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A Comparison of DNA Metabarcoding Cloacal Swabs and Stomach Contents for Shark Diet Reconstruction

Savannah J. Ryburn[1] | Eldridge Wisely[2] | Jeffrey D. Plumlee[3] | Creed C. Branham[4] | F. Joel Fodrie[1,4] | John F. Bruno[5]

[1]Environment, Ecology, and Energy Program, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA | [2]Genetics Graduate Interdisciplinary Program, University of Arizona, Tucson, Arizona, USA | [3]School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA | [4]Institute of Marine Sciences, University of North Carolina at Chapel Hill, Morehead City, North Carolina, USA | [5]The Department of Biology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Correspondence: Savannah J. Ryburn (savannahjryburn@gmail.com)

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ABSTRACT

Metabarcoding fecal matter is increasingly common in dietary studies across a variety of taxa. This approach assumes that enough prey DNA remains detectable in the fecal DNA (fDNA) as the prey DNA becomes degraded while passing through the digestive tract. However, as prey DNA is degraded during digestion, diet reconstruction based on metabarcoding of fDNA could become incomplete, i.e., species for which DNA was highly degraded would not be detected. The purpose of this study was to test the use of cloacal swabs and metabarcoding fDNA for shark diet reconstruction. To do this, both stomach contents and cloacal swabs were collected from the same individual sharks and metabarcoded using two previously published primer sets targeting teleost fishes and crustaceans. Samples were collected from four coastal species of sharks in a temperate estuary: bonnethead (*Sphyrna tiburo; n* = 10), blacknose (*Carcharhinus acronotus; n* = 4), blacktip (*C. limbatus; n* = 4), and Atlantic sharpnose (*Rhizoprionodon terraenovae; n* = 5). We determined how well the fDNA represented the corresponding prey DNA from the stomach contents (stDNA) and found no statistical difference in taxonomic richness or diversity when comparing the two sample types. Our results indicate that the less invasive, non-lethal method of DNA metabarcoding cloacal swabs provided higher taxonomic resolution than more common methods for studying trophic ecology (i.e., morphological stomach contents identification and trophic biomarkers such as stable isotope analysis) with no statistical difference in overall diet description between the two sample types.

1 | Introduction

The diets of many marine predators are poorly understood due to the difficulty in observing predation events and because the traditional methodologies for diet reconstruction (e.g., morphologic stomach contents identification) have poor taxonomic resolution. Diet is often measured via gut content analysis, which is collected with either lethal stomach contents dissection (Giménez et al. 2017; de la Cocheret Morinière et al. 2003; Ibáñez et al. 2021; Rohner et al. 2013) or gastric lavage (Weideli et al. 2023; Yick et al. 2012; Frisch et al. 2016). However, gastric lavage is rarely used on animals such as sharks, with only 13 studies using this method on sharks over the past 40 years (Myers et al. 2025). Although gastric lavage is claimed to be non-lethal, little research has been conducted to measure the recovery and survival rate of wild-caught sharks after the invasive procedure (Myers et al. 2025). Therefore, morphological stomach content analysis can be impossible and/or unethical to use for protected, rare, or long-lived species and is often imprecise (i.e., lacks

taxonomic resolution). Moreover, identifying prey species from partially digested gut contents is difficult, often leaving remnants of digested contents completely unidentified or only categorized to a higher level taxonomic grouping (i.e., above the species or genus level, e.g., "teleost fish") (Tavares 2008; Hoffmayer and Parsons 2003; Poulakis et al. 2017). Therefore, the taxonomic precision of morphological stomach contents identification is limited because it generally can only provide a crude description of diet. Traditional stomach content analysis based on morphology can also lead to the underrepresentation of soft-bodied organisms that lack body parts that are resistant to digestion in the stomach, e.g., shells and bony skeletons (Sekiguchi and Best 1997; Jackson and Place 1992; Medved et al. 1988; Bush and Holland 2002). Additionally, many dietary studies reliant on stomach contents alone, especially ones focused on sharks, have found a large proportion of their samples to have empty stomachs (Bush and Holland 2002; Joyce 2002; Pethybridge et al. 2011; Hoffmayer and Parsons 2003), resulting in unproductive lethal sampling and the need for large sample sizes to provide any description of diet. The analysis of trophic biomarkers, such as stable isotopes and fatty acids, is also frequently used to assess predator diets (Estrada et al. 2003; Plumlee et al. 2023). While this approach effectively estimates a predator's general trophic position by reflecting the mean trophic level of its dominant prey, it lacks the taxonomic resolution needed to identify specific prey species.

The use of DNA metabarcoding for diet reconstruction has become increasingly common (Sousa et al. 2019; Deagle et al. 2019; Ando et al. 2020). DNA metabarcoding allows for simultaneous identification of many taxa within a single sample. Studies in both terrestrial and marine systems have used metabarcoding of stomach contents and feces to characterize and redefine the diets of predators (Berry et al. 2017; Ford et al. 2016; Henger et al. 2022; Harms-Tuohy et al. 2016) that were once only studied in a broad sense (i.e., at a low taxonomic resolution). Additionally, feces can often be collected from an animal in a relatively non-invasive manner (e.g., a swab of the cloaca) or without capturing the animal at all (e.g., collecting scat from the ground).

In a novel study, van Zinnicq Bergmann et al. (2021) held controlled feeding trials with three captive juvenile lemon sharks (*Negaprion brevirostris*) and showed that the use of cloacal swabs to collect fecal matter from sharks and metabarcoding the fecal DNA (fDNA) can be successfully used for shark diet reconstruction. They also collected cloacal swabs from 21 free-ranging juvenile bull sharks (*Carcharhinus leucas*) and were able to identify 26 families of teleost fish from the fDNA. Additionally, shark cloacal swabs have been used to describe the diet of white sharks (*Carcharodon carcharias*) (Clark et al. 2023) and migratory sharks in the mid-Atlantic bright (Olin et al. 2023). These results are important steps towards validation of this method. However, in a natural setting fecal samples could potentially yield incomplete descriptions of a predator's diet due to DNA degradation of prey as it passes through the gut (Seymour et al. 2018). During digestion, if prey DNA degrades into too small of fragments, the DNA won't be amplified in PCR and detected by metabarcoding (Barnes et al. 2014; Jo 2023; Moushomi et al. 2019; Tsuji et al. 2017). Therefore, DNA degradation during digestion could lead to incomplete dietary reconstruction. Additionally, the amount of detectable DNA left in feces could vary depending on the tissue type of the prey (Thomas et al. 2014).

