CHARACTERIZING DIET OF THE LEAST TERN *STERNULA ANTILLARUM* USING DNA METABARCODING

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ABSTRACT

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A complete picture of diet composition is an essential element to understanding the ecological role of organisms. Moreover, diet studies can serve as an important tool for monitoring species and changes to the food web. One method to provide resolution when studying the diet of avian species is DNA metabarcoding of fecal samples. As such, we used DNA analysis to determine the diet of the Least Tern *Sternula antillarum* and compare results with diet analysis based on composition of fish dropped within breeding colonies. Comparisons between adult and chick fecal samples were also made across three years and within three zones of sample collection. Results show differences in diet composition between the two methods as well as across zones and years. Significant differences between prey items of adults and chicks were also identified. Metabarcoding data indicate that Least Terns are consuming Lionfish *Pterois* spp. (most likely in larval stages), a prey item that had not been previously recorded for Least Terns, and that data obtained from dropped fish might not be representative of chick diet. Differences across years and zones are likely due to shifts in the abundance and availability of prey items.

Key words: diet analysis, DNA metabarcoding, fecal samples, Least Terns, shore birds

INTRODUCTION

Understanding diet is a key component in discerning the ecological role of an organism. Dietary studies on avian species provide insights into their ecology along with temporal changes in their environment (Jordan 2005, Gaglio *et al*. 2017). Therefore, diet studies can serve as important tools for monitoring changes to the food web (Green *et al.* 2015), as diet can be an indicator of the needs of an avian species, thus providing insight into when protection of habitats and/or ecological resources is warranted. For success of the latter, accurate identification of prey items is essential (Esnaola *et al*. 2018).

Identification of avian prey items has typically been carried out using analysis of stomach contents, direct observation of feeding or food remnants in pellets, and identification of dropped fish near colonies (Atwood & Kelly 1984, Wilson *et al*. 1993). Such conventional approaches can have several limitations. For example, prey items from stomach contents and pellets are not always identifiable due to digestion. Further, dropped prey may be used to indicate a prey item, but the prey item may not actually have been consumed. Finally, direct observations are often inadequate for juvenile or larval prey that are difficult to identify, and for small prey captured and consumed underwater. An advantage of direct observations is assessment of prey size for those items observed by eye, which is not possible with molecular methods.

Given the challenges with conventional diet sampling for birds, methodological advances, such as DNA metabarcoding and high throughput sequencing, have allowed for high-resolution diet analysis in birds. Specifically, DNA metabarcoding of fecal samples can provide a non-invasive, accurate, as well as time- and cost-effective method to determine animal diets (Ando *et al*. 2020). One drawback is that DNA metabarcoding cannot readily distinguish primary from secondary prey, although abundance of read can, in some cases, indicate level of prey consumption. Regarding seabirds, Cavallo *et al*. (2018) used DNA fecal-metabarcoding to determine the prey consumed by Little Penguin *Eudyptula minor*, finding gelatinous and crustaceous plankton that had not been previously detected in 76% of the samples analyzed. Similarly, McInnes *et al*. (2016) used a similar method to determine that there was no significant difference in diet between chicks and adults of Adelie Penguins *Pygoscelis adeliae*. Likewise, Gerwing *et al.* (2016) revealed that Semipalmated Sandpipers *Calidris pusilla* function as generalist foragers, revealing several novel prey items. Each of these studies provided a greater understanding of the prey items consumed by these animals.

To date, no molecular studies have focused on the diet of a commonly encountered, migratory seabird, the Least Tern *Sternula antillarum*, the focus of this study. The Least Tern breeds on barren sandy substrates along rivers and coasts in North America (Thompson *et al*. 1997). Currently, there are three Least Tern subspecies described that correspond to their geographic distribution, although molecular studies indicate that the separation into subspecies is questionable (Draheim *et al*. 2010).

The diet of the Least Tern has been conventionally studied since 1950, with findings indicating that individuals primarily feed on small fish,

with occasional invertebrate prey items (Atwood & Kelly 1984). A deeper understanding of the diet utilized by the Least Tern, however, could provide valuable data for conservation efforts. In this study, DNA metabarcoding of fecal samples was utilized to examine and compare fish prey items of Least Terns from breeding colonies over a three-year period. Fecal-metabarcoding results were also compared to dropped fish data collected simultaneously. Fecal samples from adults and chicks were compared from a selection of sites.

METHODS

Study site

This study was conducted along the 42.5-km stretch of sandy beach along the mainland shore of Harrison County, Mississippi, and at a gravel rooftop nesting site in Jackson County, Mississippi (Fig. 1), during the breeding seasons (May–August) of 2017–2019. The Harrison County shoreline was created in front of a seawall that separates the 50–130 m beachfront from a four-lane highway. The seawall also serves as a multi-use trail for pedestrians and cyclists. Beaches are groomed on a weekly or biweekly basis throughout the year, and sections are renourished with dredged sand during the winter, as occurred in 2017/18. Some beaches contained constructed sand dunes ~1 m high that had been planted with Sea Oats *Uniola paniculata*. Habitat across the highway from the beach ranged from commercial developments (malls and hotels) to open parkland with mature Live Oaks *Quercus virginiana*. The gravel rooftop on a commercial building was a flat, 0.15-ha (1500 m^2) in area with a surface of tar and pea gravel. Audubon staff monitored this site in 2018 and 2019 and were given permission to access the roof for breeding surveys.

