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Elucidating shark diets with DNA metabarcoding from cloacal swabs

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Abstract

Animal dietary information provides the foundation for understanding trophic relationships, which is essential for ecosystem management. Yet, in marine systems, high-resolution diet reconstruction tools are currently under-developed. This is particularly pertinent for large marine vertebrates, for which direct foraging behaviour is difficult or impossible to observe and, due to their conservation status, the collection of stomach contents at adequate sample sizes is frequently impossible. Consequently, the diets of many groups, such as sharks, have largely remained unresolved. To address this knowledge gap, we applied metabarcoding to prey DNA in faecal residues (fDNA) collected on cotton swabs from the inside of a shark's cloaca. We used a previously published primer set targeting a small section of the 12S rRNA mitochondrial gene to amplify teleost prey species DNA. We tested the utility of this method in a controlled feeding experiment with captive juvenile lemon sharks (*Negaprion brevirostris*) and on free-ranging juvenile bull sharks (*Carcharhinus leucas*). In the captive trial, we successfully isolated and correctly identified teleost prey DNA without incurring environmental DNA contamination from the surrounding seawater. In the field, we were able to reconstruct high-resolution teleost dietary information from juvenile *C. leucas* fDNA that was generally consistent with expectations based on published diet studies of this species. While further investigation is needed to validate the method for larger sharks and other species, it is expected to be broadly applicable to aquatic vertebrates and provides an opportunity to advance our understanding of trophic interactions in marine and freshwater systems.

KEYWORDS
12S, diet reconstruction, elasmobranch, faecal, faecal analysis, fDNA, predator

1 | INTRODUCTION

Ecological network analysis and ecosystem-based fisheries management are contingent on a holistic understanding of the diet of constituent species (Casey et al., 2019; Pikitch et al., 2004; Shabtay et al., 2018). However, accurate and efficient methods to assess diet composition are currently lacking for many large-bodied aquatic predators such as sharks, whose feeding behaviour is difficult to observe directly. Shark diet assessment and reconstruction generally rely on morphological stomach content analysis, obtained through

invasive methods such as stomach eversion and gastric lavage, or lethal sampling (Barnett et al., 2010; Bornatowski et al., 2014; Cortés & Gruber, 1990; Matich et al., 2020; Papastamatiou et al., 2006; Rosende-Pereiro et al., 2019; Trystram et al., 2017). These methods are necessary but undesirable for threatened and/or protected species (Hammerschlag & Sulikowski, 2011; Heupel & Simpfendorfer, 2010). Moreover, morphological stomach content analysis is labour-intensive, requires extensive taxonomic knowledge and lacks resolution as prey items are often digested, making them difficult or impossible to identify. This also introduces a bias towards prey that are slower to digest (Baker et al., 2014; Gosselin et al., 2017). Alternatively, obtaining gross information on diet through the non-lethal and less invasive analysis of biochemical tracers that integrate consumed prey information in consumer tissues over a large time span (weeks to months), such as bulk and compound-specific stable isotopes and fatty acids, is limited in that both methods require intensive sampling of both predator and prey species (Munroe et al., 2018; Nielsen et al., 2018). They also rely on well-resolved trophic discrimination factors or calibration coefficients to make gross dietary inferences that are rarely resolved to constituent species (Hussey et al., 2012; Meyer et al., 2019; Nielsen et al., 2018). Indeed, for generalist predators that feed across multiple trophic levels, such as many shark species, species-level resolution of prey often remains uncertain when using chemical tracers (Layman et al., 2012; Nielsen et al., 2018).

There is a growing momentum in the application of high-throughput sequencing technology in conjunction with DNA metabarcoding in diet reconstruction studies, which allows for the large-scale, high-resolution characterization of species composition in stomach contents and faecal samples (Brassea-Pérez et al., 2019; Carroll et al., 2019; Dunn et al., 2010; Finucci et al., 2016; Gordon et al., 2019; Monterroso et al., 2019). However, the collection of shark faeces is challenging because defecation events are rarely observed and typically result in faecal particles suspended in seawater, making them difficult to collect. Moreover, once faeces have been released into the water, or on skin or other surfaces, environmental DNA (eDNA) contamination may be introduced (Meekan et al., 2009; Poulakis et al., 2017). Consequently, even in the rare instance of sampling faecal matter during a defecation event, it becomes difficult to determine whether detected prey DNA originates from the faecal matter or from eDNA present in the surrounding seawater. The development of an accurate, efficient, and minimally invasive method for the collection and metabarcoding of faecal DNA (fDNA) that concurrently reduces or eliminates eDNA contamination could bridge a major part of the current knowledge gap concerning the diets of different shark species. Sharks expel both urinary and solid waste through the cloaca, which may create an opportunity to sample fDNA directly from a shark sometime around a defecation event instead of opportunistically collecting a faecal sample released by the animal during a capture and tagging event (Hancock et al., 2019; Poulakis et al., 2017).

Here, we aimed to assess the applicability of the collection of traces of faecal material from a shark's cloaca using cotton swabs, and subsequently metabarcoding of fDNA extracted from these samples, in order to assess the animals' diet. Our first objective was to ground truth the method by performing a controlled feeding experiment with captive juvenile lemon sharks (*Negaprion brevirostris*, Poey 1868) to determine whether a known diet can reliably be identified by fDNA metabarcoding and if eDNA contamination from surrounding seawater can be excluded. We focus on a single primer set targeting a small fragment of the 12S rRNA region to identify a teleost prey species that is not part of the natural diet of *N. brevirostris* in this size class. Our second objective was to test the utility of the method on free-ranging juvenile bull sharks (*Carcharhinus leucas*, Müller and Henle 1839) using the same primer set to determine whether the method could reliably be used to obtain prey signatures that are consistent with previously published diet studies and locally available prey.