Few studies have measured how digestion influences prey DNA detection (but see: Devloo-Delva et al. 2019; Thomas et al. 2014; Snider et al. 2022) and none have done so in sharks. When comparing the stomach contents of piscivorous fish (*Plectropomus* spp.), Devloo-Delva et al. (2019) found that highly degraded stomach contents revealed lower prey species richness than less degraded stomach contents. In contrast, Snider et al. (2022) compared stomach contents to fecal samples in seaside sparrows (*Ammospiza maritima*) and found similar measures of taxonomic richness and diversity in both sample types and concluded that sample type did not influence the description of diet.

Sharks play integral roles in marine ecosystems, yet for many species, fundamental biological information such as diet remains limited. The purpose of this study was to further test the use of cloacal swabs and metabarcoding fDNA for shark diet reconstruction. To do this, we took samples from four different shark species and compared the DNA results of prey found in the upper and lower gastrointestinal tracts by metabarcoding DNA from stomach contents (stDNA) and fDNA collected with cloacal swabs of the same individual sharks. We also morphologically identified the stomach contents from these same sharks and compared the results to the stDNA and fDNA results. We hypothesized that (1) the stDNA would yield more amplifiable prey DNA than the fDNA because the stomach contents were less digested and therefore less degraded than the fecal samples; (2) both sample types, fDNA and stDNA, would provide similar measures of taxonomic richness (i.e., number of different taxa present) and composition (i.e., list of different taxa present); therefore, the description of diet would not be affected by sample type.

2 | Methods

2.1 | Shark Capture

Four species of sharks including the bonnethead (*Sphyrna tiburo*), blacknose (*Carcharhinus acronotus*), blacktip (*C. limbatus*), and Atlantic sharpnose (*Rhizoprionodon terraenovae*) were collected from Back Sound and Onslow Bay, North Carolina (N 34.683, W 76.617). Fishing occurred between June and September of 2020, primarily in the shallow coastal waters dominated by salt marsh-oyster-seagrass complexes. The sharks were caught using standardized longline and gillnet surveys during the same fishing efforts as Plumlee et al. (2023) and Ryburn (2025). Bait comprised predominantly of sciaenids spot (e.g., *Leiostomus xanthurus* and Atlantic croaker *Micropogonias undulatus*) collected from trawl surveys in Onslow Bay. Animal care and use were approved and overseen by the University of North Carolina at Chapel Hill (UNC) IACUC (19–137.0-B). Most of the sharks used for this study were incidental mortalities (52%) as part of UNC's long-term shark survey programs. Once captured, all sharks were brought back to the laboratory at the UNC Institute of Marine Sciences (IMS) whole on ice for dissection and stomach sample collection. Sharks that were not incidental mortalities were placed in a cooler of salt water chilled with ice

immediately after capture. The decreased temperatures slowed the vitals of the shark causing less stress. Once back at the lab in an unresponsive state, death was assessed by removing each shark from the cooler and pinching the fin and skin to verify no response and checking the eye for response to light and touch before dissection.

2.2 | fDNA Collection

We used a modified version of the van Zinnicq Bergmann et al. (2021) protocol to collect the fDNA (Ryburn 2025). All cloacal swabs were collected in the field immediately after the shark was removed from the water. First, the shark was laid on top of a flat surface on the boat, ventral side facing up, with the pelvic fins spread to prevent contact between the swab and exterior skin. Using gloves, a Kimwipe soaked in 99.5% ethanol (Ruiz et al. 2024) (discarded after single use) was used to wipe and dry any excess seawater from the exterior of the shark's cloaca. A sterile, individually packaged cotton tipped swab with wood handle (Puritan 25-806 1WC FDNA; length 15.2 cm, tip diameter 0.46 cm) was inserted into the cloaca, past the sphincter (~2.5 cm) and rotated for ~10 s. Once removed, the tip of the swab was placed into a 2 mL, sterile vial and the excess handle was cut off using scissors sterilized with 99.5% ethanol. The vial containing the swab was immediately placed in a cooler on ice, separate from the sharks, and then once we returned to the laboratory the vial was stored at −80°C for about 6 months until further processing.

2.3 | stDNA Collection

Immediately upon return to the laboratory the stomach of each shark was excised by exposing the abdominal cavity and severing the esophagus and duodenum. Stomachs were then bagged and stored at −23°C for about 2 months. During the subsequent stomach contents dissection, all tools, equipment, and surfaces were sterilized with a 20% bleach solution between each shark to avoid contamination. Each stomach was thawed, emptied into a petri dish, weighed, and then run through a set of two sieves using Milli-Q water. Based on the expected and observed size of the prey typically found in shark stomach contents we chose a 1 mm sieve to separate the larger prey items from the stomach slurry. A 250 μm sieve was placed underneath the 1 mm sieve because it was able to capture the liquid stomach slurry and did not allow for any flow through. The contents that flowed through the larger 1 mm sieve but were then trapped in the second, smaller 250 μm sieve (i.e., stomach slurry) were kept as the stDNA. We pipetted 0.5 mL of each stomach slurry into two separate, sterile vials and stored at −80°C for about 6 months until further processing. The contents that were too large to flow through the 1 mm sieve were morphologically identified to the lowest taxon possible using a dissecting scope.

2.4 | fDNA and stDNA Extraction

The fDNA on the swabs was extracted using the DNeasy Blood and Tissue Kit (www.qiagen.com) (Clark et al. 2023), following the manufacturer's protocol but using a 24–48 h digestion at 70°C. The stDNA was extracted using the DNeasy PowerSoil Kit (www.qiagen.com) following the manufacturer's protocol. There was enough stomach slurry trapped in the smaller sieve that we were able to collect two vials of stomach slurry for each individual shark. We were then able to conduct two extractions on each stomach, allowing us to have replicates of each stomach. When logically feasible, replicates are always preferable to be able to infer the maximal species richness present. For each shark, the two replicate stomach slurry samples were extracted separately. All DNA was eluted to 100 μL and frozen at −20°C until further processing. Single-use disposable gloves were used at all stages of sample collection and laboratory procedures. All surfaces and laboratory equipment were cleaned with a 20% bleach solution before and after each use.