The number of breeding colonies in the study area ranged from 10 to 17 per year, with some colonies unoccupied in some of the years. Breeding activity and breeding success, and therefore opportunities to collect dropped fish or fecal samples, also varied by site and year. As a result, rather than attempting to model site- and year-specific patterns in diet, breeding colony locations were designated into one of three larger zones (Zones 1–3; Fig. 1) based on hypothesized differences in foraging habitat available to birds breeding in each zone. Zone 1 contained the western half of Harrison County beach colonies, with likely foraging areas including the Mississippi Sound north and west of Cat Island, and St. Louis Bay. Zone 2 contained the eastern half of Harrison County, with a 7-km gap between Zones 1 and Zones 2, and with available foraging area in the Mississippi Sound north of Ship Island. This area is less constricted by barrier islands than Zone 1 and is farther away from the freshwater influxes of St. Louis Bay and Lake Pontchartrain. The Jackson County rooftop colony comprised all of Zone 3 due to its isolated nature and inland location relative to the beach colonies; suspected foraging areas included Fort Bayou to the north and the mouth of the Biloxi River to the south.

Fig. 1. Study sites were located along the 42.5-km stretch of sandy beach along the mainland shore of Harrison County, Mississippi, and at a gravel rooftop nesting site in Jackson County, Mississippi. Least Tern *Sternula antillarum* fecal samples were collected during the 2017–2019 breeding seasons. Locations are colored for year sampled and are divided into Zones 1, 2, and 3.

All beach areas were open to public use and easily accessed by parking bays located at regular intervals along the highway. To establish specific areas used by Least Terns, Audubon biological staff scouted the entire length of beach one to two times per week (starting surveys at sunrise and continuing until 12h00 or later, depending on the number of active colonies and number of staff available), looking for male-female courtship and formation of nest scrapes. An area with scrapes was marked with red flags on metal stakes to alert the beach groomers to avoid the area. Staff monitored flagged areas every 3–5 d, and if terns began laying eggs, the area was roped and signed on three sides (left open along the high tide line) using 3-m wooden stakes, nylon cord, and metal signs encouraging the public to stay out of the area. Staff attempted to maintain a 20-m buffer between the outermost nests in the colony and the rope (except along the northern seawall).

Audubon staff conducted surveys at colonies throughout the breeding seasons, visiting at least once per week until all adults and fledglings had dispersed. Staff counted the number of active nests, flightless chicks, and fledglings using either an exterior or interior survey protocol. Staff conducted exterior surveys by standing outside the symbolic fencing at one corner of the colony and scanning the interior with binoculars (8 × 42) and scope (60× power). Staff conducted interior surveys by walking systematically through the entire colony, keeping a count of all nests, chicks, and fledglings observed. All colonies were surveyed using a combination of interior and exterior protocols throughout the season. The exterior protocol was employed whenever an interior survey would cause excessive disturbance (though staff would enter these colonies at other times for the purpose of checking nest plots), such as in the case of large colonies, colonies with a large number of flightless but mobile chicks, days with high winds or other inclement weather, hot and sunny days, or when time and logistics otherwise necessitated an exterior survey.

Sample collection

To determine diet composition, both dropped fish and fecal samples were collected from May to July 2017–2019 from 16 Least Tern coastal nesting sites in Mississippi, USA (Fig. 1). Both fecal samples and dropped fish were collected opportunistically during the weekly breeding surveys, as well as during banding efforts in 2019. During breeding surveys, observers collected any fish or invertebrates that could have been dropped by a Least Tern (items roughly < 10 cm in length) found inside the colony interior. Dropped fish were then stored in a freezer and later identified, and their total length (cm) and weight (g) was measured. Least Terns react aggressively toward humans that approach nests or chicks, dive-bombing, vocalizing, and defecating on the intruders. To collect fecal samples, observers examined each other for fresh feces after completing each breeding survey and collected fecal samples from clothing using tweezers (sterilized with ethanol between uses). During 2019, samples were also taken from adults and chicks in-hand during banding if they defecated onto clothing or equipment. Samples were preserved in glass vials containing 96% ethanol. Tubes were stored in a freezer at -20 °C until analysis. Thirty-two (32 adults, 0 chicks) fecal samples were collected from six sites in 2017, 16 fecal samples (16 adults, 0 chicks) were collected from six sites in 2018, and 35 fecal samples (25 adults, 10 chicks) were collected from eight sites in 2019.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from whole fecal samples using the DNeasy® Blood and Tissue Kit (Qiagen) following the protocol of the manufacturer. A negative control was used during the extraction step to ensure DNA contamination did not occur. A 163–185 base pair (bp) fragment of the mitochondrial 12S ribosomal RNA (rRNA) region was amplified using the universal primers MiFish-U-F 5′-GTCGGTAAAACTCGTGCCAGC-3′ and MiFish-U-R 5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′ (Miya *et al*. 2015, Miya *et al*. 2020) with added adapters for high-throughput sequencing. Polymerase chain reaction (PCR) mix for 12.1 µL reaction consisted of 2 µL of DNA template, 6 µL Kapa HiFi HotStart ready mix, 0.7 µL of each primer (10 µM), 0.2 µL bovine serum albumin (BSA), and 12.5 µL water (ddH2O). The PCR cycling protocol consisted of denaturation at 95 °C for 3 min followed by 35 cycles at 98 °C for 30 s, 65 °C for 15 s, and 72 °C for 5 s, followed by a final elongation at 72 °C for 5 min. PCR was conducted in duplicate to increase sample volume for high-throughput sequencing. PCR products were visualized under ultraviolet (UV) light using 2% agarose gel electrophoresis to verify adequate amplification of the target DNA. PCR duplicates amplified from each sample were pooled and quantified using the Qubit dsDNA BR Assay Kit (Sigma-Aldrich). Negative controls (sterile deionized water) were included for each sample that was amplified by PCR to ensure that samples were not contaminated during the amplification process. Libraries were normalized using the SequalPrep Normalization Plate Kit (Invitrogen, Life Technologies) before pooling samples. Libraries were quantified using quantitative PCR (qPCR) and the KAPA Illumina Library Quantification Kit on a LightCycler Real-Time PCR system (Roche Sequencing) according to manufacturer's guidelines. Libraries were run on a NovaSeq 6000 using V1.5 chemistry for Paired End 250 base pair run (Illumina) at the Hubbard Center for Genomics, Sequencing Core Facility (Durham, New Hampshire, USA).