2 | MATERIALS AND METHODS

2.1 | Experimental study

2.1.1 | *Negaprion brevirostris* capture and husbandry

Three male juvenile *N. brevirostris* (Table S1) were collected from a tidal creek system in Cape Eleuthera, The Bahamas (24°49′46.43″N, 76°19′41.49″W), on October 15 and 16, 2018. All individuals were caught using either seine netting or rod and reel fishing, followed by transportation to the Cape Eleuthera Institute's covered, open-sided outdoor wet-laboratory facility. Sharks were individually housed in 13,000-L (3.7 m diameter × 1.3 m depth) continuous flow-through seawater tanks and exposed to ambient water conditions and a natural photoperiod (latitude 25°N; Bouyoucos et al., 2017). Fresh aerated seawater (flow rate: 1 L/s; time to fill a holding tank: 2 h 41 min) was supplied from an intake at 1.5 m depth, located ~200 m offshore in a hard-bottom mangrove creek habitat with nearby patch reefs.

2.1.2 | Controlled feeding experiment and fDNA collection

Following capture, the sharks were housed for 14 or 15 days between October 15 and 29, 2018. Once daily between 7:30 and 8:00 AM, from the day after capture until the day of release, individual sharks were offered 6% of their body weight (instead of the recommended 2%) in *Euthynnus alletteratus* (little tunny) filets in order to reduce food retention time, accelerate the onset of faecal production, and to extend the time period during which faeces would be produced (Cortés & Gruber, 1990; Wetherbee & Gruber, 1990; Table S1). This pelagic teleost species is not a natural prey item of juvenile *N. brevirostris*, whose diet has previously been linked to mangrove communities (Cortés & Gruber, 1990; Newman et al., 2010). Consequently, it is highly unlikely that *E. alletteratus* would have been consumed under natural conditions, and therefore be detected prior to the onset of

the feeding experiment. Offered prey that was not consumed was removed from the tank within 60 min. Cloacal swab samples were collected for 13 days starting on the day of capture. All three sharks were sampled daily between 11:00 AM and 2:00 PM for fDNA. The sharks were placed in a cooler partially filled with seawater from their tank, held upside down in tonic immobility (a temporal state of paralysis; Kessel & Hussey, 2015), and pelvic fins were spread apart while the posterior half of the shark was held out of the water. This enabled clear access to the cloaca and prevented contact between the swab and the exterior skin and surrounding water. To further reduce the risk of contamination from fish eDNA that might be present in the tank water, the external cloacal area was dabbed dry with a paper towel (discarded after single use) prior to sample collection. A sterile, individually packaged cotton tipped swab with wood handle (Puritan 25–806 1WC FDNA; length 15.2 cm, tip diameter 0.48 cm) was inserted ~1 cm into the cloaca and rotated against the inside wall for ~5 s. Following removal, the tip was cut off from the excess handle using sterilized scissors (leaving ~2 cm of the handle for handling the swab in the laboratory) and stored for further processing (as described under 'fDNA and eDNA processing and extraction'). Upon completion of the experiment, the sharks were released in good condition at the same location as where they were captured.

2.1.3 | eDNA controls

To test for potential contamination of the cloacal swabs with teleost eDNA from the surrounding seawater in the controlled feeding experiment, we characterized the eDNA species composition of four water samples from the tanks in which the sharks were housed. Water samples (2 L each) were collected with sterile collection bottles, from two tanks, on two consecutive days concurrently with the cloacal swab sample collection (i.e., between 3.5 and 6.5 h after the sharks had been fed). Vacuum filtration was carried out directly on site with a peristaltic pump, filtering the water through hydrophilic polyethersulfone (PES) filters (Pall Corporation; 47 mm diameter; 0.45-µm pore size). After filtration, filters were stored at −20°C until further processing as described under 'fDNA and eDNA processing and extraction.'

2.2 | Field application

Field trials were conducted on a well-studied population of juvenile *Carcharhinus leucas* in the Shark River Estuary in the Florida Everglades National Park (ENP), USA, in the 2019 wet season (April–November; Table S2). Sharks were caught using 500-m longlines, fitted with 50 15/0 circle hooks baited with mullet (*Mugil* spp.), attached by 2-m gangions of 400-kg test monofilament line [as described by Heithaus et al. (2009)]. Sharks were brought onboard and placed inside a cooler partially filled with water collected directly from the sampling site. The water was changed prior to the start of the workup of each shark. Sex was determined and length measurements were taken to the nearest centimetre for each animal, and weight was measured to the nearest 0.5 kg. Cohort and age class were estimated based on size at capture (Matich & Heithaus, 2015). Subsequently, a cloacal swab sample was collected as described above, after which the sharks were released.

2.3 | fDNA and eDNA processing and extraction

The fDNA cotton swab samples and the filters containing eDNA sample filtrates were stored in sterile 5.0-ml cryogenic screw cap vials containing silica beads. The silica beads function as a desiccant, drying out the swabs and filters, preventing the DNA from degrading (Bakker et al., 2017). Subsequently, all samples were initially preserved in a cooler filled with ice and then stored at −20°C until extraction. Sterile tweezers and pliers were used to handle all samples. Prior to extraction, the wooden ends were removed from the cotton swabs and filters were cut in half. Filter and swab samples were processed on separate occasions. Genomic DNA was extracted using the DNeasy PowerSoil Kit (www.qiagen.com), following the manufacturer's protocol. DNA was eluted into 100 µl and frozen at −20°C until further processing. At all stages of sample collection and laboratory procedures, single-use disposable gloves were used. All surfaces, field sampling, and filtration and laboratory equipment were cleaned with a 50% bleach solution and all laboratory work was carried out inside a laminar flow hood. Swab and filter DNA extraction and library preparation were performed at Jonah Ventures (www.jonahventures.com) in dedicated controlled eDNA laboratories.