2.5 | Metabarcoding Laboratory Protocol

A protocol detailing all steps, reagents, primer sequences, and cycling conditions for the metabarcoding procedure from the first PCR until sending for MiSeq sequencing is available at protocols.io DOI: https://doi.org/10.17504/protocols.io.ewov1qxokgr2/v3 (Wisely 2023). We amplified the MiFish-U (Miya et al. 2015) region of the mitochondrial 12S rRNA gene and the Crustacean_16S (Berry et al. 2017) mitochondrial rRNA gene region in all of our samples. We chose these two primer sets because the diets of bonnethead, blacknose, blacktip, and Atlantic sharpnose sharks are thought to mainly be composed of teleost fishes and crustaceans (Cortes et al. 1996; Plumlee and Wells 2016; Harrington et al. 2016; Ford 2012; Matich et al. 2021). Both sets of primers had Illumina's Nextera-style adapters appended to the 5′ ends of both the forward and reverse primer binding regions, after 3 to 4 Ns of heterogeneity spacers for sequence diversity on the flowcell. The heterogeneity spacers also reduce the potential of tag bias introduced by the addition of the Illumina adapters on the 5′ end of the primers (Bohmann et al. 2022). All PCR reactions were performed in triplicate for each sample and primer combination using Takara High Fidelity PCR EcoDry Premix in the Bruno Lab at UNC.

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Equal volumes (10 μL) of each PCR reaction for both MiFish and Crustacean_16S primers were combined by sample for a total volume of 60 μL (i.e., 10 μL of each triplicate for each primer), except for the PCR negative controls which were pooled by primer set, resulting in 60 μL of MiFish PCR control and Crustacean_16S PCR control. Negative controls were pooled by primer so that we had representation of negative controls from all three plates of PCR for the corresponding primer in a single well. The negative control encompassed all controls for potential contamination introduced in the PCR steps for each primer set, and keeping them separated by primer (i.e., MiFish and Crustacean_16S) allowed us to use them as sequencing controls to eliminate tag-jumps. Each well containing the combined PCR products was then cleaned with a 1.5× ratio of Ampure XP beads to remove any unattached primers or primer-dimer from the first PCR reaction and eluted in 30 μL of Qiagen Buffer EB. The second PCR step applied indexed iNext primers (Glenn et al. 2019) to 2.5 μL of combined and cleaned MiFish and Crustacean_16S PCR replicates for each sample separately (average 100 ng), using a combinatorial dual indexing strategy. iNext primers 1–12 and A-P minus L and O were chosen to maintain the proper color balance on the sequencer.

Indexed amplicons were then visualized in a 2% agarose gel to ensure that there was not an excess amount of visible primer-dimer. Each sample and negative control were quantified with picogreen at the University of Arizona Genetics Core, so that a single equimolar pool containing amplicons from each sample in equal proportions could be made. A final bead cleanup using a ratio of 0.9× Ampure XP beads removed fragments shorter than 200 base pairs since our target fragments were 350–400 base pairs long after the addition of the sequencing adapters and indexes. An aliquot of 200 μL of 10 nM pooled library was sent to the High-Throughput Sequencing Facility (HTSF) at UNC for paired-end sequencing on a single MiSeq lane using 250 cycles.

2.6 | Bioinformatics

Sequences were demultiplexed by sample and then by primer set, separating the fish reads from the crustacean reads for each sample, using Cutadapt (v4.1) (Martin 2011). Adapter and primer sequences were removed using Cutadapt and Trimmomatic (v0.39) (Bolger et al. 2014). Cleaned reads for fishes and crustaceans were processed separately in Obitools3 (Boyer et al. 2016). Paired-end sequences with an alignment score over 80% were merged and retained, and the merged sequences that occurred fewer than 10 times in the dataset or were shorter than 80 base pairs were filtered out. The copy number threshold of 10 was chosen because it is the parameter setting in the tutorial accompanying this software, and because sequences occurring at low copy number in the overall dataset are more likely to be chimeric sequences (Alberdi et al. 2018), or the result of low amplification efficiency of the template DNA relative to the metabarcoding primers (Kelly et al. 2019). The obiclean function of obitools3 was used to filter out putative PCR artifacts and identify amplicon sequence variants (ASVs) for taxonomic assignment.

Taxonomic assignment databases were created by downloading all vertebrate (for fish) or invertebrate (for crustaceans) sequences in the EMBL database (December, 2022). In silico PCR was conducted on the database sequences using the ecoPCR function (Ficetola et al. 2010) of Obitools3, allowing up to 3 bp mismatches in the primer binding region, to create the final MiFish-U and Crustacean_16S databases. ASVs were assigned to taxa using the lowest common ancestor (LCA) algorithm implemented in the ecotag function of Obitools3. Sequences that matched to the database with over 97% sequence similarity in our initial taxonomic assignment step were assigned to the taxonomic classification of the lowest common ancestor of their best matches with ecotag, and subjected to further filtering in R using the *LULU* (version 0.1.0) (Frøslev et al. 2017) and *MetabaR* (version 1.0.0) (Zinger et al. 2021) R packages.