Sequence data processing

The resulting demultiplexed reads were denoised, duplicated, and chimeras were filtered out using QIIME 2 (Boylen *et al*. 2019) and QIIME 2 plugin DADA2 (Callahan *et al*. 2016). Sequences with phred quality values below 20 were removed. Quality Control quality plots were used to remove low quality regions (QC > 30) from forward and reverse reads. Reads were then truncated at 240 bp for the forward reads, and at 220 bp for the reverse reads. All resulting amplicon sequence variants (ASV) were 250 bp long. A cut-off of 95% was used to assign the ASV to Operational Taxonomic Units (OTU) using QIIME 2 and nucleotide BLAST (Johnson *et al*. 2008; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch); however, only hits with 98% sequence similarity or higher were considered valid for species level. Manual Blast was also conducted in cases where OTUs corresponded to species not reported in the Gulf of Mexico. If a different hit was obtained manually, the hit with the highest percentage of similarity was chosen. Non-prey items (e.g., Least Tern, human, dog) were excluded from the analysis. The data have been deposited in GenBank under BioProject ID: PRJNA947766.

Data analysis

Data analysis and statistical tests were conducted in R 4.1.1 using the "vegan" package version 2.5-7 (Oksanen *et al*. 2020). Given the unequal number of reads per OTU per sample, samples were rarified based on sequencing depth using the *rrarefy* function. The rarefied dataset was used in all downstream analysis. Number of reads assigned to each OTU per sample were then converted to

percentages to obtain relative read abundance (RRA) after Hellinger transformation; this measurement is used to reduce bias that causes overrepresentation of prey items present in small quantities (Deagle *et al.* 2018). Frequency of occurrence (FOO) was also calculated for each prey item to provide an overall diet summary. Matrices of community dissimilarity were generated for the number of reads per sample based on the Bray-Curtis index. Differences between fish diet composition by year (2017, 2018, 2019) and zone (Zones 1-3) were tested with permutational multivariate analysis of variance (PERMANOVA) with 999 permutations using the *adonis2* function. Similarity percentage analysis (SIMPER) tests were conducted using the *simper* function to show which OTUs contributed the most for the dissimilarity/similarity composition among different groups (year, zone).

A comparison of fecal samples from adults (n = 20) and chicks (n = 10) was also conducted, but only for 2019, given that samples from chicks were not collected in previous years. To facilitate the comparison, only samples having both chick and adult feces from the same location (Zone 3) were included. Statistical analyses comparing samples from adults and chicks were not conducted given the low sample size.

Barplots of RRA of prey items per sample, and overall fish diet composition by year and zones, were constructed to visualize diet trends across samples and years. OTUs that represented less than 2% of the total prey items were clustered into the category "others."

RESULTS

Metabarcoding

Of the 104 fecal samples collected, 83 showed positive amplification of the expected PCR product and were sent for sequencing. Quality control resulted in 1 877 866 final reads; sample 17-OC1 was removed due to low read count (> 50 reads). Metabarcoding revealed a total of 21 fish prey species (Table 1; Appendix 1, available online: rarefied dataset of samples per location) over three years, from 13 fish families, across 16 sites, with an average number of 2.95 (± 0.98 standard deviation [SD]) taxa per sample (Appendix 2, available online; fish per sample). The number of prey items recovered by year ranged from eight to 12. Nineteen taxa were identified to species, and two only to genus. Of the OTU's identified to genus, one corresponded to Lionfish *Pterois* spp., which showed 100% sequence similarity to Red Lionfish *P. volitans* and Luna Lionfish *P. lunulata*. Thus, discrimination between the two species using metabarcoding was not possible. However, *P. lunulata* has not been reported in the Gulf of Mexico, indicating that this identity is more likely *P. volitans*. The second case corresponded to *Eleotris* spp., originally identified as Spotted Sleeper *E. picta* based on blast results but with low percentage of sequence similarity (< 90%). However, *E. picta* has not been reported for the Gulf of Mexico. Moreover, the only *Eleotris* known to inhabit the Gulf of Mexico (i.e., Spiny-cheek Sleeper *E. pisionis*) does not have a 12S reference barcode, limiting the possibility to identify this species with molecular data at this time.

