TPWD) long-term fishery-independent monitoring program from 2023 to 2024 (Martinez-Andrade 2018). Gillnets were generally selective for larger, more mobile species such as alligator gar and bull shark, while bag seines captured Atlantic stingray effectively in similar locations as

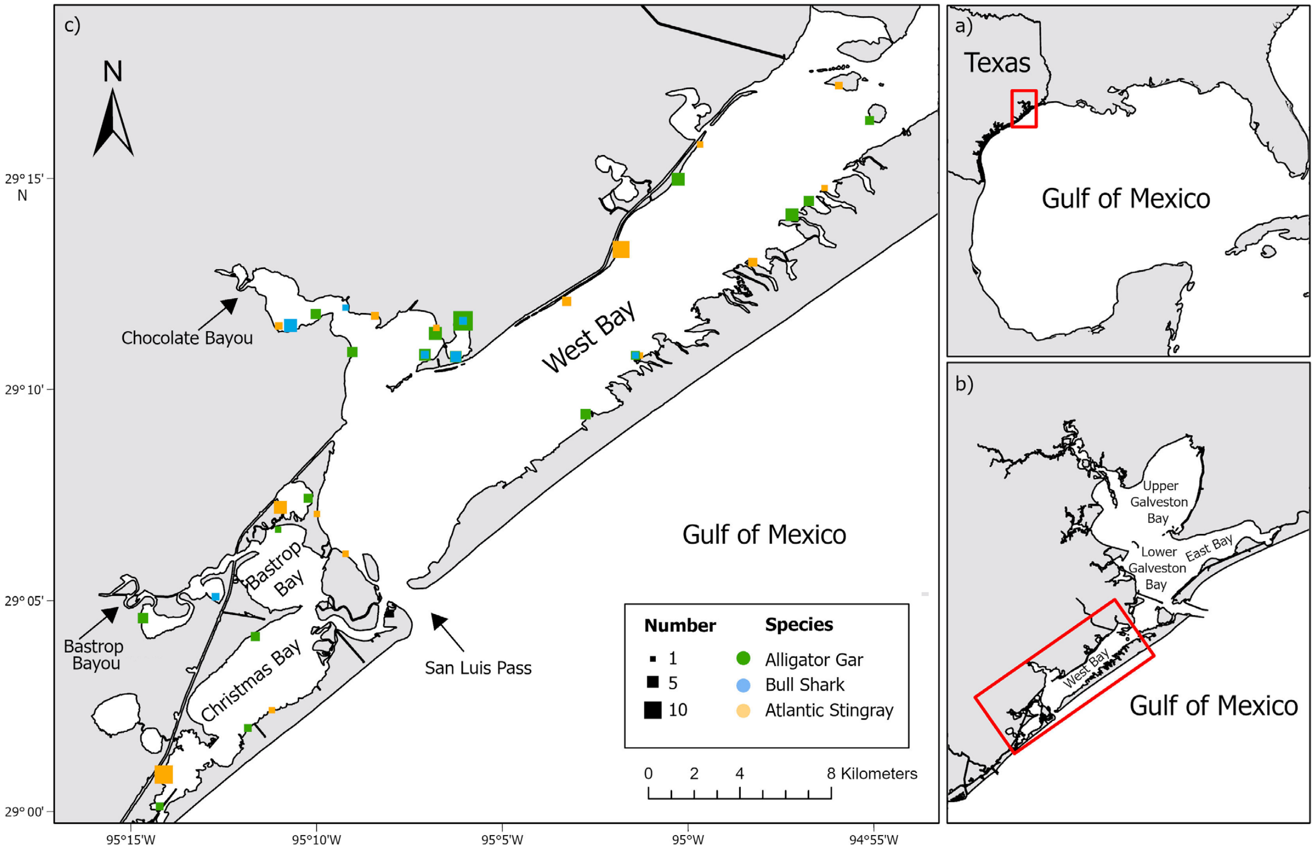

Fig. 1. (a) Location of the Galveston Bay Complex (GBC), Texas, USA, in the Gulf of Mexico and (b) West Bay in the GBC. (c) Sample collections in West Bay for each consumer species and primary producers

gillnets (along shorelines). All alligator gar, bull shark, and Atlantic stingray individuals found dead upon capture were either immediately dissected or frozen until dissection could be conducted (<1 mo frozen). After recording size (total length [TL] or disc width [DW]), sex and maturity were determined by macroscopic examination following Núñez & Duponchelle (2009) for bony fishes (alligator gar) and Stehmann (2002) for elasmobranchs (bull shark and Atlantic stingray). Life stages were classified as juveniles (immature, developing) or adults (reproductively capable). Because all bull sharks captured were immature, specimens were divided into 2 size classes based on TL: intermediate 90 cm < TL ≤ 120 cm and large: TL > 120 cm (Branstetter & Stiles 1987).

Stomach contents were extracted and stored in 75% ethanol until identification. Prey items in stomachs were visually identified to the lowest possible taxonomic resolution, and individual items were counted and weighed (dry weight). For fDNA analysis, cloacal swabs were collected using a sterile, individually packaged cotton-tipped swab inserted ~3 cm into the cloaca and rotated against the inside wall for ~5 s. After removal, the tip was cut off from the excess handle using sterilized scissors and stored in sterile 5.0 ml cryogenic screw cap vials containing silica beads. All fDNA samples were stored at −20°C until extraction.

Swab extraction and library preparation were performed by Jonah Ventures (www.jonahventures.com) in dedicated controlled eDNA laboratories. The MiFish-U (Miya et al. 2015) and UniCOI (Leray et al. 2013) primer sets were used for the amplification of DNA from cloacal swabs. The MiFish-U primer set was specifically developed for amplifying fish DNA, while the UniCOI primer set targets a broad range of organisms, including invertebrates. The MiFish-U primer was chosen for both alligator gar and bull shark, as both species are primarily piscivorous (Tin-Han & Wells 2021, Marsaly et al. 2023, Livernois et al. 2024). For Atlantic stingray, which primarily consumes benthic invertebrates and small fish (Cook 1994, Bradley 1996), both primer sets were used. Extended methods for DNA extraction, library preparation and sequencing, and bioinformatic analysis are detailed in Text S1 in the Supplement at www.int-res.com/articles/suppl/meps15051_supp.pdf.

Tissue samples for SIA were collected from dorsal muscle and were stored frozen at −20°C until further analyses. Tissue samples were oven-dried at 60°C, homogenized, and lipid and urea were extracted (Kim & Koch 2012) to control for interspecific variation that could confound carbon and nitrogen stable isotopes ratios (Post et al. 2007). Lipids and urea were extracted from dried, homogenized muscle tissue using petroleum ether and deionised (DI) water, respectively, with a Dionex Automated Solvent Extractor (ASE). Each sample was packed in an ASE cell between pre-combusted (450°C for 4 h) 30 mm GF/B filters, and any remaining space was filled with clean sand. The cell was then rinsed 3 times with 100% petroleum ether at 100°C, 1500 psi (~103 bar), and 60% rinse volume for 5 min, followed by 3 rinses with DI water using the same settings (Livernois et al. 2024). Samples were removed from the cell, oven-dried at 60°C for 12–24 h and homogenized with a mortar and pestle. Finally, ~1.2 mg of each sample was packaged in 5 × 9 mm tin capsules and shipped to the University of California–Davis Stable Isotope Facility (UC-Davis SIF) with precision ≤0.10‰ for $\delta^{13}C$, ≤0.13‰ for $\delta^{15}N$, and ≤0.35‰ for $\delta^{34}S$. The stable isotope composition is reported in the conventional delta (δ) per mille notation (‰), relative to Vienna Pee Dee belemnite ($\delta^{13}C$), atmospheric N_2 ($\delta^{15}N$), and Vienna-Canyon Diablo troilite ($\delta^{34}S$).

2.3. Data analysis

All statistical analyses were performed in R v.4.1.0 and statistical significance was set at $\alpha = 0.05$ (R Core Team 2024).

2.3.1. Stomach content analysis (SCA)

A vacuity index (%V) was calculated as the percentage of empty stomachs for each species and each subgroup to assess feeding frequency (Hyslop 1980). Differences in vacuity index between species and within subgroups were evaluated using Fisher's exact test, which is appropriate for small sample sizes. Cumulative prey curves were used to determine if the number of stomachs with identifiable prey was adequate to describe the diet of the species and subgroups (fall, spring, female, male, immature/intermediate, and mature/large). The final 4 curve points for each species and for each subgroup were statistically compared with the zero-slope line using a Student's *t*-test to assess saturation (Bizzarro et al. 2007).