Using the *MetabaR* package, sequences were filtered based on ASVs found in negative controls and taxonomic identification sequence similarity thresholds. The parameters for filtering were determined for this dataset by visualizing the distribution of each characteristic used for quality control in the *MetabaR* package. Low quality outliers for every criterion mentioned above were flagged and removed. Although none of the ASVs flagged as contaminants by *MetabaR* were identified in the initial taxonomic assignment step as belonging to any taxon of interest, we used the Megablast algorithm (Morgulis et al. 2008; Zhang et al. 2000) on the NCBI web server (accessed 12-2-2024) to create a table of putative contaminant identities (Data S3). Samples with less than 2000 total reads (i.e., those in the lower tail of the distribution histogram of sequencing read depth (Data S4)) and any sample with 10% or more of its reads determined to be contaminants and ASVs which were not assigned to at least 98% sequence identity were removed. Finally, reads occurring at a threshold below 1% of the reads in each Crustacean_16S sample and below 4% in each MiFish sample were flagged as potential tag-jumps and were removed based on the incidence of reads from each primer occurring in the other's negative controls. After filtering, the effectiveness of the *MetabaR* filtration parameters was confirmed by verifying that the negative controls contained no remaining ASVs. Reads from each shark species in the study were removed from their respective samples, and finally, the curated and filtered fish and crustacean ASVs were merged back together into a final fish and crustacean combined dataset for downstream analyses. The *phyloseq* (version 1.42.0) (McMurdie and Holmes 2013) R package was employed to aggregate ASVs from each prey species and conduct exploratory visualizations. Read counts per prey taxon were then normalized to median sequencing depth and converted to percentages of occurrence in each sample for statistical analysis. All bioinformatics code is available at https://github.com/EWisely/NC_Sharks (v1.0.3).

2.7 | Statistical Analysis

All statistical analysis was performed in R (version 4.2.2; www.R-project.org), using RStudio (version 2022.12.0 + 353). We only included paired samples that had both the fecal and stomach slurry sample from the same individual shark successfully pass the quality control and filtering steps ($n = 23$) in the analysis. A Levene's test and a paired *t*-test were used to compare the number of ASV reads between the fDNA and stDNA using the *car* package (version 3.1–2) (Fox

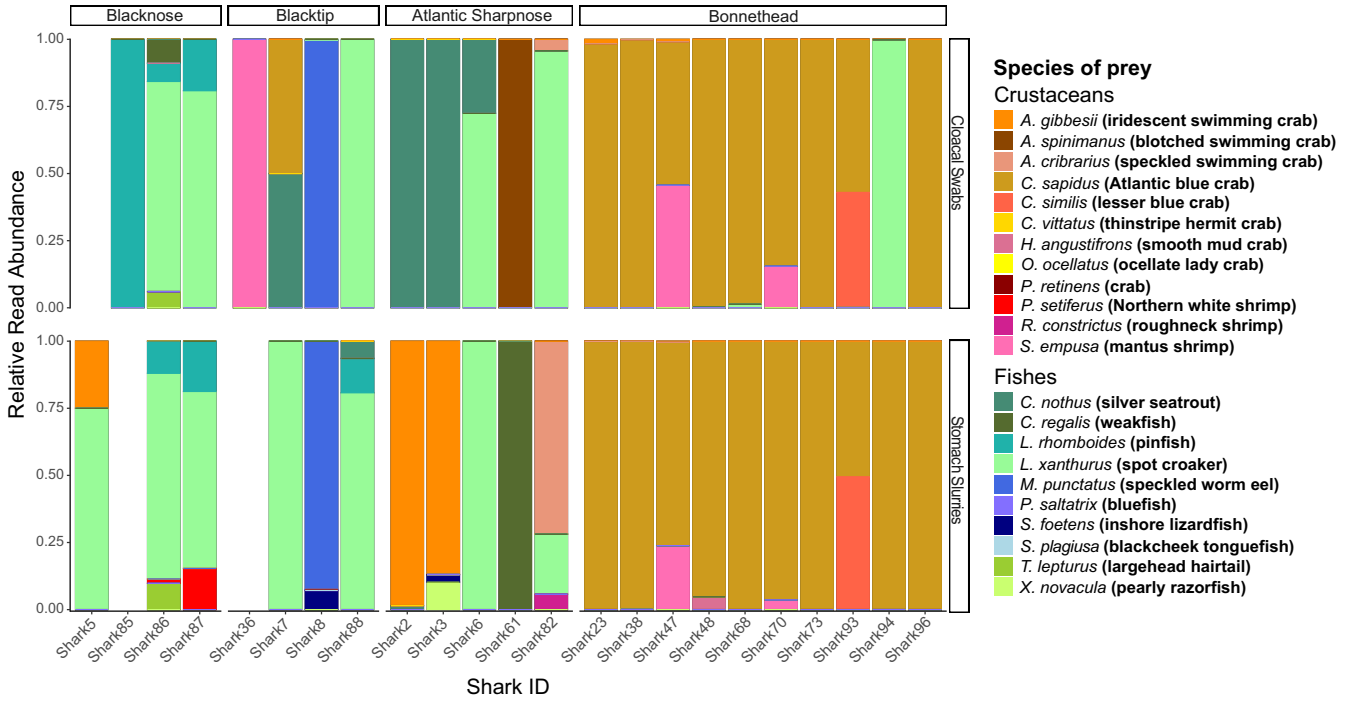

FIGURE 1 | Normalized relative read abundance of the most common 20 species of prey found in all sharks separated by shark species ($n = 23$). The x-axis represents individual sharks, with the two materials analyzed: cloacal swabs (fDNA) represented in the top of the plot and stomach slurries (stDNA) represented in the bottom of the plot. For the cloacal swab of shark 5 and the stomach slurries of sharks 85 and 36, the DNA found was either not included in the 20 most common species of prey found or could not be classified to species level.

and Weisberg 2018) and the *rstatix* package (version 0.7.2) (Kassambara 2019). We used the *vegan* package (version 2.6-4) (Oksanen 2015) for the calculation of the sample-based prey rarefaction curves. The Shannon-Wiener diversity index and richness between sample types were each compared with the shark species as the independent factor using ANOVAs with the *stats* package (version 4.2.2) (R Core Team and contributors worldwide). For these three comparisons (i.e., rarefaction curves, Shannon-Wiener diversity index, and richness) we focused on prey that were taxonomically identified to genus and/or species because all data passing our 97% sequence similarity threshold were able to be identified to at least genus and a majority were identified to species. We calculated the Sørenson similarity index between each individual shark's paired fDNA and stDNA samples using the *phyloseq* package (McMurdie and Holmes 2013). A permutational multivariate analysis of variance (perMANOVA) was used to compare the dietary differentiation found in the cloacal swabs and the stomach slurries among the four different shark species with *adonis2* (Oksanen et al. 2015) using Jensen Shannon distance (i.e., measurement of the similarity between two probability distributions) of relative prey read abundance (compositional transformation in *phyloseq* (McMurdie and Holmes 2013)) in the *vegan* package (Oksanen et al. 2015).