2.4 | Library preparation and sequencing

Concentrations of a subset of extracted genomic DNA were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific), prior to PCR amplification. The MiFish-U primer set (Miya et al., 2015), targeting an ~171-bp fragment of the mitochondrial 12S rRNA region, was used for the amplification of fDNA and eDNA from cloacal swabs and water filters, respectively. This primer set was specifically designed for the amplification of fish DNA (thus inherently it will also amplify the DNA of a number of elasmobranch species) and was chosen for this study because both *N. brevirostris* and *C. leucas* are primarily piscivorous (Newman et al., 2010; Poulakis et al., 2017; Snelson et al., 1984; Snelson & Williams, 1981). The MiFish-U primer set is known to be highly specific for the amplification of teleost DNA, with very little cross-amplification of other groups of animals (except for some elasmobranchs; Collins et al., 2019; Miya et al., 2015). Therefore, we expected that using this particular set would maximize the number of fDNA sequence reads that could be obtained using a single primer set. Both forward (5′-GTCGGTAAAACTCGTGCCAGC-3′) and reverse (5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′) primers contained a 5′ adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25-µl PCR was prepared according

to Promega PCR Master Mix specifications, which included 12.5 µl Master Mix, 0.5 µM of each primer, 1.0 µl of gDNA and 10.5 µl DNase/RNase-free water. All PCR amplifications were done in triplicate reactions using the following PCR profile: initial denaturation at 95°C for 3 min, followed by 45 cycles of 20 s at 98°C, 30 s at 60°C and 30 s at 72°C, and a final elongation at 72°C for 10 min. To determine amplicon size and PCR efficiency, each reaction was assessed by electrophoresis, running the products through a 2% agarose gel and visualized on a UV light platform. Amplicons were subsequently cleaned by incubation with Exo1/SAP for 30 min at 37°C, followed by inactivation at 95°C for 5 min. A second PCR was performed on the cleaned amplicons from the first-stage PCR to give each sample a unique 12-nucleotide index sequence, yielding a total length of 346 bp. The indexing PCR included Promega Master mix, 0.5 µM of each primer and 2 µl of template DNA. The PCR profile included an initial denaturation of 95°C for 3 min, followed by eight cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. To test for successful barcoding, the indexed PCR products were visualized on a 2% agarose gel. As the samples were collected on separate sampling campaigns, two Illumina libraries containing the swab samples from the two different species were built on separate occasions. All samples were run alongside other libraries from unrelated projects, equalizing the sequencing depth across all samples by pooling an equal number of samples for each run. Sample library pools were sequenced on an Illumina MiSeq platform at the CU Boulder BioFrontiers Sequencing Center using the v2 500-cycle kit. Necessary quality control measures were performed at the sequencing centre prior to sequencing.

2.5 | Bioinformatic processing and statistical analysis

Sequences were processed using the JAMP pipeline (https://github.com/VascoElbrecht/JAMP). Raw reads were demultiplexed with 'iu-demultiplex' version 2.3 (https://github.com/merenlab/illumina-utils). Forward and reverse reads from each sample were paired end merged with USEARCH version 11.0.667 (Edgar, 2010). Forward and reverse primers were trimmed and reads with sequence length 130–210 bp were retained using CUTADAPT version 1.18 (Martin, 2011). Quality filtering was carried out using expected error filtering (max ee =0.5) as implemented in USEARCH (Edgar & Flyvbjerg, 2015). Subsequently, instead of operational taxonomic unit clustering, reads affected by sequencing and PCR errors were removed using the unoise3 algorithm with an alpha value of 5 (Edgar, 2016). Denoising was applied to each individual sample and exact sequence variants (ESVs) were compiled in an ESV table including sequences and read counts for each sample. Taxonomy assignment was performed by mapping each ESV against the MITOFISH database (http://mitofish.aori.u-tokyo.ac.jp/) containing complete and partial mitochondrial DNA (mtDNA) reference data, using Usearch_global with maxaccepts 0 and -maxrejects 0 to ensure mapping accuracy. A value of 0 ensures all potentially matching sequences are aligned and the best one is selected. With the default settings, only the top potential hits would be compared, leading to less accurate results if many similar sequences are in the reference database. Consensus taxonomy is generated from the hit tables, by first considering 100% matches followed by going down in 1% steps until hits are present for each ESV. In the respective 1% bracket, taxonomy present in at least 90% of the hits is reported. An NA is reported if several taxa match the ESV. To reduce errors caused by misidentified taxa, the bracket is increased to 2% if matches of 97% or higher are present, and no family-level taxonomy is returned. All resulting prey sequences where subsequently blasted in GenBank to ensure that potential prey species were not missed because some western Atlantic teleost 12S sequences that are deposited in GenBank are not yet available in the MITOFISH database. Where an ESV could subsequently not be assigned to the genus and/or species level, the vernacular name of the family was recorded where possible, based on the genus or species known to occur in our study site (Kline et al., 2014; Trexler & Goss, 2009). All statistical analyses were performed in R version 3.4.0 (https://www.R-project.org/). The VEGAN package version 2.4–081 in R was used for the calculation of the sample-based prey species accumulation curve. A regression analysis was performed to examine if the cloacal swab sample size was large enough to adequately describe the diet of the juvenile *C. leucas* in this system.