The importance of various prey species to the diets of alligator gar, bull shark, and Atlantic stingray was assessed by calculating the percent frequency of occurrence (%FO), average percent number (%N), average percent weight (%W), prey-specific number (%PN), and prey-specific weight (%PW; Hyslop 1980, Brown et al. 2012). The prey-specific index of relative importance (%PSIRI) was then used to measure the relative importance of each prey group in the diet of the 3 study species (Brown et al. 2012). Stomachs that were empty or contained only slurry and/or detritus were not considered when calculating diet measures. These indices were calculated as follows:

Frequency of occurrence (%FO):

$$\%\mathrm{FO}_i = \frac{n_i}{n} \times 100 \tag{1}$$

Average percent abundance ($\%\mathrm{N}_i$, $\%\mathrm{W}_i$):

$$\%\mathrm{A}_i = \frac{\sum_{j=1}^{n} \%\mathrm{A}_{ij}}{n} \tag{2}$$

Prey-specific abundance ($\%\mathrm{PN}_i$, $\%\mathrm{PW}_i$):

$$\%\mathrm{PA}_i = \frac{\sum_{j=1}^{n} \%\mathrm{A}_{ij}}{n_i} \tag{3}$$

Prey-specific index of relative importance ($\%\mathrm{PSIRI}_i$):

$$\%\mathrm{PSIRI}_i = \frac{\%\mathrm{FO}_i \times (\%\mathrm{PN}_i + \%\mathrm{PW}_i)}{2} \tag{4}$$

where n is the total number of stomachs containing prey, n_i is the number of stomachs containing prey i, and $\%\mathrm{A}_{ij}$ is the percent abundance (by number or weight) of prey category i in stomach sample j (Brown et al. 2012).

To measure the trophic niche breadth, the standardized Levin's index (Ba) was calculated:

$$\mathrm{Ba} = \frac{\frac{1}{\sum \mathrm{P}_{ij}^2} - 1}{n - 1} \tag{5}$$

where P_{ij} is the proportion of the prey j in the diet of the species i, and n is the number of prey categories. This index ranges from 0 (minimum niche breadth) to 1 (maximum niche breadth).

Dietary niche overlap (i.e. the similarity in trophic resources used) was estimated with the Schoener overlap index (Marshall & Elliott 1997):

$$\mathrm{P}_{jk} = 100 \times \sum_{i=1}^{n} \min\left(\%\mathrm{PSIRI}_{ij}, \%\mathrm{PSIRI}_{ik}\right) \tag{6}$$

where n is the total number of prey species, P_{jk} is the Schoener overlap between the 2 groups j and k, and $\%\mathrm{PSIRI}_{ij}$ and $\%\mathrm{PSIRI}_{ik}$ are the PSIRIs of prey item i in the diet of groups j and k, respectively.

Trophic position (TPj) was defined for each individual according to Cortés (1999):

$$\mathrm{TP}_j = 1 + \sum_{j=1}^{n} \mathrm{DC}_{ji} \times \mathrm{TP}_i \tag{7}$$

where TP_j is the trophic position (level) of predator j, DC_{ji} is the proportion of prey category i in the diet of predator j, n is the total number of prey categories, and TP_i is the trophic position of prey group i. Trophic positions of prey categories were assigned based on literature values (Table S1). This calculation was applied to each individual predator based on its specific diet composition, allowing estimation of species-level mean and standard deviation in trophic position. The assumptions of normality and homogeneity of variances were tested using the Shapiro-Wilk test and Levene's test, respectively. Once assumptions were confirmed ($p > 0.05$ for all tests), differences in trophic position among species were analyzed using analysis of variance (ANOVA), followed by post hoc comparisons with Tukey's honestly significant difference (HSD) test (Sawyer 2009, Kucuk et al. 2016).

To evaluate differences among the diets of the 3 predators, we used the weight of food items (%W) grouped into 9 prey categories: Polychaeta, Bivalvia, Decapoda, Clupeidae, Mugilidae, Sciaenidae, Ariidae, Other fish spp., and Aves. For clarity, functional group terminology is applied to describe these prey categories, with Annelida (Polychaeta), Arthropoda (Bivalvia, Decapoda), and Chordata (Teleostei, Aves) representing key functional groups within the broader taxonomy. Standardized %W values for the 9 prey categories were used to build a dissimilarity matrix using the Bray-Curtis dissimilarity index. %W was used for the statistical analyses since this measure best reflects the nutritional contribution of prey (Macdonald & Green 1983). The Bray-Curtis dissimilarity index was employed as it effectively quantifies dietary differences by prioritizing the abundant prey categories, which are the primary contributors to dietary composition (Marshall & Elliott 1997). Given the low number of stomachs collected (see Table 1), gravimetric data pooling was designed to reduce the number of prey categories in the samples with zero values, thus increasing the efficiency of multivariate analysis (White et al. 2004). Prior to conducting permutational multivariate analysis of variance (PERMANOVA), a non-parametric permutational analysis of multivariate dispersions (PERMDISP) was conducted using the function 'permdisp' of the R package 'vegan' (Oksanen et al. 2024), to test the homogeneity of multivariate dispersions of data and thereby to assess PERMANOVA robustness (Anderson 2006). Next, PERMANOVA was run based on the Bray-Curtis dissimilarity matrix in the 'adonis' function of the 'vegan' R package (Oksanen et al. 2024) to test for statistical differences among species and subgroup diets. PERMANOVA is highly robust when applied to Bray-Curtis dissimilarity, making it a suitable method for analyzing the composition of prey groups (Lek et al. 2011). For pairwise differences between species diet compositions, PERMANOVA tests were run for all species pairs using the ‘adonis.pair’ function of the ‘EcolUtils’ R package (Salazar 2018), with Bonferroni correction to set significance levels for p-values. When significant differences in diet composition were observed, similarity percentage (SIMPER) analysis was conducted using the R function ‘simper’ of the package ‘vegan’ (Oksanen et al. 2024) to detect which prey categories were the main contributors to the dissimilarity observed. SIMPER performs pairwise comparisons to quantify the average contribution of each prey category to the overall dissimilarity between groups, allowing for the identification of the specific dietary components that drive the observed differences between groups (Clarke 1993). Nonmetric multidimensional scaling (nMDS) was performed using the ‘vegan’ package (Oksanen et al. 2024) to observe possible groupings or separation in diet among predators, as identified by the PERMANOVA test, providing a clear, visual representation of the similarities and dissimilarities between dietary compositions, with stress values less than 0.2 indicating a reliable 2-dimensional representation of the data structure (Clarke & Warwick 2001).

2.3.2. Fecal DNA (fDNA)

Due to the small sample size and unequal distribution of cloacal swabs across different groups, we did not evaluate differences in diet based on season, size, and/or sex for fDNA. The overall relative contribution of taxa to the diet of each species was estimated via the calculation of frequency of occurrence (%FO) across all samples. Relative read abundance (RRA) for each taxon was also recorded within and across samples. While RRA has been used as an indicator of relative species biomass within environmental samples (i.e. Zhang et al. 2022), we chose to exclude these estimates from our analyses. The broad nature of predatory fish diets and extreme morphological variation between observed prey species detected in this study suggest that DNA concentration within samples is unlikely to reliably reflect the relative abundance of prey consumed (Deagle et al. 2019). For this reason, all downstream analyses were restricted to presence/absence data and %FO of prey species. All non-Animalia taxa (i.e. Chromista and Fungi) detected were considered to result from incidental ingestion from the sediment interface or the ambient water column and were excluded from the analysis.

The %FO of prey species were grouped into larger prey categories including 3 invertebrate groups (Polychaeta, Bivalvia, and Decapoda) and 5 fish groups (Clupeidae, Mugilidae, Sciaenidae, Ariidae, and Other fish spp.). Histograms generated were used to present the relative %FO of prey categories. The same analytical framework applied to stomach content analysis was used to assess differences in diet composition among species. To summarize, %FO of prey species was then used to build a dissimilarity matrix using the Bray-Curtis dissimilarity index. The Bray-Curtis dissimilarity index was applied to %FO due to its ability to handle zero values effectively by ignoring joint absences. This dissimilarity matrix was then employed to perform a PERMANOVA followed by pairwise comparisons to assess significant differences in diet composition both among species and between each pair of species (Oksanen et al. 2024). Following the PERMANOVA tests, a SIMPER analysis was conducted to identify prey categories that contribute most significantly to the observed dissimilarity in the diets among species (Oksanen et al. 2024).