We considered both relative read abundance (RRA) and percent of occurrence (POO) in our analyses. RRA is the proportion of reads assigned to a specific taxon within a sample, relative to total reads within that sample. POO is the percentage of samples in which the presence of a particular taxon was recorded, weighing the presence of each identified taxon as one and the absence of detection as zeros, in an effort to negate technical and biological biases inherent in the method. However, the dietary importance of rarely consumed species may be exaggerated in POO analyses (Deagle et al. 2019). Statistical analysis R Markdown code is available at https://github.com/sryburn95/GitHub_cloacalswab_v_stomach.

3 | Results

A total of 48 sharks (i.e., 17 bonnethead, seven blacknose, five blacktip, and 19 Atlantic sharpnose) were caught, 28 of which had stomach contents. Of those, we successfully obtained both the fDNA and stDNA (Figures 1 and 2) from 23 sharks: 10 bonnethead (100.4 ± 3.3 cm mean STL), four blacknose (113.9 ± 5 cm mean STL), four blacktip (116.4 ± 1.9 cm mean STL), and five Atlantic sharpnose sharks (81.7 ± 9.8 cm mean STL). Among the 23 successfully metabarcoded sample pairs, 21 stomachs had prey items that could be morphologically identified (Data S2).

For cloacal swabs, a total of 19 prey taxa were identified: 74% identified to species and all were identified to genus and family (cloacal swabs; mean ASV reads per prey species: 33,359.36 ± 20,568.54, range 1–278,060; Data S1). Bonnetheads had three prey species (*Leiostomus xanthurus*, *Symphurus plagiusa*, and *Penaeus setiferus*) and one genus (*Stephanolepis*) detected exclusively in cloacal swabs (Figure 3). Blacktips also had three prey species (*Symphurus plagiusa*, *Callinectes sapidus*, and *Squilla empusa*) along with one genus (*Penaeus*) unique to cloacal swab results. For Atlantic sharpnose sharks, cloacal swabs had a single prey species (*Achelous spinimanus*) not detected in the stomach slurry or morphological analysis, while blacknose sharks had no prey taxa unique to cloacal swabs.

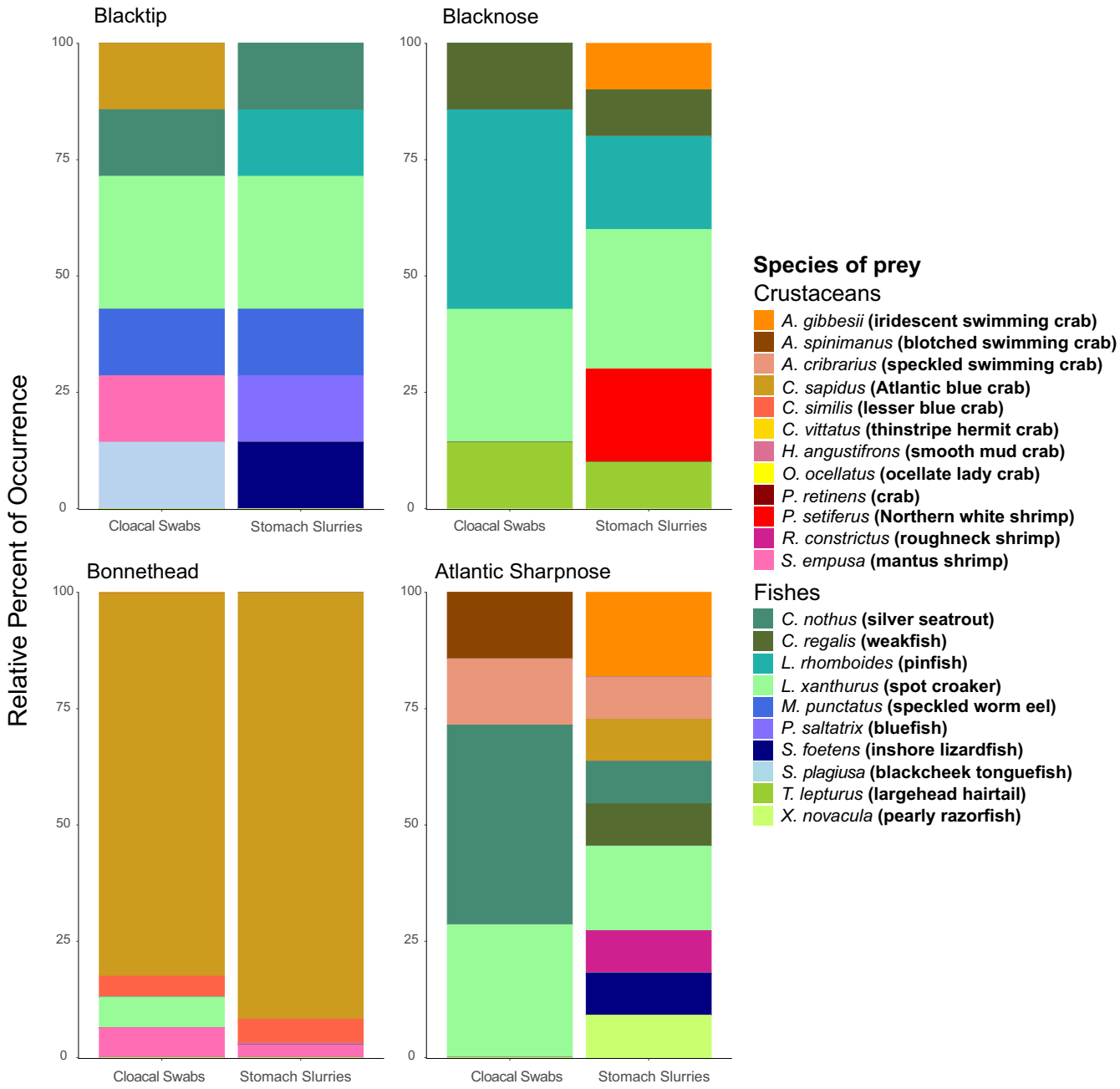

FIGURE 2 | Relative percent of occurrence of the most common 20 species of prey found in all sharks ($n = 23$). The x-axis represents the two material types analyzed: cloacal swabs (fDNA) on the left and stomach slurries (stDNA) on the right, separated by the four shark species.