3 | RESULTS

3.1 | Controlled feeding experiment

While cloacal swab collection was initiated on the first day of capture, the sharks did not start feeding until the third or fourth day, after which feeding continued daily until the end of the trial (Figure 1; Table S1). *Negaprion brevirostris* DNA (1,060,112 total sequence reads) was recovered from every sample from all individuals across the entire trial, indicating that sampling, sample storage and DNA processing workflows were successful. No teleost DNA was detected in the swab samples in the days prior to the initiation of feeding, providing a negative control for the experiment, indicating that no teleost eDNA contamination from the water had occurred. Once the sharks started feeding, teleost DNA was detected, and all teleost sequence reads were assigned to *Euthynnus alletteratus* (53,619 sequence reads total across all positive swabs, see Table S3 for summaries of bioinformatics stats). The first appearance of *E. alletteratus* reads on swabs varied between 3.5 and 6.5 h (shark 1 and 2) and 2 days (shark 3) after feeding. *E. alletteratus* reads were present in swab samples intermittently thereafter, from days 4 or 5 to the end of the feeding trial (Table S4.A). Once feeding commenced, 5/10 swabs from shark 1, 2/10 from shark 2, and 3/11 swabs from shark 3 contained *E. alletteratus* sequences. The number of sequence reads per individual and per sample was variable but increased towards the end of the experiment for all

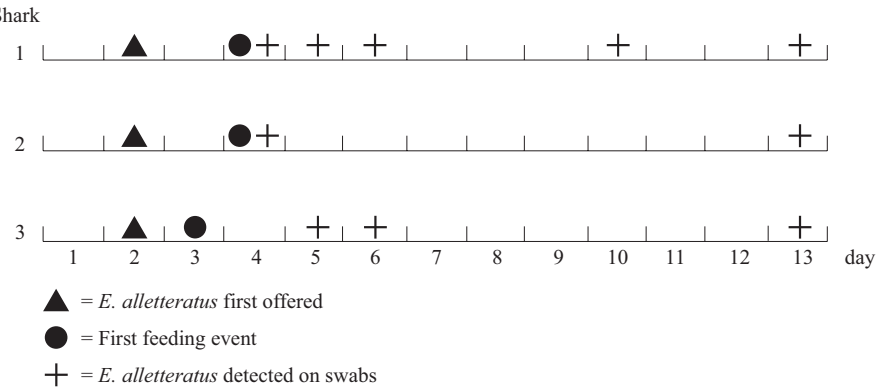

FIGURE 1 Schematic overview of the results of the controlled feeding experiment for three juvenile lemon sharks, *Negaprion brevirostris*, held in captivity. Day 1 denotes the day of capture

three individuals. The proportion of recovered *N. brevirostris* reads relative to those recovered from *E. alletteratus*, averaged across individuals and samples, was 0.84 (range, 0.72–0.97; Table S4.A). Four water samples from the tanks housing the sharks were collected, filtered, extracted and sequenced (6292 total sequence reads) in order to profile potential eDNA contamination in the swabs. One eDNA sample contained no teleost eDNA, one contained only *E. alletteratus* and two contained *E. alleratus* with up to three other local teleost species (*Halichoeres bivittatus* [748 reads], *Atherinomorus stipes* [112 reads], *Stegastes* sp. [451 reads] ; Table S4.B).

3.2 | *Carcharhinus leucas* fDNA

A total of 21 juvenile *C. leucas* (110.22 ± 14.8 cm stretched total length [STL], mean ± *SD*; 90–132 cm, range) were caught and sampled for fDNA (see Table S2 for catch data). All swabs contained host and/or prey DNA (Figure 2 and Table S5). A total of 1,850,298 sequence reads, distributed over 37 ESVs (i.e., prey species) were recovered from the 21 swabs (Figure 3, Table S5; Table S3 for summaries of bioinformatics stats). Host DNA was present in 18 out of the 21 cloacal swabs and accounted for only 0.65% (11,972 sequence reads) of the total number of recovered sequence reads. Similarly, prey DNA was recovered from 18 cloacal swabs (Figure 2). The three swab samples that did not contain prey DNA did contain host DNA. The 37 different ESVs consisted of 20 orders comprising 26 families of which seven could be identified down to the species level. DNA from one elasmobranch prey species (family Dasyatidae) was also detected (Table S5).

The majority of the detected teleost prey families/species of the sampled juvenile *C. leucas* (Table S5) were native to the ENP and/or the Gulf of Mexico, while three species are invasive to the ENP (*Naso lituratus*, *Hypophthalmichthys nobilis*, *Cyprinus carpio*). The majority of prey sequence reads were assigned to Ariidae (catfishes: 56.8%, present in 15 samples that contained teleost prey DNA; Figure 2 & 3, Table S5). The second most abundant prey family was Mugilidae (mullets: 20.6%, present in six samples) followed by Serranidae (sea basses: 7.2%, present in six samples). Combined, these three families made up the bulk of the recovered diet (84.6%), while the other unique ESVs (spread over 12 samples) individually made up <3% of all sequence reads (Figure 2 & 3, Table S5), with the lowest read count (119 sequence reads) belonging to a species from the family Serranidae. Three samples contained only catfish DNA (sharks 1, 5 and 11), whereas the recovered diet from three other sharks (12, 13 and 15) consisted of multiple prey species (n = 26, n = 19, and n = 24, respectively) from fresh, estuarine and marine environments (Figure 3, Table S5). A diet species accumulation curve (Figure 4) shows that 21 samples is not yet sufficient to fully capture the species richness of juvenile *C. leucas* diets in the system because the curve has yet to reach a plateau and the slope of the regression line through the last four data points is greater than 0 (p = .002). A total of 11 negative extraction controls per each run were sequenced in parallel with the swab samples and none of these negative controls contained any teleost or elasmobranch reads.

4 | DISCUSSION

Here we show that cloacal swabs for the collection of faecal matter, and subsequent analysis by metabarcoding of fDNA, can be used to reconstruct shark diets. By first conducting a controlled feeding experiment with captive juvenile *Negaprion brevirostris* that were fed a known diet, we show that fDNA can detect prey items without incurring contamination from teleost eDNA that may be present in the surrounding seawater. We then show that cloacal swabs collected from juvenile *Carcharhinus leucas* in the field identified prey species that are a well-known dietary component for this species, highlighting the potential applicability of fDNA swab metabarcoding as a diet reconstruction method.