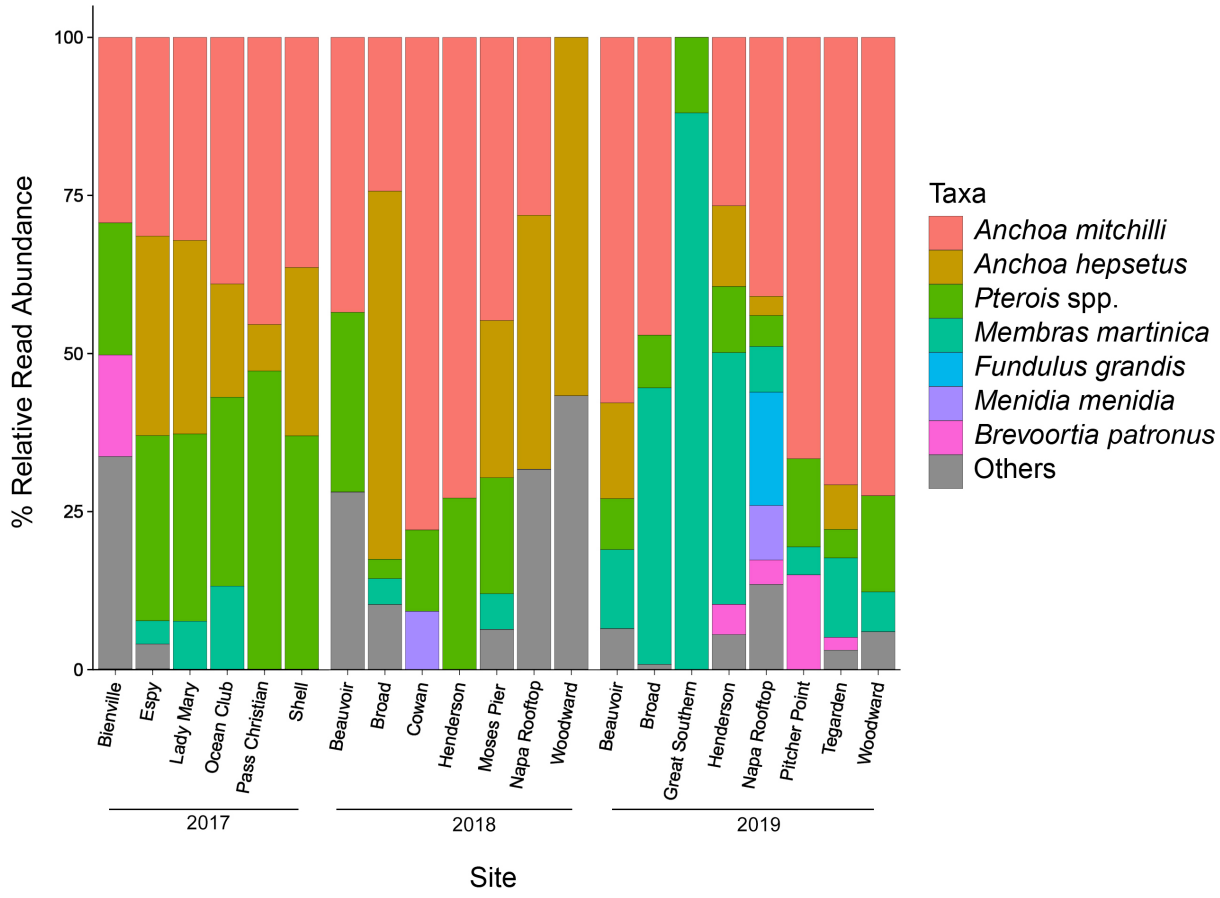

Fig. 2. Fish prey revealed using DNA metabarcoding of fecal samples. Colors are specific to species detected and are indicative of percent of relative read abundance (RRA).

Of the 21 OTU's identified, four taxa accounted for 91% of total prey items (Fig. 2). The taxa having the most representation were Bay Anchovy *Anchoa mitchilli* and Broad-striped Anchovy *A. hepsetus*. These accounted for 45% and 18% of overall RRA and were present in 85% and 62% of the samples (%FOO), respectively. The next most abundant taxa detected were (1) Lionfish *Pterois* spp., accounting for 13.6% of RRA and present in 73% of the samples; and (2) Rough Silverside *Membras martinica*, accounting for 12.7% of RRA and present in 33% of samples. Gulf Killifish *Fundulus grandis*, Atlantic Silverside *Menidia menidia*, Gulf Menhaden *Brevoortia patronus*, and Brook Silverside *Labidesthes sicculus* represented 5% of RRA, while 13 taxa (Table 1) represented < 1% each of the prey items.