2.3.3. Stable isotope analysis (SIA)

Muscle tissue $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values of the 3 predators were used to build a similarity matrix using Euclidean distances. We chose Euclidean distances to construct the dissimilarity matrix for stable isotope data because the values represent continuous data, and Euclidean distance is well-suited for measuring the absolute differences between these continuous variables (Krislock & Wolkowicz 2012). Prior to conducting permutational multivariate analysis of variance (PERMANOVA) for SIA, the same analytical framework applied for both SCA and fDNA was used to test the homogeneity of multivariate dispersions across the data. PERMANOVA was then used to test for differences in the isotopic composition among the 3 species. Individual PERMANOVAs were then conducted to examine each species separately, with season, life stage, and sex included as variables.

To investigate isotopic niche space and isotopic niche overlap among species, stable isotope values ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) were used to create hypervolumes representing each species' multidimensional isotopic niche. Specifically, the ‘hypervolume’ package (V.3.1.4, Blonder et al. 2014, Blonder 2018) was used to seed Gaussian kernel density estimations, generating a cloud of points along $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ axes for each species. Each hypervolume included 95% of the total probability density (quantile threshold = 0.05, Blonder et al. 2014). The size of each hypervolume was calculated, representing the relative breadth of each species' isotopic niche. The degree of overlap between each pair of species' hypervolumes (isotopic niches) was determined using the Sørensen overlap index (proportion overlapping, Blonder et al. 2014). The Sørensen overlap index quantifies the similarity between the isotopic niche hypervolumes of 2 species by calculating the proportion of shared volume relative to the combined total volume occupied by both species. This index ranges from 0 (i.e. no overlap) to 1 (i.e. complete overlap). The Sørensen overlap index was employed as a robust and interpretable metric to quantify niche overlap, facilitating the assessment of the degree to which species' trophic niches coincide within isotopic space.

Trophic position (TPSI) of individual species was estimated from nitrogen stable isotope values following the scaled Δ^{15}N framework approach based on a dietary δ^{15}N value-dependent model (Hussey et al. 2014a,b). Relative TPSI was calculated as follows:

$$\mathrm{TPSI} = \frac{\log\left(\delta^{15}\mathrm{N}_{\mathrm{lim}} - \delta^{15}\mathrm{N}_{\mathrm{base}}\right) - \log\left(\delta^{15}\mathrm{N}_{\mathrm{lim}} - \delta^{15}\mathrm{N}_{\mathrm{TPSI}}\right)}{k} + \mathrm{TPSI}_{base} \quad (8)$$

where $\mathrm{TPSI}_{\mathrm{base}}$ is the TP of the baseline species, $\delta^{15}\mathrm{N}_{\mathrm{lim}}$ the saturating isotope value, k represents the rate at which $\delta^{15}\mathrm{N}_{\mathrm{TP}}$ approaches $\delta^{15}\mathrm{N}_{\mathrm{lim}}$, and $\delta^{15}\mathrm{N}_{\mathrm{TPSI}}$ is the δ^{15}N value of the consumers. The $\delta^{15}\mathrm{N}_{\mathrm{lim}}$ and k values of 21.93 and 0.14, respectively, were retrieved from a meta-analysis of experimental isotope data (Hussey et al. 2014a). The mean (±SE) δ^{15}N value of seagrass and saltmarsh vegetation (*Spartina alterniflora*, *Halodule wrightii*, and *Thalassia testudinum,* $\delta^{15}\mathrm{N}_{\mathrm{base}} = 5.56 \pm 0.76$, $\mathrm{TPSI}_{\mathrm{base}} = 1$) was used, obtained from samples collected in West Bay and the adjacent waterbodies (Livernois et al. 2024).

The assumptions of normality and homogeneity of variances were tested using the Shapiro-Wilk test and Levene's test, respectively. Once assumptions were confirmed ($p > 0.05$ for all tests), differences in trophic position among species were analyzed using ANOVA, followed by post hoc comparisons with Tukey's HSD test (Sawyer 2009, Kucuk et al. 2016).

3. RESULTS

3.1. Stomach content analysis

A total of 150 stomachs were examined across 3 species: alligator gar (n = 79), bull shark (n = 19), and Atlantic stingray (n = 52; Table 1). Of the analyzed stomachs, 69 stomachs were empty: 39 (49.4%) from alligator gar, 8 (42.1%) from bull sharks, and 22 (42.3%) from Atlantic stingrays. The vacuity index was significantly higher for alligator gar compared to bull sharks and Atlantic stingrays (Fisher's exact test, $p < 0.05$ for all tests). When considering season, the vacuity index was higher during the fall for alligator gar, while it was lower during the spring for bull sharks (Fisher's exact test, $p < 0.05$ for all tests). For Atlantic stingrays, no significant seasonal differences in vacuity index were observed (Fisher's exact test, $p = 0.12$). There were no significant differences in vacuity index between juveniles and adults for alligator gar (Fisher's exact test, $p = 0.43$). Small juvenile bull sharks had significantly higher vacuity indices than sharks >120 cm TL (Fisher's exact test, $p = 0.02$), while juvenile Atlantic stingrays exhibited a lower vacuity index compared to adults (Fisher's exact test, $p = 0.003$). Males exhibited significantly higher vacuity indices than females across all studied species (Fisher's exact test, $p < 0.05$ for all tests). Prey cumulative curves suggested that low samples sizes likely prevented us from capturing a large part of the diet

Table 1. Total number of stomachs (% of empty), sampling season, life stage, number and percentage by sex, mean size, Levin's index (Ba), and trophic position (TP) of alligator gar, bull shark, and Atlantic stingray collected in West Bay, TX, USA. Sizes are displayed as total length for alligator gar and bull shark, and disc width for Atlantic stingray

Species	Number of samples	Season		Life stage		Sex		Mean size (range), cm	Ba	TP Mean ± SD
		Fall	Spring	Juvenile/ intermediate size class	Adult/ large size class	Female	Male			
Alligator gar	79 (49.4%)	23 (73.9%)	56 (39.9%)	27 (48.1%)	52 (50.0%)	34 (32.4%)	45 (62.2%)	102.91 (145.45–74.40)	0.37	3.8 ± 0.3
Bull shark	19 (42.1%)	5 (0.0%)	14 (57.1%)	13 (38.5%)	6 (50.0%)	5 (0.0%)	14 (57.1%)	109.32 (146.20–90.30)	0.26	3.4 ± 0.4
Atlantic stingray	52 (42.3%)	28 (53.3%)	24 (29.2%)	12 (66.7%)	40 (35.0%)	36 (36.1%)	16 (56.3%)	24.25 (38.60–16.20)	0.63	3.1 ± 0.2

composition of male bull sharks and immature Atlantic stingrays as asymptotes were not reached (Fig. S1, Table S2).

Stomach content analysis revealed that alligator gar, bull shark, and Atlantic stingray fed on multiple taxa (Table 2). For alligator gar, a total of 65 prey items distributed across 2 functional groups were identified: Arthropoda (Decapoda) and Chordata (Teleostei). Of identifiable teleost fishes, the relative importance of prey based on %PSIRI was highest for Clupeidae (48.4%), followed by Mugilidae (21.8%), with other less-represented genera including Sciaenidae (13.8%), Ariidae (10.5%), and Other fish spp. (4.4%). Penaeidae were also found in the diet of alligator gar, contributing 1.1% to the overall prey composition. For bull shark, a total of 17 prey items were identified within the main functional group Chordata (Teleostei and Aves). Bull shark %PSIRI for fishes was highest for Mugilidae (36.6%), followed by Ariidae (24.3%) and Sciaenidae (22.5%). A small bird was also present in 1 stomach (6.6%). For Atlantic stingray, a total of 48 prey items within 3 functional groups were identified: Annelida (Polychaeta), Arthropoda (Bivalvia and Decapoda), and Chordata (Teleostei). Penaeidae were the most important prey items for Atlantic stingray with %PSIRI of 39.6%, with other prey items including Panopeidae (21.7%), Portunidae (15.7%), Bivalvia (11.5%), and Polychaeta (7.3%). Sciaenidae were also found in a few Atlantic stingray stomachs (4.2%).

The trophic niche breadth of Atlantic stingray estimated from SCA was higher (Ba = 0.63) than those of alligator gar and bull shark (Ba = 0.37 and 0.26, respectively; Table 1). The Schoener index based on %PSIRI showed a 56% overlap in the trophic niches of bull shark and alligator gar. In contrast, Atlantic stingray experienced a lower niche overlap with alligator gar (11.5%) and bull shark (9.1%). Mean trophic position estimates varied among species (ANOVA, $F_{2,78} = 4.27$, $p = 0.017$), with bull shark (TP = 3.8 ± 0.3) followed by alligator gar (TP = 3.4 ± 0.4) and Atlantic stingray (TP = 3.1 ± 0.2). Species trophic positions were all significantly different from one another (HSD, $p < 0.05$ for all tests; Table 1).