4.1 | Validating cloacal swab fDNA metabarcoding under controlled conditions

The predictions of the controlled feeding experiment were that (i) the only DNA recovered from cloacal swab samples would be that of the host species and its known prey, (ii) that prey DNA would be detected only after the onset of feeding and (iii) that eDNA contamination was not the source of prey DNA detected on the swabs. The results confirm these expectations. All cloacal swabs

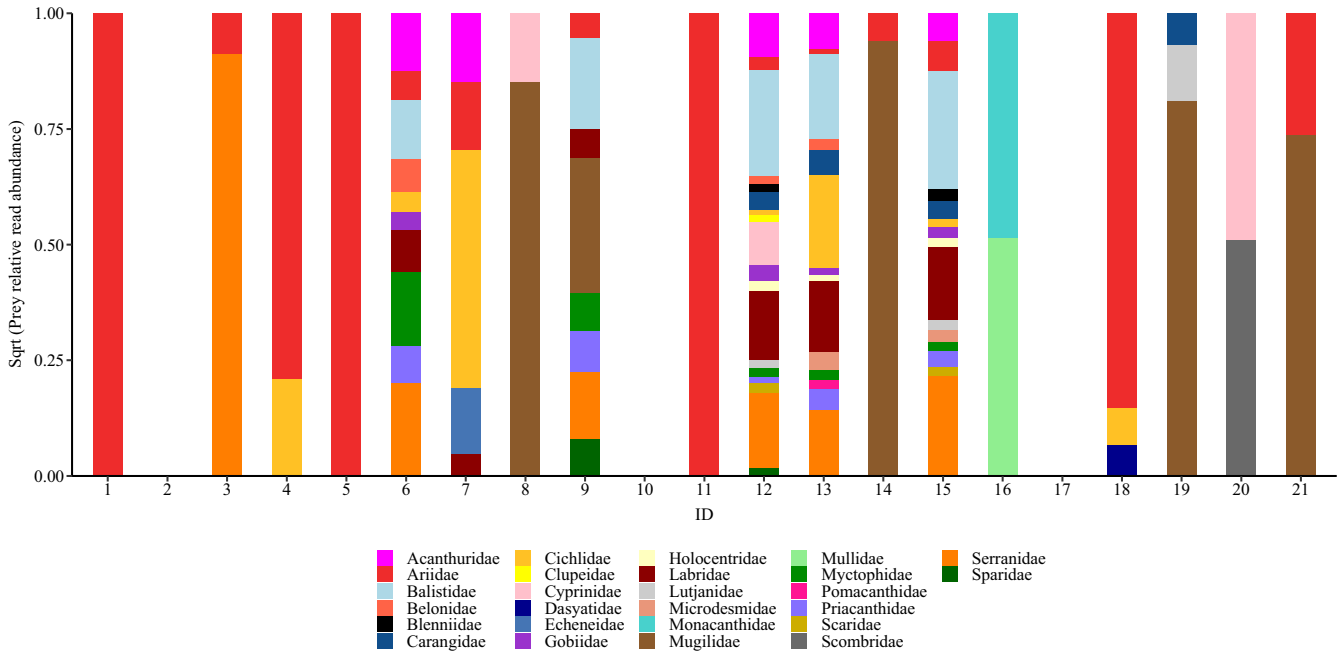

FIGURE 2 Cloacal swab sample results showing the prey diversity (family taxonomic level) for each individual juvenile bull shark, *Carcharhinus leucas*, sampled within the Shark River estuary in the Florida Everglades National Park, FL, USA. In shark samples #2, 10 and 17 no fDNA was detected and therefore are not shown in the graph [Colour figure can be viewed at wileyonlinelibrary.com]

collected in the juvenile *N. brevirostris* controlled-feeding experiment contained either only host DNA or both host DNA and known prey DNA. It is most likely that host DNA originates from epithelial cells that are unavoidably collected during swabbing because they were recovered from 100% of the swab samples. In the days before the sharks started eating the offered filets, no teleost DNA was detected in swabs, but the consistent presence of host DNA provided a positive control for the workflow. Once the sharks started feeding, *Euthynnus alletteratus* was the only teleost species that was detected in the swab samples in addition to the host DNA. It is highly unlikely that eDNA contamination from the surrounding water was the source of these DNA sequence reads. If *E. alletteratus* DNA on the cloacal swabs originated from the surrounding tank water rather than from the ingested food, it would most likely have been detected in most, if not all, swab samples collected during the experiment instead of only on ~33% of them. This is further supported by the characterization of the eDNA profile of the tank water in which three reef-associated teleost species were found across four water samples. If eDNA contamination from the water had been the source of *E. alletteratus* rather than faecal material inside the cloaca, the DNA of the additional three teleost species recovered from the eDNA water samples would almost certainly have been detected on the swab samples as well (and others given that they originate from outside seawater pumped through the tanks), especially given the relatively high contributions observed in some of the eDNA samples. These results indicate that the precautions implemented to avoid eDNA contamination during sampling (holding the posterior part of the shark out of the water and drying the cloacal area with paper tissue) are sufficiently rigorous to avoid such contamination.