Fish diet composition by year

We observed differences from Least Tern fecal samples when comparing fish consumption by year. *Anchoa mitchilli* represented 35% of the RRA for 2017, 45% of the RRA for 2018, and 56% of the RRA for 2019. *A. hepsetus* also showed an increase for the first two years, from 25% to 35% of RRA, but decreased sharply for 2019, representing only 5% of RRA. In contrast, *Pterois* spp. abundance decreased by year, accounting for 30% of RRA for 2017 but only 6% and 3% for 2018 and 2019, respectively. On the other hand, *Membras martinica* accounted for 22% of RRA for 2019, but < 10% for previous years. Importantly, *Menidia menidia* was found in 2018 and 2019 samples, while Gulf Killfish *Fundulus grandis* was only recovered from 2019 samples (Table 1; Fig. 3A). Fish prey items by year were significantly different when comparing RRA for each year (PERMANOVA; $R^2 = 0.19785$, $P < 0.0001$). Three taxa (*A. hepsetus*, *A. mitchilli*, and *Pterois* spp.) contributed the most to the changes observed by year.

Fish diet composition by zone

Anchoa mitchilli was recovered as the most abundant fish prey in fecal samples for the three zones (Fig. 3B). However, RRA of *A. hepsetus* was three times higher for Zone 1 compared to Zones 2 and 3 (24.5%, 13.4%, and 7%, respectively; Fig. 3B). Another difference was evident in observed *Pterois* spp., which was most abundant in Zones 1 and 2 with RRAs of 17% and 12%, respectively, and with an RRA of < 1% for Zone 3. A similar pattern was found for *Membras martinica*, which was mostly recovered in Zone 1 and Zone 2, with RRAs of 11% and 18%, respectively, and with an RRA of only 2.5% for Zone 3. Prey items belonging to *Fundulus grandis* and *Labidesthes sicculus* were only identified with metabarcoding data for Zone 3. Similarly, *Menidia menidia* was predominately recovered for Zone 3. Differences in recovery of fish prey items by zone were significantly different (PERMANOVA; $R^2 = 0.08786$, $P < 0.001$).

Adults vs. chicks (2019)

There were significant differences in fish prey items of adults and chicks in 2019 (Fig. 4). *Anchoa mitchilli* represented 69% of the RRA for adults but only 30% of RRA for chicks ($\chi^2 = 30.7273$, $P = 0.00001$). Moreover, *A. hepsetus* was only recovered from adult samples. Notably, the prey item with the highest RRA in chicks was *Membras martinica* with a representation of 40%, unlike adults which consistently consumed *Anchoa* spp. as the most abundant prey. *Menidia menidia* was only recovered in chick pellets and

TABLE 1
Summary of number of reads, relative read abundance (RRA), and frequency of occurrence (FOO), per year and overall

Taxa	Number of reads	RRA per year (%)			FOO per year (%)			RRA overall (%)	FOO overall (%)
		2017	2018	2019	2017	2018	2019		
Anchoa mitchilli	1 071 942	34.7	46.0	56.0	96.9	75.0	80	45.9	85.5
Anchoa hepsetus	151 825	25.6	35.3	4.9	84.4	68.8	40	18.7	62.7
Pterois spp.	93 446	29.2	6.6	2.8	96.9	56.3	60	13.7	73.5
Membras martinica	280 028	7.6	2.2	22.2	25.0	12.5	51.4	12.7	33.7
Fundulus grandis	73 889	0	0	5.8	0	0	8.6	2.4	3.6
Menidia menidia	36 862	0	0.5	2.6	0	6.3	5.7	1.2	3.6
Brevoortia patronus	16 333	0.3	0	1.5	3.1	0	11.4	0.8	6.0
Labidesthes sicculus	18 671	0	0	1.6	0	0	5.7	0.7	2.4
Opisthonema oglinum	4 935	0.7	1.9	0	6.3	6.3	0	0.6	3.6
Anchoa lyolepis	3 766	0	2.7	0	0	12.5	0	0.5	2.4
Lepomis macrochirus	1 613	1.3	0	0	3.1	0	0	0.5	1.2
Gambusia holbrooki	13 047	0	0	1.1	0	0	2.9	0.5	1.2
Harengula jaguana	2 353	0	2.3	0	0	12.5	0	0.4	2.4
Menticirrhus americanus	7 207	0	0	0.7	0	0	8.6	0.3	3.6
Symphurus plagiusa	1 397	0	1.3	0	0	6.3	0	0.3	1.2
Cyprinella leedsi	1 322	0	1.2	0	0	6.3	0	0.2	1.2
Acipenser oxyrinchus	917	0.6	0	0	3.1	0	0	0.2	1.2
Mugil curema	6 692	0	0	0.5	0	0	5.7	0.2	2.4
Lucania parva	3 194	0	0	0.2	0	0	2.9	0.1	1.2
Eleotris spp.	2 486	0	0	0.1	0	0	2.9	0.1	1.2
Cynoscion regalis	547	0	0	0.1	0	0	2.9	0	1.2