We identified 25 prey taxa in the stomach slurries: 80% identified to species and all were identified to genus and family (stomach slurry; mean ASV reads per prey species: 37,702.30 ± 21,379.78, range 6–424,272; Data S1). Bonnetheads had six prey species (*Cynoscion nothus*, *Ovalipes ocellatus*, *Lagodon rhomboides*, *Hexapanopeus angustifrons*, *Clibanarius vittatus*, and *Pinnixulala retinens*) along with one genus (*Brevoortia*) that was unique to the stomach slurries (Figure 3). Atlantic sharpnose sharks had five unique prey species (*Cynoscion regalis*, *Synodus foetens*, *Xyrichtys novacula*, *Callinectes sapidus*, and *Rimapenaeus constrictus*) and one genus (*Stephanolepis*) detected only in stomach slurries. Blacktips had three prey species (*Potatomus saltatrix*, *Lagodon rhomboides*, and *Synodus foetens*) exclusive to stomach slurries. Blacknose sharks had two unique prey species (*Penaeus setiferus* and *Achelous gibbesii*) and two genera (*Stephanolepis* and *Pinnixa*) found only in stomach slurries. All prey taxa found in the cloacal swabs and stomach slurries were also identified to order except for those in the Sciaenidae family since the order is still taxonomically disputed.

The 23 paired samples produced 1,396,719 reads of ASVs with a mean count per sample of 30,363 ± 6,167. There was an average of 5.34 ASVs per prey taxon (range: 1–49). Cloacal swabs had 28% fewer ASV reads per sample (21,757 ± 11,125 reads) than stomach slurries (38,969 ± 5,010 reads; paired *t*-test: $t = 4.92$, $df = 45$, $p < 0.0001$). Stomach slurries and cloacal swabs had equal variation in the number of ASV reads per sample (Levene's test: $F = 0.0154$, $df = 1$, $p = 0.9018$).

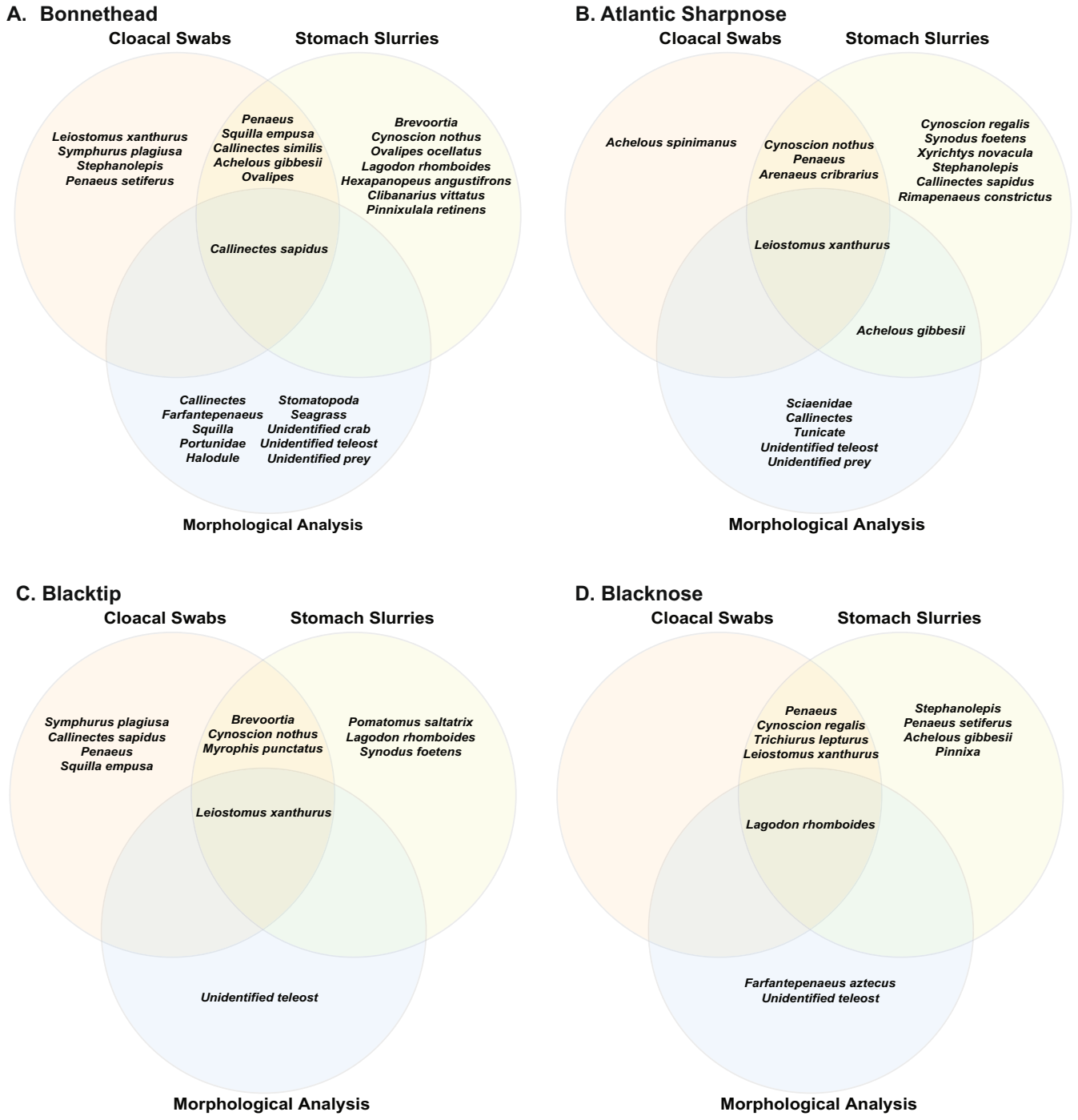

FIGURE 3 | Venn diagrams of prey taxa identified with metabarcoding cloacal swabs, metabarcoding stomach slurries, and morphological identification of stomach contents for each shark species. Figure includes results from all 23 sharks.