Minimum food transit time in juvenile *N. brevirostris*, defined as the time it takes to observe faeces for the first time following a meal, has previously been indicated to be ~12 h (Wetherbee & Gruber, 1990). Yet, in our study the onset of prey DNA detection in cloacal swabs ranged from 3.5 to 48 h after the sharks accepted the offered filets for the first time. These differences may be indicative of variability in food retention and digestion times between individuals, but it may also be a result of the type of sampling method used (i.e., the collection of expelled faecal matter as opposed to fDNA collection using a cloacal swab). In Wetherbee and Gruber (1990) faecal production was detected only after defecation took place, whereas cloacal swabs have potentially detected *E. alletteratus* DNA either prior to defecation or sometime after. However, only ~32.3% of the swabs contained *E. alletteratus* DNA, with variable numbers of sequence reads among swabs and individuals. *N. brevirostris* do not defecate continuously. Instead, faecal matter builds up behind the cloaca prior to defecation and there is considerable individual variability in defecation frequency (Wetherbee & Gruber, 1990). Accordingly, while traces of fDNA are left behind, the temporal window to collect fDNA with cloacal swabs is likely to be relatively small.

4.2 | Testing the method under field conditions

Our study in the coastal Everglades was not designed to be a comprehensive investigation of the diet of juvenile *C. leucas*, but rather to assess the execution of the sampling approach in a field setting, and with the expectation that, at least, prey items known a priori to occur in the diet of this species would be detected. The cloacal

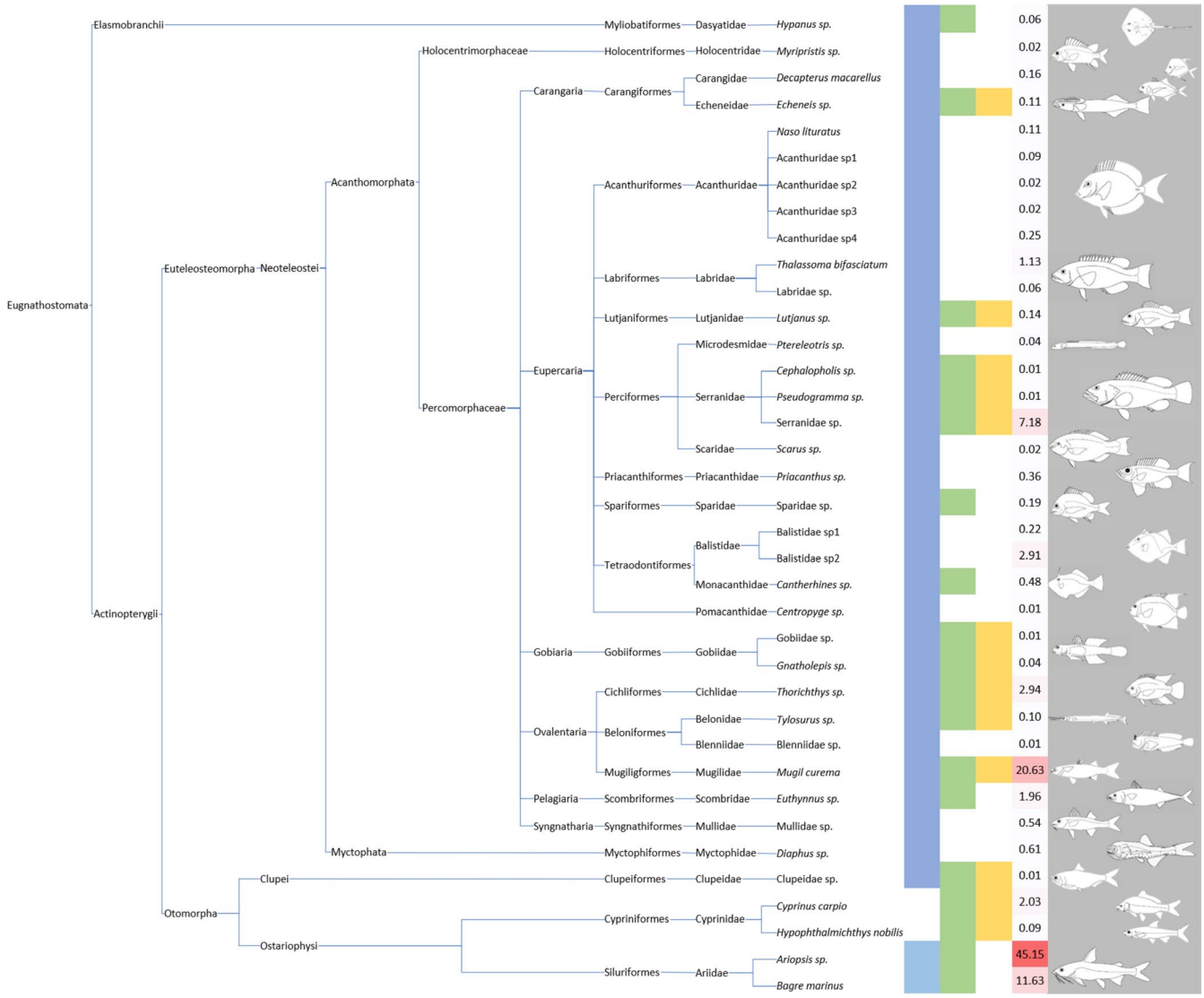

FIGURE 3 Cladogram showing the prey diversity (unique Exact Sequence Variants (ESV)) and their frequencies (relative read abundance) over all collected bull shark cloacal swabs. Colors denote the environment in which the prey species can be found (blue = marine, green = brackish, yellow = freshwater), as well as their relative importance in the diet of the bull sharks (darkest red = most important). Image attributions: FishBase (www.fishbase.orgwileyonlinelibrary.com]). Taxonomic denominations were extracted from The Fish Tree of Life (www.fishtreeoflife.orgwileyonlinelibrary.com]) [Colour figure can be viewed at wileyonlinelibrary.com]