The diets of alligator gar, bull shark, and Atlantic stingray showed significant differences based on Bray-Curtis dissimilarity of standardized %W values for prey categories (PERMANOVA, $F_{2,79} = 5.73$ $p < 0.001$; Table S3), with distinct diets observed between each predator pair (PERMANOVA, $p < 0.05$ for all tests). There were no significant differences in diet

Table 2. Stomach content composition of alligator gar, bull shark, and Atlantic stingray in West Bay, TX, USA, expressed as frequency of occurrence (FO), percent prey-specific number (PN), percent number (N), percent prey-specific weight (PW), percent weight (W), and prey-specific index of relative importance (PSIRI). Values are expressed for all individuals sampled that presented identifiable prey. Dash (—): not found. Superscript numbers correspond to the prey categories to which each item belongs, with classifications as follows: 1, Polychaeta; 2, Bivalvia; 3, Decapoda; 4, Clupeidae; 5, Mugilidae; 6, Sciaenidae; 7, Ariidae; 8, Other fish species; 9, Aves

Prey taxon	Alligator gar (%)						Bull shark (%)						Atlantic stingray (%)					
	FO	PN	N	PW	W	PSIRI	FO	PN	N	PW	W	PSIRI	FO	PN	N	PW	W	PSIRI
Polychaeta^1	—	—	—	—	—	—	—	—	—	—	—	—	13.3	59.3	7.9	50.7	6.7	7.3
Bivalvia^2	—	—	—	—	—	—	—	—	—	—	—	—	16.7	77.1	12.9	62.9	10.5	11.5
Portunidae^3	—	—	—	—	—	—	—	—	—	—	—	—	30	57.8	17.3	47.2	14.2	15.7
Panopeidae^3	—	—	—	—	—	—	—	—	—	—	—	—	30	70.2	21.1	74.8	22.4	21.7
Penaeidae^3	5.5	20	1.1	18.5	1	1.1	—	—	—	—	—	—	60	60	36	72	43.2	39.6
Clupeidae^4	55	85.3	46.9	90.7	43	48.4	12.5	77.8	9.7	80.6	10.1	10	—	—	—	—	—	—
Anchoa mitchilii^8	25	15	3.7	20.3	6.1	4.4	—	—	—	—	—	—	—	—	—	—	—	—
Mugilidae^5	37.5	62	23.2	54	21	21.8	37.5	96.4	36.2	98.5	36.9	36.6	—	—	—	—	—	—
Micropogonias undulatus^6	10	25	2.5	15	1.5	2	12.5	86.2	10.8	86.9	11.2	11.1	3.3	55.3	1.8	50.7	1.7	1.7
Sciaenops ocellatus^6	7.5	55.2	4.1	73.6	5.4	4.8	6.5	89.6	5.6	91.3	5.7	5.7	—	—	—	—	—	—
Cynoscion spp.^{6}	5	45.7	2.3	61.8	3.5	2.7	6.5	89.7	5.6	92.3	5.8	5.7	—	—	—	—	—	—
Leiostomus xanthurus^6	17.5	23.6	4.1	2.54	3.8	4.3	—	—	—	—	—	—	6.7	40	3	35	2.3	2.5
Ariidae^7	25	40.3	10	44.2	10.4	10.5	25	96.3	24.1	98.3	24.6	24.3	—	—	—	—	—	—
Aves^9	—	—	—	—	—	—	6.3	100	6.3	100	6.3	6.6	—	—	—	—	—	—

across season, life stage, or sex for any species when analyzed separately (PERMANOVA, p > 0.05 for all tests).

The nMDS analysis revealed that while there was diet overlap among the 3 predators, especially between alligator gar and bull shark, there was evidence for dietary differences supporting the PERMANOVA results (Fig. 2). SIMPER revealed that the differences in diet indicated by PERMANOVA were linked to contrasting proportions of prey categories among the 3 species (Table S4). The greatest dissimilarity was observed between the diets of bull shark and Atlantic stingray (52.11% of overall dissimilarity), with Decapoda and Mugilidae as the main drivers of dissimilarity (42.5 and 23.1%, respectively). The dissimilarity between the diets of alligator gar and Atlantic stingray was also relatively high (51.3%). This difference was primarily driven by Decapoda and Clupeidae (37.6 and 23.4%, respectively). The overall diet dissimilarity between alligator gar and bull shark was the lowest (37.4%), with Clupeidae (37.8%) contributing the most to dissimilarity followed by Mugilidae (27.7%) and Ariidae (10.5%).

3.2. fDNA analysis

fDNA sequences were obtained from 18 specimens: 6 alligator gar, 6 bull sharks and 6 Atlantic stingrays (Table 3). All swabs contained host and/or prey DNA. In total, 568 939 sequences were determined, and the mean number of analyzed reads of each sample was 31 607 (n = 18). Host DNA was present in 15 of the 18 cloacal swabs, with a %FO of 46% (259 410 sequence reads) of the total number of recovered sequence reads. Prey DNA was recovered from 16 cloacal swabs, with the 2 samples that did not contain prey DNA having host DNA. The phylum Chordata (Teleostei, %FO = 87.1) comprised most of the sequence reads, followed by Arthropoda (Amphipoda and Decapoda, %FO = 9.6) and Annelida (Polychaeta, FO = 3.3%). Non-teleost prey were only identified in the Atlantic stingray, as DNA sequencing for invertebrates was only conducted for this species.

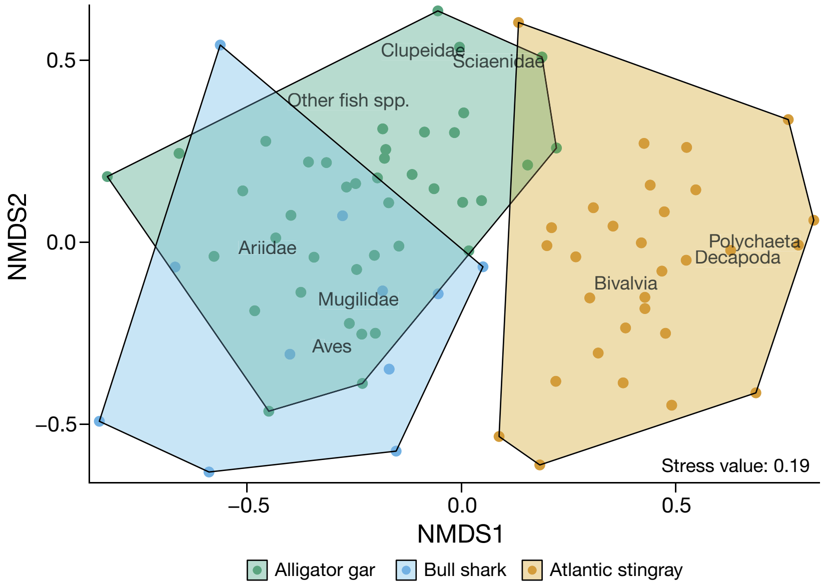

Fig. 2. Nonmetric multidimensional scaling (nMDS) of prey categories identified in the stomach contents of alligator gar, bull shark, and Atlantic stingray collected in West Bay, TX, USA

Cloacal swabs from alligator gar contained DNA from 6 fish species, with

Table 3. Total number of swab samples collected (n), number of individuals with host and/or prey DNA, sampling season, life stage, number by sex, mean size, and number of DNA sequences read from alligator gar, bull shark, and Atlantic stingray collected in West Bay, TX, USA. Sizes are displayed as total length for alligator gar and bull shark, and disc width for Atlantic stingray