Of the 48 shark stomachs, 28 (58%) contained prey items that were morphologically identified. A total of 81 prey items were collected from 21 of the 23 shark stomachs that were also successfully metabarcoded, with an average of two prey items found and morphologically identified per stomach (Data S2). We morphologically identified 32% of the prey items as either unidentifiable teleost, unidentifiable crab, or unidentifiable prey, and only five taxa could be identified to species (i.e., Atlantic blue crab (*Callinectes sapidus*), Atlantic spot croaker (*Leiostomus xanthurus*), iridescent swimming crab (*Achelous gibbesii*), pinfish (*Lagodon rhomboides*), and brown shrimp (*Farfantepenaeus aztecus*); Table 1; Figure 3). Brown shrimp was the only species identified morphologically but not detected in the DNA metabarcoding results. Bonnetheads had 10 prey categories (*Callinectes*, *Farfantepenaeus*, *Squilla*, *Portunidae*, *Halodule*, *Stomatopoda*, seagrass, unidentified crab, unidentified teleost, and unidentified prey) detected exclusively through morphological analysis (Figure 3). Atlantic sharpnose sharks had five unique prey categories (*Sciaenidae*, *Callinectes*, tunicate, unidentified teleost, and unidentified prey) identified only by morphology. Blacknose sharks had two prey categories (*Farfantepenaeus aztecus* and unidentified teleost) identified solely by morphology, while

TABLE 1 | Morphologically identified prey items identified to the lowest taxonomic rank possible from 21 of the 23 shark stomachs that had prey items present.

Prey items					Abundance			
Order	**Family**	**Genus**	**Species**	**Other**	**Bonnethead**	**Atlantic sharpnose**	**Blacktip**	**Blacknose**
Decapoda	Portunidae	*Callinectes*	*Sapidus*		21			
Acanthuriformes	Sciaenidae	*Leiostomus*	*xanthurus*			2	5	
Decapoda	Portunidae	*Achelous*	*gibbesii*			1		
Spariformes	Sparidae	*Lagodon*	*rhomboides*					5
Decapoda	Penaeidae	*Farfantepenaeus*	*aztecus*					1
Decapoda	Portunidae	*Callinectes*			5	1		
Stomatopoda	Squillidae	*Squilla*			1			
Decapoda	Penaeidae	*Farfantepenaeus*			1			
Alismatales	Cymodoceaceae	*Halodule*			1			
Decapoda	Portunidae				2			
Acanthuriformes	Sciaenidae					1		
Stomatopoda					1			
				Seagrass	3			
				Tunicate		4		
				Unidentified prey	1	1		
				Unidentified crab	1			
				Unidentified teleost	1	6	4	12

Note: Abundance refers to the number of times a particular prey taxa was identified.

blacktips had one prey category (unidentified teleost) detected only through morphological analysis. Of the 81 prey items, 23 (28%) were morphologically identified as "unidentifiable teleost" and 21 (26%) were identified as Atlantic blue crab (Table 1).

Bonnetheads had three prey species (*Squilla empusa*, *Callinectes similis*, and *Achelous gibbesii*) and two genera (*Penaeus* and *Ovalipes*) detected in both cloacal swabs and stomach slurries, as well as one species (*Callinectes sapidus*) present across all three sample types (cloacal swabs, stomach slurries, and morphological analysis) (Figure 3). Atlantic sharpnose sharks had two prey species (*Cynoscion nothus* and *Arenaeus cribrarius*) and one genus (*Penaeus*) shared between cloacal swabs and stomach slurries, along with one species (*Leiostomus xanthurus*) identified by all three methods. Blacktips had two prey species (*Cynoscion nothus* and *Myrophis punctatus*) and one genus (*Brevoortia*) found in both cloacal swabs and stomach slurries, plus one species (*Leiostomus xanthurus*) detected across all sample types. Blacknose sharks had three prey species (*Cynoscion regalis*, *Trichiurus lepturus*, and *Leiostomus xanthurus*) and one genus (*Penaeus*) shared between cloacal swabs and stomach slurries, with one species (*Lagodon rhomboides*) present in all three sample types.

A rarefaction curve (Figure 4) was made for each shark species comparing the unique prey taxa identified to genus or species in the cloacal swabs to those in the stomach slurries and showed that taxonomic richness was greater in the stomach slurry for all species of sharks except for the blacktip. Although the stomach slurry had more identified taxa (i.e., greater species richness; Table 2; Data S1), there was no statistically significant difference in the detected richness at the species or genus level among sample