swab sampling approach was successful in the field in that we were able to recover teleost sequence reads from most of the sharks. The metabarcoding results were generally consistent with previously published diet data for this species and size range in similar systems. The four predominant ESVs (84.6% of all sequence reads) obtained from 18 out of the 21 cloacal swabs were assigned to two species of catfish (Ariidae), white mullet (*Mugil curema*), and a species from the family Serranidae, which are all abundant in the Shark River Estuary and are known prey items for juvenile *C. leucas* (O'Connell et al., 2007; Poulakis et al., 2017; Snelson et al., 1984; Snelson & Williams, 1981). Stomach content analyses have previously shown that *C. leucas* consume a wide variety of prey species including teleosts, molluscs, crustaceans and other elasmobranchs. In estuarine systems, such as the Everglades, juvenile *C. leucas* may feed in freshwater, brackish and/or coastal marine habitat types (Cliff & Dudley, 1991; Matich & Heithaus, 2015; Snelson et al., 1984; Snelson & Williams, 1981; Trystram et al., 2017). Additionally, within the Shark River Estuary, stable isotope and acoustic tracking analyses have revealed ontogenetic niche shifts, as well as relatively high levels of individual dietary specialization for foraging from particular food webs (i.e., marine vs. freshwater/estuarine) among juvenile *C. leucas* (Matich et al., 2011, 2017; Matich & Heithaus, 2015). Such behaviours may be consistent with the relatively large number of rarer ESVs (n = 33) that were infrequently detected in the cloacal swabs of the juvenile *C. leucas*, relative to the dominant ESVs. These included a variety of prey families/species (ranging from freshwater to marine species), including known invasive species and some teleost prey items that were not specifically known to be part of the diet of juvenile *C. leucas*.

While the diversity of ESVs detected in a minority of swabbed *C. leucas* was higher than expected from previous stomach content

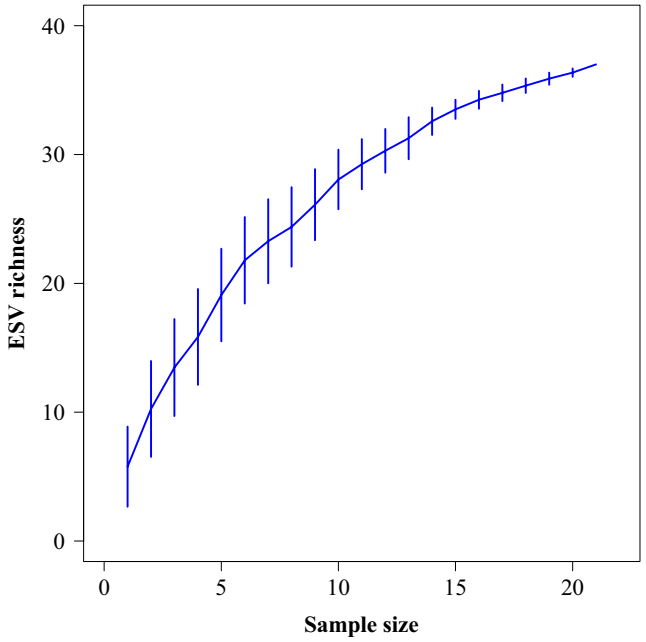

FIGURE 4 Species accumulation curve showing prey richness (unique exact sequence variants [ESVs]) as a function of the number of juvenile bull sharks, *Carcharhinus leucas*, sampled. Error bars indicate standard errors after 100 permutations [Colour figure can be viewed at wileyonlinelibrary.com]

analyses, results like these are not unprecedented. Consumer diets from highly diverse communities are often equally diverse, resulting in a large number of rare dietary prey ESVs (Casey et al., 2019; Leray et al., 2013). Generally, molecular analysis of faecal matter results in higher taxonomic resolution compared to traditional methods such as morphological stomach content analysis, and consequently, reveals a higher prey diversity (Berry et al., 2015; Bessey et al., 2019; Jeanniard-Du-Dot et al., 2017; Sousa et al., 2019; Thomas et al., 2017). In sharks, DNA metabarcoding of stomach contents previously showed a prey species richness accumulation at nearly double the rate compared to morphological stomach content analysis of the same samples (Barnett et al., 2010; Dunn et al., 2010). Such studies have led to a number of unexpected new ecological findings (Bessey et al., 2019; Granquist et al., 2018; Leray et al., 2015; Riemann et al., 2010; Sousa et al., 2019). For example, DNA from mesopelagic fishes (family Myctophidae) was unexpectedly detected in the stomachs of three mobula ray species (Bessey et al., 2019). Similarly, in this study, DNA of a myctophid species was detected, among other marine teleost species, in the swab samples from five different *C. leucas*, with a total of 11,365 sequence reads (sharks 6, 9, 12, 13 and 15; Table S5). The recovered sequence matches a species from the genus *Diaphus* with a maximum percentage identity of 96%. Regardless, it cannot currently be determined whether the sequence reads from this family originated from ingestion or contamination. While more work is needed to properly interpret these results, such as using statistical modelling approaches to quantify and correct for the abundance of false positive samples (Ficetola et al., 2016; Lahoz-Monfort et al., 2016), improvement of reference sequence databases for teleost prey species will certainly provide more clarity.

4.3 | Methodological considerations

DNA metabarcoding from cloacal swabs for the investigation of dietary composition holds great potential for resolving trophic relationships in ecological communities, offering several advantages relative to other methods. The collection of cloacal swabs, in contrast to other methods, is minimally invasive. Moreover, the collection of a cloacal swab is generally much less time-consuming than collecting stomach contents and faster than collecting most tissues for stable isotope or fatty acid analysis, which is advantageous when dealing with stress-sensitive species. In such instances, the application of cloacal swabs may present the only viable alternative to the more invasive procedures without imposing extra risk to the animal. We recovered prey DNA from ~33% of sampling events in *N. brevirostris* known to have fed recently in an experimental setting, and ~86% of *C. leucas* sampled in the field. This success rate is comparable to or exceeds other approaches for diet sampling involving stomach contents because sharks with empty stomachs (a large fraction of specimens collected by baited hook-and-line) and those that evert their stomach during capture may still provide dietary information by applying this method (Brunnschweiler et al., 2011; Joyce et al., 2002; Lowe, 1996). While biochemical tracers generally can be used on 100% of sampled individuals, diets can only be reconstructed with very low taxonomic resolution, if at all, using these methods.