Species	Number of samples		Season		Life stage		Sex		Mean size (range), cm	DNA sequence reads
	Total	With host and/or prey DNA	Fall	Spring	Juvenile/ intermediate size class	Adult/ large size class	Female	Male		
Alligator gar	6	6	3	3	2	4	3	3	116.78 (139.60–86.40)	79291
Bull shark	6	6	3	3	0	6	3	3	133.17 (152.40–112.00)	187895
Atlantic stingray	6	6	0	6	0	6	6	0	28.32 (31.80–26.00)	301753

striped mullet *Mugil cephalus* recovered from 3 individuals (%FO = 50.0; Fig. 3, Table S5). Cloacal swabs from alligator gar also contained fDNA from 2 other prey categories, including Clupeidae (%FO = 33.3) and Sciaenidae (%FO = 66.7). Similarly, cloacal swabs from bull sharks contained DNA from 10 fish species, with striped mullet and white mullet *M. curema* recovered from 5 and 1 individual, respectively, together representing a %FO of 83.3 for Mugilidae. Other groups identified in the cloacal swabs of bull shark individuals included Clupeidae (%FO = 50.0), Sciaenidae (%FO = 83.3), and Ariidae (%FO = 50.0%), with DNA from various species such as Atlantic menhaden *Brevoortia tyrannus*, red drum, and sea catfishes (*Ariopsis felis* and *Bagre marinus*). Southern flounder *Paralichthys lethostigma* was identified in 2 bull shark cloacal swabs. Cloacal swabs from Atlantic stingrays contained DNA from 7 fish species, with notable detections of Sciaenidae (%FO = 66.7), including species such as spot *Leiostomus xanthurus*, Atlantic croaker *Micropogonias undulatus*, and silver perch *Bairdiella chrysoura*. Additionally, other fish groups were identified including Clupeidae (%FO = 66.7) and Other fish spp. (%FO = 33.3), with DNA from species such as pinfish *Lagodon rhomboides* (%FO = 16.7) and bay anchovy *Anchoa mitchilli* (%FO = 16.7). DNA sequencing for invertebrates in Atlantic stingray also revealed the presence of Bivalvia (%FO = 83.3), Decapoda (%FO = 66.7), Polychaeta (%FO = 33.3), and others. Decapods were represented by snapping shrimp (*Alpheus* sp., %FO = 16.7) and the porcelain crab *Petrolisthes armatus* (%FO = 16.7).

The PERMANOVA based on Bray-Curtis dissimilarity of %FO values for prey categories revealed a significant difference in prey composition among the 3 species (PERMANOVA, $F_{2,16} = 39.32$, $p < 0.001$). Pairwise comparisons revealed significant differences in prey composition between alligator gar and bull shark (PERMANOVA, $F_{1,11} = 24.00$, $p < 0.01$), alligator gar and Atlantic stingray (PERMANOVA, $F_{1,11} = 62.94$, $p < 0.01$), and bull shark and Atlantic stingray (PERMANOVA, $F_{1,11} = 20.42$, $p < 0.01$). The dissimilarity between alligator gar and Atlantic stingray (56.7%) was largely attributed to bivalves (27.8%), followed by Decapoda (22.2%) and Mugilidae (16.7%; Table S6). The dissimilarity between bull shark and Atlantic stingray (52.8%) was primarily explained by the same prey categories as those driving the dissimilarity between alligator gar and Atlantic stingray. The lowest dissimilarity (51.1%) was observed between alligator gar and bull shark, with Ariidae as the main contributor (33.4%), followed by Mugilidae (22.2%) and Other fish spp. (22.0%).

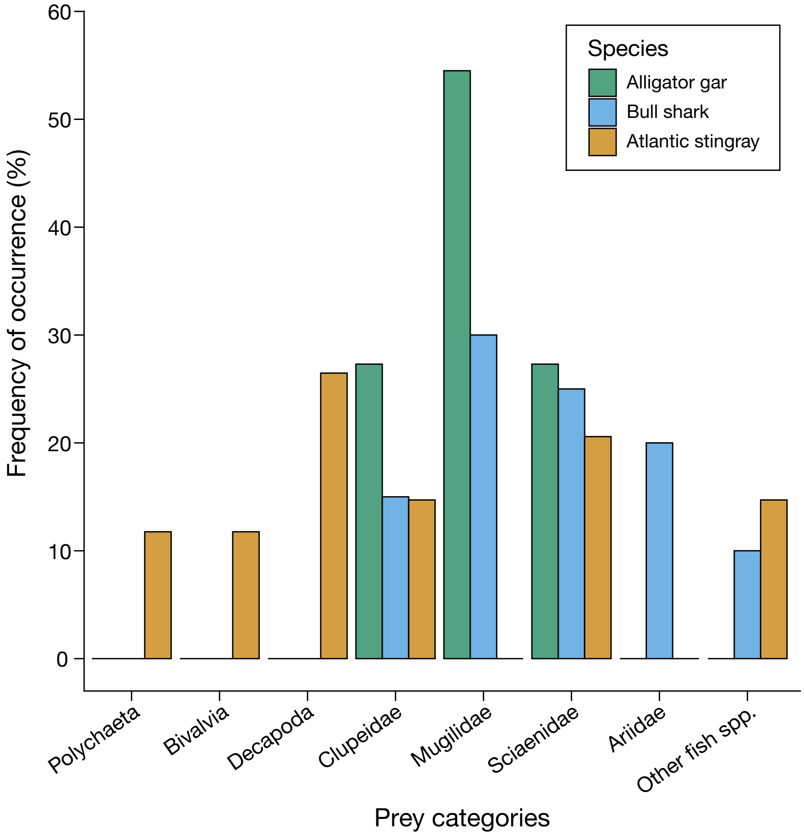

Fig. 3. Frequency of occurrences (FO%) for the prey categories identified in cloacal swabs for fecal DNA of alligator gar, bull shark, and Atlantic stingray

3.3. SIA

Muscle tissue δ^{13}C, δ^{15}N, and δ^{34}S values were measured for 79 alligator gar, 19 bull sharks, and 52 Atlantic stingrays (Table 4). There were significant differences among predatory species for both δ^{13}C and δ^{34}S values (PERMANOVA, $p < 0.05$ for all tests; Table S7), but no differences for δ^{15}N values (PERMANOVA, $F_{2,148} = 8.90$, $p = 0.571$). Alligator gar exhibited more enriched δ^{13}C values compared to Atlantic stingray (PERMANOVA, $p = 0.001$). There was no significant difference in δ^{13}C values between bull shark and either alligator gar or Atlantic stingray (PERMANOVA, $p > 0.05$

Table 4. Total number of white muscle samples collected (n), mean size, stable isotope values ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$), and trophic position estimated from nitrogen stable isotope values (TPSI) of alligator gar, bull shark, and Atlantic stingray. Sizes are displayed as total length for alligator gar and bull shark, disc width for Atlantic stingray

Species	n	Mean size (range), cm	$\delta^{13}C$ mean ± SE	$\delta^{15}N$ mean ± SE	$\delta^{34}S$ mean ± SE	TPSI mean ± SD
Alligator gar	79	102.59 (145.50–71.80)	−17.28 ± 0.18	15.52 ± 0.19	10.70 ± 0.16	4.02 ± 0.57
Bull shark	19	114.07 (156.90–90.30)	−18.05 ± 0.29	15.88 ± 0.28	12.52 ± 0.30	4.40 ± 0.11
Atlantic stingray	52	25.99 (38.60–14.80)	−18.50 ± 0.26	15.43 ± 0.22	13.05 ± 0.36	3.92 ± 0.80

for all tests). The $\delta^{34}S$ values were more enriched for alligator gar compared to bull shark and Atlantic stingray (PERMANOVA, $p < 0.01$ for all tests), with no significant differences in $\delta^{34}S$ values between bull shark and Atlantic stingray (PERMANOVA, $F_{1,70} = 0.71$, $p = 0.43$).

When PERMANOVA was performed on each species separately, significant differences between subgroups were found. For alligator gar, significant differences in $\delta^{13}C$ values were observed between seasons, with individuals captured in the fall showing more enriched $\delta^{13}C$ values (mean ± SE, $\delta^{13}C = -16.62 \pm 0.35$‰; Table S8) compared to those captured in the spring ($\delta^{13}C = -17.55 \pm 0.19$‰, PERMANOVA, $F_{1,78} = 6.17$, $p = 0.015$). $\delta^{15}N$ and $\delta^{34}S$ also varied between life stages for alligator gar and sexes, respectively, with juveniles showing more depleted $\delta^{15}N$ values ($\delta^{15}N = 14.76 \pm 0.19$‰) compared to adults (n = 52, $\delta^{15}N = 15.91 \pm 0.26$‰), and females exhibiting more depleted $\delta^{34}S$ values ($\delta^{34}S = 10.32 \pm 0.24$‰) than males ($\delta^{34}S = 10.99 \pm 0.20$‰; PERMANOVA, $p < 0.05$ for all tests). There were significant differences in $\delta^{15}N$ values between sexes for bull shark, with females displaying more depleted $\delta^{15}N$ values ($\delta^{15}N = 15.17 \pm 0.38$‰) than males ($\delta^{15}N = -16.67 \pm 0.20$‰, PERMANOVA, $F_{1,18} = 11.1$, $p = 0.007$). Differences between sexes were found for Atlantic stingray, with females displaying more enriched $\delta^{34}S$ values ($\delta^{34}S = 13.50 \pm 0.40$‰) than males ($\delta^{34}S = 12.04 \pm 0.73$‰) (PERMANOVA, $F_{1,51} = 4.00$, $p = 0.047$).