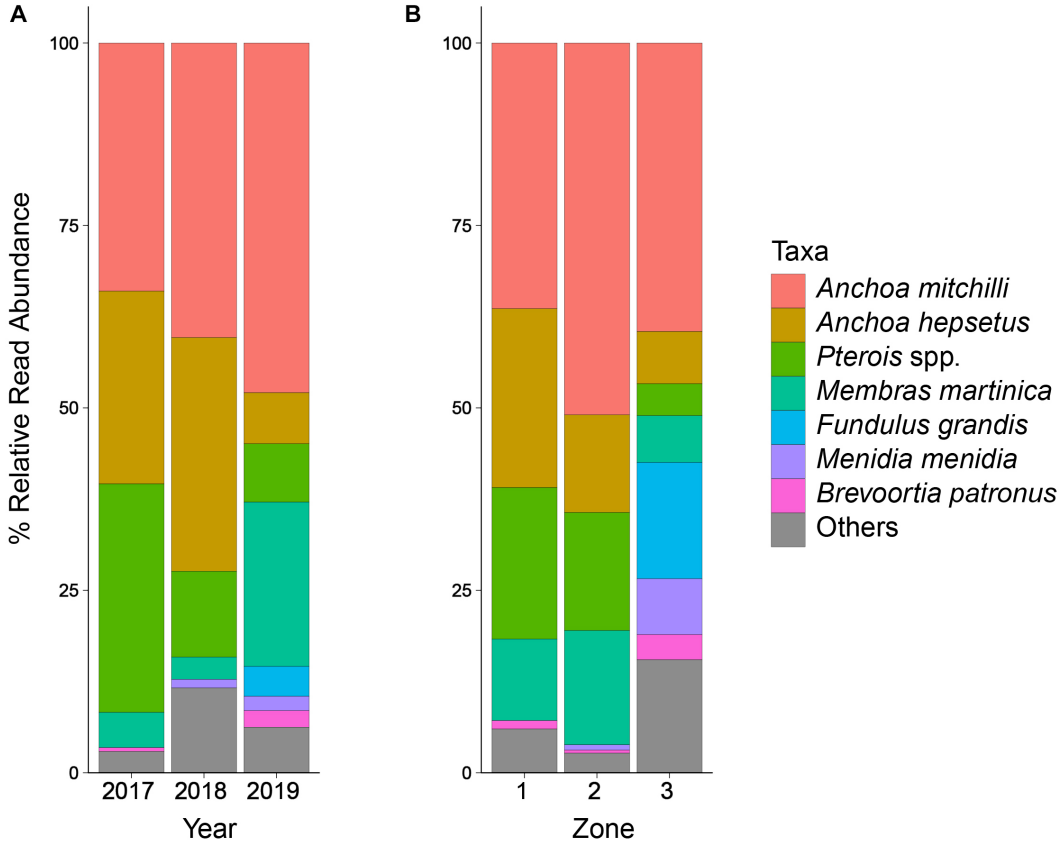

Fig. 3. Relative read abundance (RRA) of diet composition: (A) prey items by year, (B) prey items by zone. Colors are specific to species detected and are indicative of percent of RRA.

was not recovered from adult pellets that shared the same location as chick samples. Although Gulf Menhaden *Brevoortia patronus* was frequently recorded as dropped fish at nesting sites, it was not recovered with metabarcoding data from chick samples.

Dropped fish vs. metabarcoding

Among dropped fish, 32 different prey items were evident, while 21 prey items were recovered with DNA metabarcoding (Table 2). However, only six common species were recorded among dropped fish. The most abundant prey items, such as *Anchoa* spp., were identified by both methods; however, other prey items, such as *Pterois* spp., which was very abundant in 2017 (30% of RRA in adults), have not been previously reported as part of the Least Tern diet and were not recorded among dropped fish. Additionally, there were cases where different species of the same genus were recorded differently depending on the method used. For example, dropped fish included Western Mosquitofish *Gambusia affinis* and Inland Silverside *Menidia beryllina*, while Eastern Mosquitofish *G. holbrooki* and Atlantic Silverside *M. menidia* were recovered with DNA metabarcoding. These differences may be due to misidentification and also could be due to genetic reads lacking in online databases. It is not clear whether one or both species of the same genus constitute prey items of Least Terns.

DISCUSSION

This study investigated the diet of Least Terns at colonies located in the Mississippi Sound area using DNA metabarcoding of fecal samples. Results revealed a diversity of fish prey that varied by time and location. Additionally, diet differed when comparing breeding adults to chicks. Notably, DNA metabarcoding revealed several prey taxa not recovered in previous Least Tern diet studies (Atwood & Kelly 1984, Carreker 1985, Wilson *et al.* 1993, Thompson *et al.* 1997) and increased specificity of prey identification when compared to dropped fish. Though dropped fish totaled 32 prey species, DNA metabarcoding of fecal samples totaled only 21 species, suggesting that Least Terns may potentially be catching fishes they are not consuming. These fishes are likely too large for consumption.