However, like any other diet analysis method available, cloacal swab fDNA metabarcoding does have its limitations (Alberdi et al., 2019; Sousa et al., 2019). One of the caveats is that secondary predation (prey consumed by prey), or incidentally ingested items that are present in the water column, cannot be distinguished from intentionally ingested prey items (Bessey et al., 2019). Cannibalism will be obscured by host DNA, which is an issue for resolving diet for any species where this is common (Pompanon et al., 2012). Nor does the method allow for the determination of the prey's size or life stage, or the quantitative analysis of consumed items beyond relative abundances, although progress toward this goal is expected (Deagle et al., 2019; Monterroso et al., 2019). Furthermore, additional specialized primer sets are needed to target different prey groups (e.g., teleosts, crustaceans and/or mammals) in shark cloacal swab samples. Importantly, cloacal swab fDNA metabarcoding is inherently sensitive to the time of defecation, meaning that it may only pick up fDNA within a limited time frame prior to, and after, defecation. Finally, it is important to note that digestion rate plays a crucial role in prey detection of tissue-based extractions, often leading to an under-representation of those taxa whose tissue is more rapidly digested (Sousa et al., 2016). Without knowledge on specific digestion rates, it remains difficult to pinpoint over what exact time frame a prey item is consumed and for how long its signal remains detectable. However, correction factors can be estimated to account for differential digestibility rates, and derived results have been shown to be more robust when compared to morphological methods (Sousa et al., 2019).

4.4 | Future outlook

The controlled feeding experiment with captive juvenile *N. brevirostris* was successful in the detection of known prey items without incurring eDNA contamination. However, experiments to further our understanding of the obtained results are recommended. For example, experiments designed to better understand the relationship between food retention time and fDNA signal decay are vital to improve the interpretation of results obtained from future field applications of this method. In addition, the method would benefit from experiments that investigate the effects of mixed diets on fDNA recovery and composition, and from those that examine the relationship between prey biomass and sequence read abundance. While the method was successfully field-tested on juvenile *C. leucas* with only ~14% (3/21) swabs containing no detectable prey DNA, future studies should explore whether cloacal swab fDNA metabarcoding applied to larger sharks and other large vertebrate species can also recover prey DNA sequences successfully. Finally, due to the temporal differences with biochemical tracer data, it will be important to verify the correlation between short-term feeding and the biochemical tracer values of slow-turnover tissues by applying both types of method to the same individuals (Thomson et al., 2012).

The accuracy of the method presented here is contingent on the availability of well-stocked and curated reference databases (Cristescu, 2014; Devloo-Delva et al., 2019). Our study is an excellent example of the need for local sequence libraries. The 12S primer set used in this study offers very high specificity for teleosts (and a number of elasmobranchs), but lacks adequate references (Collins et al., 2019; Miya et al., 2015), in particular for the western Atlantic Ocean and the Gulf of Mexico. This became apparent from the relatively low number of ESVs assigned to the species level (Table 1). When comparing the prey ESVs against the available 12S teleost reference databases, due to a lack of teleost 12S barcodes, many sequences were assigned to a species not native to the study area. Because 12S sequences are evolutionary conserved, these matches represent local sister species (or potentially another species for which the short barcode is [almost] identical) that do have a 12S barcode sequence deposited in the reference database. Consequently, results from metabarcoding-based diet studies must be interpreted with caution until more comprehensive databases become available. Accordingly, region-specific fish barcode databases will significantly improve the resolution of molecular diet studies.

5 | CONCLUSION

Feeding ecology is a cornerstone for understanding not only predator biology, but also the functions of predators in ecosystems. Here, we introduce a minimally invasive and efficient tool for helping to elucidate the diets of sharks. Crucially, the use of this tool is not limited to small sharks and, after further investigation, may also be applied to larger sharks and other large vertebrate taxa (e.g., teleosts, reptiles and birds) for which direct observation of foraging behaviour is limited or impossible. Furthermore, additional primer sets targeting different taxonomic groups may also successfully be employed to amplify fDNA, depending on the research question, target species and geographical location. The method thus paves the way for addressing hypothesis-driven research questions related to high-resolution foraging behaviour and prey selection patterns of free-ranging animals that are difficult to observe directly. In contrast to other minimally invasive methods, fDNA analysis provides diet information that is both short-term (days) and high in taxonomic resolution. Consequently, fDNA may be used to identify species-specific trophic pathways and intra- and interspecific dietary partitioning. Ultimately, combining fDNA analysis with complementary methods to answer ecological questions will offer significant advancements from conventional investigations, and may further our understanding of trophic linkages.

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AUTHOR CONTRIBUTIONS

M.V.Z.B., B.D.P., D.D.C. and J.B. conceived and designed the study; fieldwork and sample collection were carried out by E.V.C.S., B.A.S. and B.S.T.; data analyses were conducted by M.V.Z.B., B.D.P. and J.B.; M.V.Z.B. and J.B. wrote the manuscript; all authors reviewed, commented on, and approved the manuscript.

DATA AVAILABILITY STATEMENT

Sequence counts and lemon shark sequences are listed in Tables 4A, B, and Table S5 available with the online version of this manuscript. Sequence data are archived in the Dryad repository: https://doi.org/10.5061/dryad.p515n265.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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