Similar to the niche breadths calculated from SCA, Atlantic stingray showed the widest stable isotope niche hypervolumes (Fig. 4, Table 5). Isotope niche size for Atlantic stingray was 52.96‰^{2} for $\delta^{13}C$ and $\delta^{15}N$, 75.87‰^{2} for $\delta^{34}S$ and $\delta^{15}N$, and 104.28‰^{2} for $\delta^{34}S$ and $\delta^{13}C$. Alligator gar exhibited intermediate isotopic niche sizes between bull shark and Atlantic stingray, with values of 40.58‰^{2} ($\delta^{13}C$ and $\delta^{15}N$), 34.55‰^{2} ($\delta^{34}S$ and $\delta^{15}N$), and 36.29‰^{2} ($\delta^{34}S$ and $\delta^{13}C$). Bull shark had the smallest isotopic niche sizes (30.32‰^{2} for $\delta^{13}C$ and $\delta^{15}N$, 31.74‰^{2} for $\delta^{34}S$ and $\delta^{15}N$, and 34.06‰^{2} for $\delta^{34}S$ and $\delta^{13}C$).

Sørensen's overlap based on stable isotope hypervolumes demonstrated high overlap between alligator gar and bull shark (74.04 to 66.48%; Fig. 4, Table 5). Atlantic stingray exhibited moderate to high levels of stable isotope niche overlap, ranging from 72.72 to 43.30%, with overlap consistently greater with alligator gar compared to bull shark. Additionally, Sørensen's overlap varied depending on the stable isotopes used, with overlap values generally higher for $\delta^{13}C$ and $\delta^{15}N$, followed by $\delta^{34}S$ and $\delta^{15}N$, and $\delta^{34}S$ and $\delta^{13}C$. There were no significant differences in estimates of trophic position based on $\delta^{15}N$ values among species (ANOVA, $F_{2,148} = 0.14$, $p = 0.83$; Table 1). Bull shark demonstrated the highest trophic position (mean ± SD, TPSI = 4.40 ± 0.11), followed by alligator gar (TPSI = 4.02 ± 0.57) and Atlantic stingray (TPSI = 3.92 ± 0.80; Table 4).

4. DISCUSSION

In this study, the integration of SCA, fDNA, and SIA provided a comprehensive framework for elucidating dietary composition and trophic overlaps among 3 estuarine predatory fishes. Results from SCA and fDNA were largely congruent, indicating that alligator gar and bull sharks predominantly consumed teleost fish, while Atlantic stingrays primarily fed on benthic invertebrates. The fDNA approach enhanced prey detection by revealing a wider array of prey at the species level than SCA, which was limited in most cases to family-level identification. The use of carbon, nitrogen, and sulfur isotopes enhanced the resolution of isotopic comparisons among the 3 predator species allowing for the detection of subtle differences in dietary sources and highlighting the utility of triple-isotope analysis. Together, these complementary methodologies provide a robust and nuanced understanding of predator trophic ecology, highlighting the complexity of trophic interactions within estuarine ecosystems.

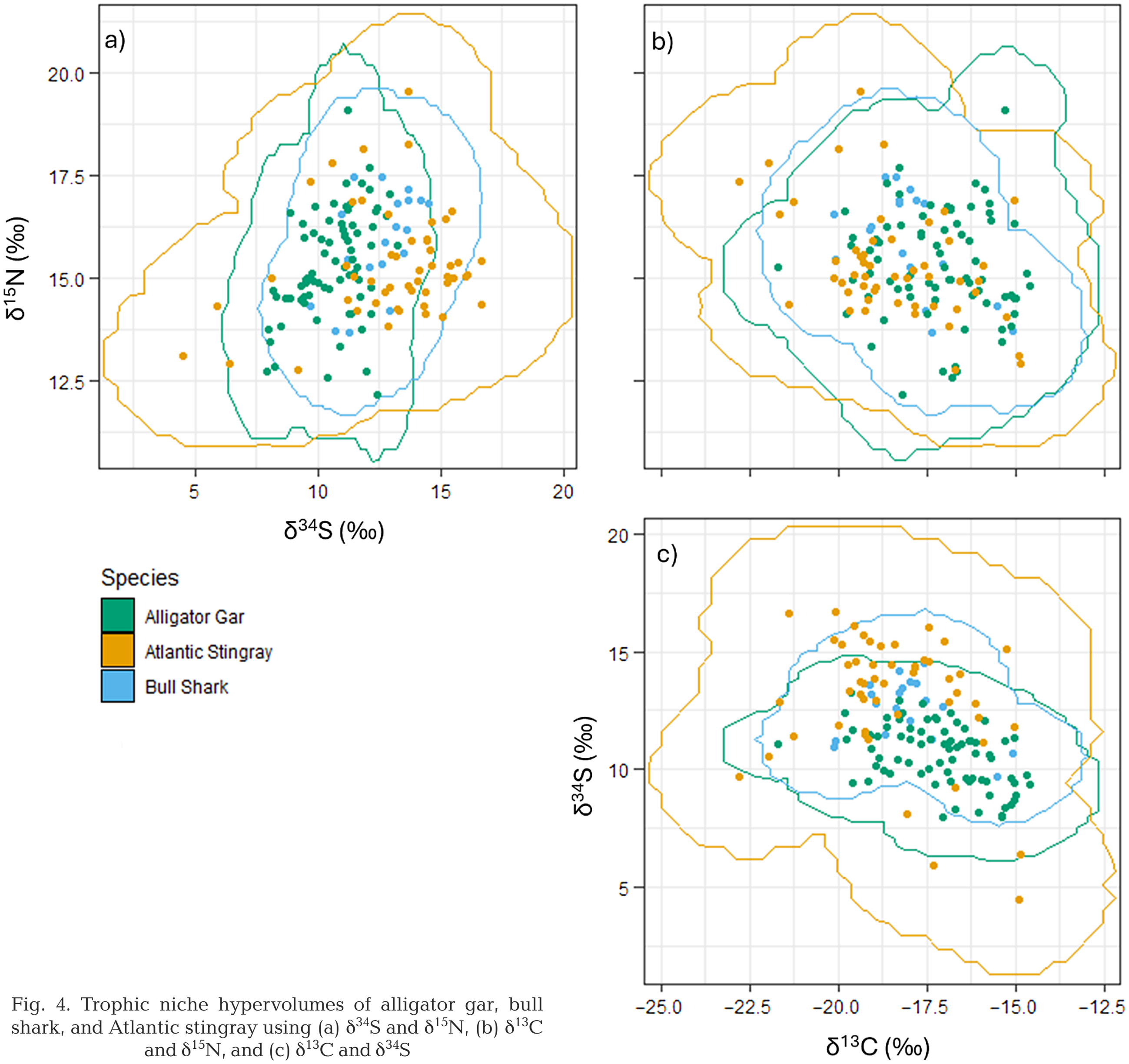

Fig. 4. Trophic niche hypervolumes of alligator gar, bull shark, and Atlantic stingray using (a) $\delta^{34}S$ and $\delta^{15}N$, (b) $\delta^{13}C$ and $\delta^{15}N$, and (c) $\delta^{13}C$ and $\delta^{34}S$

Table 5. Hypervolume size of alligator gar, bull shark, and Atlantic stingray and Sørensen's overlap in hypervolume space between each pairwise comparison using $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$

	Species	Hypervolume size (‰^{2})	Species pairwise	Sørensen's overlap
$\delta^{13}C$ and $\delta^{15}N$	Alligator gar	40.58	Alligator gar vs. bull shark	74.04
	Bull shark	30.32	Alligator gar vs. Atlantic stingray	72.72
	Atlantic stingray	52.96	Bull shark vs. Atlantic stingray	67.27
$\delta^{34}S$ and $\delta^{15}N$	Alligator gar	34.55	Alligator gar vs. bull shark	65.49
	Bull shark	31.74	Alligator gar vs. Atlantic stingray	56.17
	Atlantic stingray	75.87	Bull shark vs. Atlantic stingray	56.08
$\delta^{34}S$ and $\delta^{13}C$	Alligator gar	36.29	Alligator gar vs. bull shark	66.48
	Bull shark	34.06	Alligator gar vs. Atlantic stingray	46.17
	Atlantic stingray	104.28	Bull shark vs. Atlantic stingray	43.3