Diet variability by year and zones

Although 21 fish species were identified from fecal samples as components of the adult Least Tern diet, four fish species (*Anchoa mitchilli*, *A. hepsetus*, *Pterois* spp., and *Membras martinica*) were dominant (> 90% RRA). Adult Least Terns along the northern Gulf Coast may preferentially consume these four species. Consistent with previous studies (Carreker 1985), *A. mitchilli* was the dominant species detected across all three years in each zone, though some variation between years and zones occurred. Greater variation between years and zones was seen with *A. hepsetus*. Specifically, RRA of *A. hepsetus* decreased in 2019 (from 35% in 2017 to < 5% in 2019). Given this shift, it seems that spatio-temporal changes in abundance of fishes may have impacted diet composition and dominance of one prey item over another when comparing different years and zones. For example, *Pterois* spp. showed an RRA of 30%

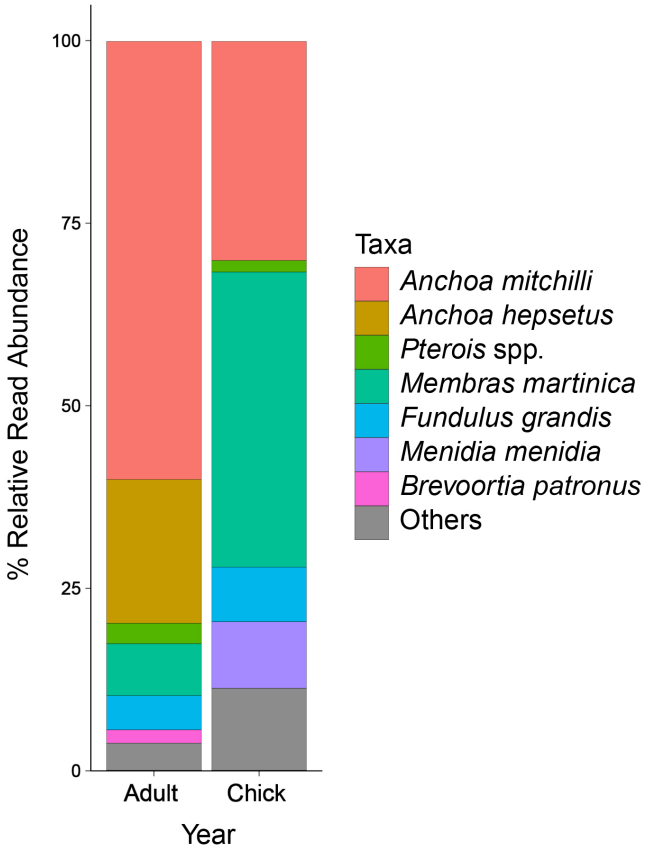

Fig. 4. Relative read abundance (RRA) of diet composition of chicks compared to adults. Colors are specific to species detected and are indicative of percent of RRA.

in 2017, but constituted < 10% of the diet for the next two years. Without direct prey sampling in the terns' foraging area, the degree to which diet reflects availability remains unknown.

A Lionfish (*Pterois* spp.) detection represents a new record for the Least Tern diet. However, given the size of adult Lionfish, it is likely that this detection reflects either prey items that were larval or juvenile Lionfish, or secondary depredation of larval Lionfish. Recurrence of Lionfish DNA in the samples based on RRA, in addition to high FOO (60%–96%) detections, suggests direct consumption and not secondary ingestion, though secondary ingestion is possible (McInnes *et al*. 2017, Deagle *et al.* 2018). Additionally, the decrease in RRA for Lionfish in 2018 and 2019 might coincide with noted declines of larval Lionfish recruitment in past years (Harris *et al*. 2020). It is well established that dietary shifts can reflect changes in fish population abundances/availability (Hebert *et al*. 2008, Panek 2021). Consequently, species like *M. martinica*, which accounted for < 5% of the Least Tern diet for 2017–2018, constituted a major prey item for 2019 (22% of the overall diet).

Additionally, differences in prey items were detected across the three spatial zones, suggesting geographic variation in diet even at a small scale. Specifically, though only represented by one site, prey items in Zone 3 were represented by an increase in brackish and freshwater species. Differences in prey items across zones could be due, in part, to the fact that different types of fishes inhabit different regions, and different populations of Least Terns experience different availability of prey items. Thomson *et al*. (1997) noted that Least Terns forage near nest sites within 100 m of the colony, and the available data indicate that birds in this study may have foraged within similar boundaries. In the current study, Zones 1 and 2 are in a region with access to saltwater fishes, whereas Zone 3 has a higher input of freshwater, which is reflected in the prey species composition for Zone 3. Specifically, species such as *Fundulus grandis* and *Labidesthes sicculus*, which are known to inhabit freshwater and brackish environments (Schofield & Fuller 2022), were only recovered in samples from Zone 3.

In 2019, the Bonnet Carre spillway, which diverts water from the Mississippi River into Lake Pontchartrain, was opened twice for a record-breaking 123 d—first during 27 Feb–11 Apr and again during 10 May–27 Jul. These openings released six trillion gallons of freshwater, resulting in lowered salinity and nutrient runoff that caused toxic algal blooms, mass oyster fatality, and dolphin mortalities in the Mississippi Sound and adjacent areas of the Gulf of Mexico (Armstrong *et al*. 2021). Freshwater influxes could well have changed the fish fauna (Hoover *et al*. 2022). These conditions likely explain the differences in prey items detected in 2019 using both dropped fish and fecal metabarcoding, such as the presence of only *Fundulus* spp. and *Labidesthes sicculus* in 2019, and the decrease in *Anchoa hepsetus* in 2019 compared to other years.