4.1. Seasonal, life stage, and sex-based trophic profiles

Overall, the assessment of season, life stage, and sex-based trophic profiles did not reveal clear distinctions between subgroup of each species, which may have been constrained by the opportunistic sampling methods employed (Martinez-Andrade 2018). Stomach contents showed no differences in diet across season, life stage, or sex for any species. Previous studies based on SCA in the western Gulf of Mexico with larger sample sizes (i.e. n >100 stomach contents) revealed distinct feeding patterns across seasons and life stages for both alligator gar and bull sharks. Alligator gar exhibited a seasonal shift in diet, with Clupeidae consumption decreasing and Mugilidae increasing from spring to fall (Marsaly et al. 2023), while bull shark consumption of Ariidae increased as Clupeidae consumption decreased with size (TinHan & Wells 2021). Therefore, our results likely highlight the limitations of SCA, particularly the need for large sample sizes to obtain reliable quantitative data (Cortés 1997). This limitation is common in many dietary studies of aquatic predators (Pethybridge et al. 2011, Rohner et al. 2013), reinforcing the need to integrate isotopic and molecular tools to overcome biases associated with SCA. In contrast to alligator gar and bull sharks, intraspecific variation in the diet of Atlantic stingrays based on SCA has not been well described, limiting the ability to compare our findings with other studies. The absence of intraspecific dietary differences in the Atlantic stingray, however, coincides with a high degree of intraspecific co-occurrence observed for this species in West Bay (Fontaine et al. 2024), as well as periodic aggregations in batoid communities, where individuals feed and rest in groups, both with conspecifics and other species (Vaudo & Heithaus 2012, Kanno et al. 2019). As such, differences among Atlantic stingrays were not expected.

SIA provided better insight into intraspecific diet variation compared to SCA, likely because it offers time-integrated information on assimilated, rather than ingested prey (Hussey et al. 2012). Alligator gar exhibited the most pronounced variation in diet, with differences among sub-groups observed in all stable isotopes (i.e. δ^{13}C, δ^{15}N, and δ^{34}S). In estuarine ecosystems, sources of organic matter along salinity gradients tend to have distinctive δ^{13}C values, with an increase in δ^{13}C from the upper estuary downstream towards the lower estuary (Fry 2002). Due to slow turnover rate of muscle tissues (i.e. on the order of many months), higher δ^{13}C values observed for alligator gar captured during the fall may therefore represent the diet integrated during the spring when alligator gar expand their habitat use throughout the GBC (Livernois et al. 2021, Fontaine et al. 2024). Tissues with faster turnover rates, such as blood plasma, may have provided better insight into seasonal variation and short-term shifts in foraging behavior that muscle tissue may obscure (Matich & Heithaus 2014). Higher δ^{15}N values for adult alligator gar may also be attributed to an ontogenetic shift to larger fish prey (Snow & Porta 2020). Size-based shifts in diet are common in teleost fishes due to morphological changes (e.g. gape, body size) which enable adults to access larger fish prey (Scharf et al. 2000). Interestingly, variation in diet across sexes, which is often overlooked (Karachle & Stergiou 2008), was observed for all study species. Males and females often exhibit morphological (e.g. body size) and behavioral (e.g. habitat use) differences across many fish populations, resulting in sex-based differences in resource use and ultimately stable isotope values (Wearmouth & Sims 2008). Sexual dimorphism is observed in alligator gar, with females having longer snouts than males (McDonald et al. 2013), potentially enhancing foraging success (Love 2002). In bull sharks, sex-based spatial segregation has been identified, with females likely segregating for reproductive reasons or to access more energy-rich prey (Werry & Clua 2013, Lara-Lizardi et al. 2022). Similar dietary differences linked to sex-based segregation, size, and dental dimorphism have been reported for batoids (Ajemian & Powers 2014, de Sousa Rangel et al. 2016, Jargowsky et al. 2020). Whether sex-related variation in foraging behavior, habitat use, or both drives the observed differences in stable isotope values remains uncertain; however, our results emphasize the importance of considering sex, along with season and life stage, when assessing trophic dynamics, as these factors can significantly influence the trophic roles of predators.

In addition to the seasonal, life stage, and sex-based trophic patterns observed in this study, other ecological factors may also influence the trophic ecology of alligator gar, bull sharks, and Atlantic stingrays in the western Gulf of Mexico. For example, time-lagged abiotic factors, such as temperature and chlorophyll *a* concentration, may play a role in shaping predator diet. Marine and estuarine ecosystems often show delayed responses to environmental conditions, where the effects of chlorophyll concentrations are not immediately reflected in predator trophic dynamics, but rather with a temporal lag (Olden & Neff 2001, Wang et al. 2018). Chlorophyll *a*, a key indicator of primary productivity, accumulates over a period of time before being reflected in higher trophic positions (Wang et al. 2018), suggesting that the prey availability for these species may be influenced by conditions in previous months. Furthermore, biotic interactions, such as competition or predation, and the location of feeding, spawning, or overwintering grounds, may also affect the trophic roles and distribution of these species (Yates et al. 2018, Bouchet et al. 2019). The interplay of these additional temporal environmental variables merits further investigation to fully understand the drivers of resource use in estuarine predators.

4.2. Interspecific trophic dynamics

All 3 of the methods employed in this study indicated that alligator gar, bull sharks, and Atlantic stingrays feed on a wide range of prey items, with teleosts and decapods as the predominant prey among all predators. This generalist feeding strategy mirrors patterns observed in other estuarine predators like red drum and spotted seatrout, which also exhibit broad dietary niches influenced by prey availability and environmental conditions (Llanso et al. 1998, TinHan et al. 2018). The diet of alligator gar revealed through stomach contents was dominated by Clupeidae and Mugilidae, accounting for 70.2% PSIRI. fDNA analysis further confirmed the prevalence of Atlantic menhaden, gizzard shad *Dorosoma cepedianum*, and striped mullet, with Sciaenidae also identified through both SCA and fDNA. These findings are consistent with previous studies, which have highlighted the importance of Clupidae and Mugilidae in the diet of alligator gar (Marsaly et al. 2023, Livernois et al. 2024). A high vacuity index for alligator gar (49.4%), coupled with low DNA sequence readings, likely reflects the infrequent capture of prey and reduced fecal material, consistent with their ambush predation strategy, which involves sporadic but highly successful foraging events (Butler et al. 2019).

Based on their stomach contents, bull sharks frequently consumed Mugilidae, which is consistent with previous studies that also identified Mugilidae as a key prey group for this species (TinHan & Wells 2021, Marsaly et al. 2023). Mugilidae also had the highest %FO (30%), with both striped mullet and white mullet identified. Clupeidae and Sciaenidae were also important prey categories for bull sharks, though Clupeidae were only detected through fDNA. This dietary pattern resulted in a 56% diet overlap with alligator gar. A notable difference in the diet of bull sharks was the significant presence of Ariidae in both stomach contents and DNA sequencing; ariids were less prominent or absent in alligator gar. The presence of ariids suggests that juvenile bull sharks are demersal foragers but also feed on species in the upper water column, such as Mugilidae (Clark & von Schmidt 1965, van Zinnicq Bergmann et al. 2021).

Atlantic stingrays predominantly consumed decapods, supplemented by mollusks, polychaetes, and small fishes, confirming previous observations from the Gulf of Mexico (Cook 1994, Bradley 1996). High contributions of Decapoda (%PSIRI = 77.0) to the diet of Atlantic stingrays led to the small overlap observed with both alligator gar (11.5%) and bull sharks (9.1%). fDNA analysis identified species such as snapping shrimp and porcelain crab along with 7 fish species, highlighting the broad trophic niche of Atlantic stingrays (Ba = 0.63). The high proportion of empty stomachs in Atlantic stingrays (42.3%), along with the high number of these prey in stomachs when non-empty (i.e. up to 13 shrimp in 1 stomach), suggests an ambush predation strategy. In this study, the integration of fDNA analysis with traditional SCA enhanced dietary assessments by identifying prey items that may be underrepresented or overlooked in stomach contents alone.

Interspecific differences in stable isotope values were observed except for δ^{15}N values. As previously postulated, the respective organic sources along salinity gradients tend to have distinctive stable isotope values (Fry 2002). δ^{13}C values for fishes range from −21 to −25‰ in the upper estuary and above −20% in the lower estuary (Fuji et al. 2011). This gradient is also observed for δ^{34}S, with fishes from riverine and upper-estuarine habitats exhibiting more depleted δ^{34}S values (−5 to +5‰), while fishes from the lower, more marine portions of estuaries display more enriched δ^{34}S values (11−17‰; Fry & Chumchal 2011). δ^{13}C and δ^{34}S values of the study species closely align with those of fishes in lower estuarine and marine environments, indicating a reliance on marine-derived organic matter as a primary source of carbon and sulfur. It should be noted, however, that alligator gar exhibited significantly more depleted δ^{34}S values compared to other species, suggesting a greater reliance on freshwater-derived organic matter. This observation is consistent with habitat preferences of alligator gar, which occupy estuarine environments but are typically found near sources of freshwater inflow (Allen et al. 2017, Daugherty et al. 2017).

While uniform isotopic baselines are often assumed (Pethybridge et al. 2018, Shipley et al. 2021) in areas like West Bay where freshwater and marine ecosystems meet, multiple stable isotope baselines can persist in a relatively small geographic region (Shipley et al. 2019a). Consequently, without consistent data that account for spatiotemporal variation in stable isotope baselines, it is difficult to ascertain whether the observed differences in stable isotope values are attributable to variations in foraging habitats and/or prey consumed. Atlantic stingray displayed the largest stable isotope niche, encompassing a substantial portion of the niche space occupied by the other species, and this pattern was consistent across all stable isotope comparisons. The larger niches of Atlantic stingrays resulted in consistently greater percentages of unique niche space relative to alligator gar and bull sharks. As a result, alligator gar and bull sharks, with smaller trophic niches, experienced greater overlap with each other, which coincides with similarities in habitat suitability and frequent co-occurrence patterns (Livernois et al. 2021, Fontaine et al. 2024). While niche overlap was most notable between alligator gar and bull sharks, it was greater than 40% between any species combination, which is consistent with the findings of Marsaly et al. (2023). However, this observation contrasts with overlap values determined by Livernois et al. (2024), where niche overlap was notably lower (i.e. <50%), suggesting methodological differences in assessing niche overlap. The high overlap observed in our study does not necessarily mean that species are feeding on the same prey, as suggested by diet results, but rather reflects similarities in habitat use in West Bay. This distinction between dietary overlap and habitat-driven isotopic similarity is a recurring theme in predator ecology and highlights the importance of using multiple methods to disentangle stable isotope-derived ecological niches from actual prey consumption across different systems.

4.3. Ecological role of alligator gar, bull shark, and Atlantic stingray in West Bay

Trophic positions (TP) estimated from stomach contents were generally close to values reported for alligator gar (TP = 3.15), juvenile bull shark (TP = 3.65, Marsaly et al. 2023) and Dasyatidae (TP = 3.5, Jacobsen & Bennett 2013) and consistent with values reported using stable isotope-based trophic positions (TP = 4.02 and TP = 4.27 for alligator gar and bull shark, respectively, Livernois et al. 2024). Our estimation of trophic position based on stable isotopes was lower than that based on stomach content. SCA and SIA can produce different results for trophic position due to the different temporal integration of the diet, and in our case, the limited information available on baselines to estimate trophic position (Olin et al. 2013, Matich et al. 2021). Trophic position estimates among species ranged from 3.3 to 4.4, depending on the method and species, confirming high trophic positions of alligator gar and bull shark and the mesopredator role of Atlantic stingray.

The combination of stomach content data, DNA metabarcoding, and SIA indicates that alligator gar and bull sharks exhibited some similarities in diet and habitat uses. However, substantial differences in diet compositions, DNA sequences, and stable isotope values suggested that these 2 species occupy distinct ecological roles within estuarine food webs. Alligator gar likely play a more prominent role in trophic regulation in low-salinity habitats, while bull sharks are more influential in higher-salinity environments. These differences are critical for understanding energy flow through estuarine ecosystems, as each species interacts with different trophic positions and prey assemblages (Elliott et al. 2002). However, this distinction in ecological roles may not be absolute, as both species demonstrate considerable flexibility in their foraging behaviors. The predominance of Mugilidae and Clupeidae in their diet suggests that both alligator gar and bull sharks may face shared challenges if menhaden and/or mullet populations are overharvested in Texas estuaries, as these species are critical bait fishes for regional fisheries. This is particularly important for management strategies focused on ensuring the sustainability of key species and maintaining the integrity of food webs in estuarine ecosystems. As natural and anthropogenic disturbances alter coastal ecosystems, the flexible foraging strategies of alligator gar and bull sharks may enhance their persistence by allowing dietary adjustments to local fitness trade-offs (Marsaly et al. 2023). However, these strategies may also increase resource overlap in chronically disturbed systems, potentially altering trophic dynamics and leading to long-term consequences for competitive interactions among these 2 species (Fontaine et al. 2024). Such changes in resource use could be especially pronounced for juveniles, which may not fully exploit the broader range of resources available to larger individuals This observation reflects differences in resource use during early life stages and highlights how shifts in trophic interactions may alter community structure. Therefore, understanding trophic ecology and habitat use is essential for predicting how these species will respond to ongoing environmental changes and evaluating the potential impacts on community dynamics and ecosystem stability. This knowledge can inform effective management practices and help prioritize conservation efforts to ensure the resilience of estuarine ecosystems under changing environmental conditions.

4.4. Complementary insights from SCA, fDNA, and SIA

The integration of SCA, fDNA, and SIA offers a comprehensive and complementary toolkit for elucidating predator feeding ecology, as each method contributes unique insights into diet composition and trophic interactions (albeit with inherent limitations), thereby underscoring the necessity of combining methods to build a more accurate portrait of predator diets. SCA offers direct evidence of an integrated diet over the course of hours to days and is extremely limited in taxonomic resolution and temporal resolution due to digestion rate (Hyslop 1980, Reñones et al. 2002). This caveat has long been reported in the literature, especially for soft-bodied prey with faster rates of digestion that are commonly underrepresented (Garnett 1985, Delany & Abercrombie 1986). Conversely, fDNA provides higher taxonomic resolution than SCA by detecting prey DNA post-consumption, although the precise duration of prey signal detectability remains uncertain (de Sousa et al. 2019). This temporal resolution, combined with fine-scale prey discrimination, enables a detailed characterization of predator diet, particularly for soft-bodied or highly digestible prey that may be underrepresented or missed entirely by SCA. However, like other dietary analysis methods, fDNA metabarcoding has limitations (Alberdi et al. 2019), including the potential detection of secondary predation (Bessey et al. 2019), inability to quantify absolute intake (Deagle et al. 2019), host DNA interference (Pompanon et al. 2012), and the need for taxon-specific primers to recover prey diversity comprehensively. On the other hand, SIA complements SCA and fDNA by integrating assimilated dietary information over tissue-specific metabolic rates (i.e. faster tissue isotopic turnover rates of ~30 d), thereby reflecting trophic relationships and habitat use beyond immediate feeding events (Kim et al. 2012, Malpica-Cruz et al. 2012, Zeichner et al. 2017, Alberdi et al. 2019). With the application of multiple stable isotopes, SIA also enables the characterization of ecological niches by examining the dispersion of stable isotope values across multiple elemental axes, thereby providing a composite view of resource use and trophic variability among predators (Bearhop et al. 2004, Newsome et al. 2007). However, considerations must be made when applying SIA, as multiple factors drive isotopic variability and influence the ultimate geometry of observed niche dimensions (Shipley & Matich 2020). Together, these approaches overcome individual limitations and biases: SCA captures short-term diet composition, fDNA enhances taxonomic resolution, and SIA elucidates broader trophic interactions. This multi-method framework enhances dietary reconstruction while offering a more nuanced perspective on trophic interactions and ecological roles of predators. Therefore, we encourage integrating all of these methods together wherever possible to achieve a more comprehensive understanding of trophic interactions.

4.5. Conclusion

This study provides novel insights into the feeding ecology of alligator gar, bull shark, and Atlantic stingray across a salinity gradient by integrating 3 complementary approaches. The results reveal feeding preferences of alligator gar and bull sharks for teleosts and of Atlantic stingrays for crustaceans. Additionally, differences in stable isotope values highlight distinct resource use among these species, reflecting their unique ecological roles within estuarine ecosystems. Intra-specific assessment remains preliminary, as the number of samples was low, therefore limiting the robustness of the conclusions. Further studies should be conducted to better understand seasonal, ontogenetic, and sex-related changes in diet and habitat use, especially for Atlantic stingrays, where such information remains scarce. These findings contribute to a deeper understanding of trophic dynamics in estuarine ecosystems and provide a valuable foundation for future research on the ecological interactions and adaptive responses of these predators to natural and anthropogenic disturbances.

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