Finally, it is important to mention that, in this study, dropped fish are not directly comparable to prey items detected in feces because samples do not necessarily come from the same bird, despite being recorded from similar locations and time points. Thus, dropped fish data can be used to help support and/or validate findings of the metabarcoding approach.

Diet comparison of chicks vs. adults

Differences in diet existed in chick fecal samples compared to breeding adult samples in Zone 3 during 2019 only. Other zones and year comparisons were not made because of a lack of fecal samples from chicks. Specifically, the diet of adults from Zone 3 was dominated by *Anchoa mitchilli* and *A. hepsetus*, whereas the diet of chicks was dominated by *Membras martinica* and *A. mitchilli*. *A. hepsetus* was absent from the chick diet, indicating that unlike in adults, *Anchoa* spp. might not represent the taxa contributing to the majority prey items consumed by juveniles. The lack of *A. hepsetus* is difficult to explain because both *A. hepsetus* and *A. mitchilli* are similar in shape, though on average, *A. mitchilli* is shorter in length (10 cm) than *A. hepsetus* (14 cm). It is possible that the length of *A. hepsetus* prevents consumption by chicks. Additionally, *Brevoortia patronus* was recorded in the dropped fish and in the adult diet of Least Terns, but it was not recovered in the chick diet. This may be due to the fact that *B. patronus* is a deep-bodied fish and, similar to *A. hepsetus*, its size makes it difficult for chicks to ingest.

CONCLUSION

This study represents the first use of both metabarcoding and dropped fish to identify the diet of Least Terns and highlights the advantage of using DNA sequencing as a mechanism for providing greater resolution in elucidating diet composition. Changes in diet over time may correlate with changes in fish abundance, which could have significant long-term implications on the trajectory of colonies of Least Terns. Future studies which sample the available fish fauna will provide further insight into the diet of Least Terns.

TABLE 2
A comparison of results from dropped fish vs. DNA metabarcoding

Taxa	2017		2018		2019	
	DNA	Dropped fish	DNA	Dropped fish	DNA	Dropped fish
Anchoa hepsetus	✓	✓	✓	✓	✓	✓
Anchoa lyolepsis	×	×	✓	×	×	×
Anchoa mitchilli	✓	✓	✓	✓	✓	✓
Acipenser oxyrinchus	✓	×	×	×	×	×
Ariopsis felis	×	×	×	×	×	✓
Brevoortia patronus	✓	✓	×	✓	✓	✓
Caranx hippos	×	×	×	×	×	✓
Citharichthys spilopterus	×	×	×	✓	×	×
Cynoscion arenarius	×	×	×	✓	×	✓
Cynoscion regalis	×	×	×	×	✓	×
Cyprinella leedsi	×	×	✓	×	×	×
Cyprinodon variegatus	×	×	×	✓	×	×
Eleotris spp.	×	×	×	×	✓	×
Elops saurus	×	×	×	×	×	✓
Fundulus grandis	×	×	×	×	✓	×
Fundulus spp.	×	×	×	×	×	✓
Fundulus pulvereus	×	×	×	×	×	✓
Gambusia affinis	×	×	×	✓	×	×
Gambusia holbrooki	×	×	×	×	✓	×
Harengula jaguana	×	✓	✓	✓	×	×
Labidesthes sicculus	×	×	×	×	✓	×
Lagodon rhomboides	×	✓	×	✓	×	✓
Leiostomus xanthurus	×	×	×	✓	×	×
Lepomis macrochirus	✓	×	×	✓	×	×
Lepomis microlophus	×	×	×	✓	×	×
Lepomis miniatus	×	×	×	✓	×	×
Lolliguncula brevis	×	×	×	✓	×	×
Lucania parva	×	×	×	×	✓	×
Membras martinica	✓	✓	✓	✓	✓	✓
Menidia beryllina	×	×	×	✓	×	✓
Menidia menidia	×	×	✓	×	✓	×
Menticirrhus americanus	×	×	×	×	✓	×
Menticirrhus littoralis	×	×	×	✓	×	×
Micropogonias undulatus	×	×	×	✓	×	×
Micropterus salmoides	×	×	×	×	×	✓
Mugil cephalus	×	×	×	✓	×	✓
Mugil curema	×	×	×	×	✓	×
Myrophis punctatus	×	×	×	✓	×	×
Opisthonema oglinum	✓	×	✓	✓	×	×
Poecilia latipinna	×	×	×	×	×	✓
Pomatomus saltatrix	×	×	×	✓	×	×
Scomberomorus maculatus	×	✓	×	✓	×	✓
Symphurus plagiusa	×	×	✓	×	×	×
Trachinotus carolinus	×	✓	×	×	×	×
Trinectes maculatus	×	×	×	✓	×	×

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