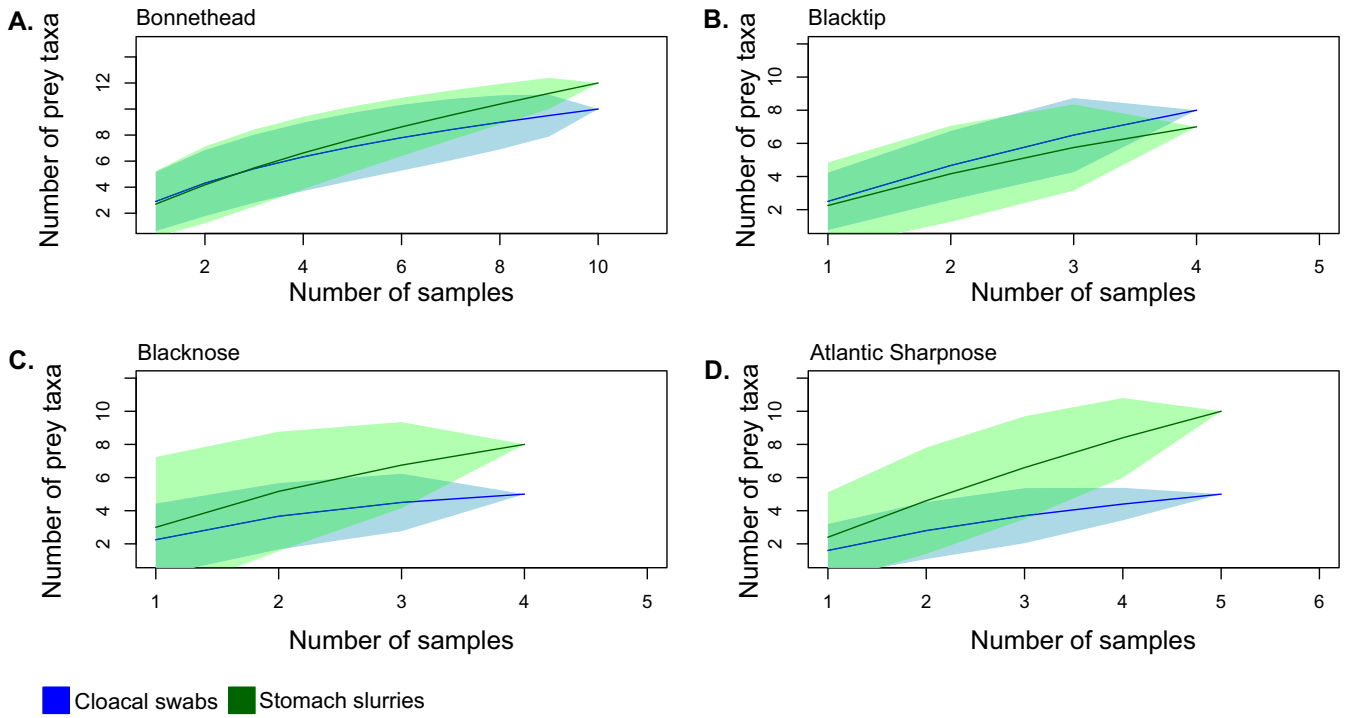

FIGURE 4 | Rarefaction curves depicting prey richness (i.e., number of unique prey taxa classified to the genus and species levels) as a function of the number of sharks sampled for each species (i.e., bonnethead $n = 10$, blacktip $n = 4$, blacknose $n = 4$, and Atlantic sharpnose $n = 5$). Color depicts sample material type, cloacal swabs (i.e., fDNA) and stomach slurries (i.e., stDNA). Error bars indicate the 95% confidence intervals after 100 permutations.

TABLE 2 | Number of prey identified to the species level and the number of reads in the cloacal swabs and the stomach slurry per shark species.

Shark species	Number of sharks sampled	Mean STL (cm)	STL range (cm)	Cloacal swabs		Stomach slurry	
				Number of prey species	Number of reads	Number of prey species	Number of reads
Atlantic Sharpnose	5	81.7 ± 9.8	43–96.2	4	17,141	9	187,990
Blacknose	4	113.9 ± 5	105.4–125.4	4	39,925	6	122,774
Blacktip	4	116.4 ± 1.9	112–120	6	34,433	6	106,033
Bonnethead	10	100.4 ± 3.3	81.1–113.7	6	402,324	10	479,500

Note: Table includes results from the 23 paired successful cloacal swabs and the 23 successful stomach slurries.

types with shark species as an effect (genus level: ANOVA; df = 3, $F = 0.379$, $p = 0.769$) (species level: ANOVA; df = 3, $F = 0.956$, $p = 0.433$). There was also no statistically significant difference in the detected diversity between sample types based on the number of reads (ANOVAs: genus level; Shannon–Wiener diversity: df = 3, $F = 0.221$, $p = 0.88$; species level; Shannon–Wiener diversity: df = 3, $F = 0.441$, $p = 0.727$). It is also important to note that none of the rarefaction curves reached a plateau (Figure 4). Therefore, it is possible that taxa richness is underrepresented.

The degree of similarity of measured prey composition for cloacal swab and stomach slurry samples collected from the same individuals ranged from perfect overlap (Sørenson similarity index = 1) to no overlap (Sørenson similarity index = 0) (Figures 5 and 6).

A nonmetric multidimensional scaling (NMDS) plot (Figure 7) based on Jensen Shannon distance of relative prey read abundance (compositional transformation in *phyloseq*) was created to visualize the degree of overlap in prey composition when the prey was categorized at the genus level between the two sample types (i.e., fDNA and stDNA) separated by shark species. There was no statistical difference when comparing the prey composition between sample types (perMANOVA: pseudo $F = 1.007$, $R^2 = 0.013$, df = 1, $p = 0.398$). However, prey composition did vary among the four shark species (perMANOVA: pseudo $F = 10.886$, $R^2 = 0.435$, df = 3, $p = 0.001$).

4 | Discussion

Our results indicate that DNA metabarcoding of cloacal swabs is representative of shark stomach contents and an effective tool to assess shark diets. We predicted that (1) the stDNA would yield more identifiable DNA than the fDNA because the stomach contents would be less degraded and that (2) both sources of DNA would provide similar measures of taxonomic richness and composition. Therefore, the description of diet would not be affected by sample type. As we predicted, there were significantly more reads of ASVs produced from the stomach slurries, suggesting some DNA degradation in the intestines. However, the fact that there was no difference in taxonomic richness and composition (between sample types) suggests that prey DNA degradation was not so severe as to meaningfully affect the diet reconstruction.

Our findings were similar to those of Snider et al. (2022) who also found no statistical difference in taxonomic diversity between the stomach contents and fecal matter of seaside sparrows. Even though richness and composition did not differ significantly, we did not expect or find prey composition estimated from the stomach contents and cloacal swabs sampled from the same individuals to be identical. The two sample types were collected simultaneously and thus likely represented different feeding events, possibly days apart due to the time required for the prey DNA to move through the digestive system (Figure 8). Consequently, we found genera that were unique to the stomach slurries (e.g., *Pomatomus*) and the cloacal swabs (e.g., *Symphurus*) (Figure 3). Sharks feed intermittently (Cortés and Gruber 1990) and have a slow digestion rate compared to carnivorous teleosts (Cortés and Gruber 1992; Wetherbee et al. 1990). The gastric evacuation (i.e., rate of digestion) in sharks can be variable ranging from 5.4 h in juvenile scalloped hammerhead sharks (*Sphyrna lewini*) (Bush and Holland 2002) to 92.3 h in young sandbar sharks (*Carcharhinus plumbeus*) (Medved et al. 1988). Residual DNA may also persist in the gut

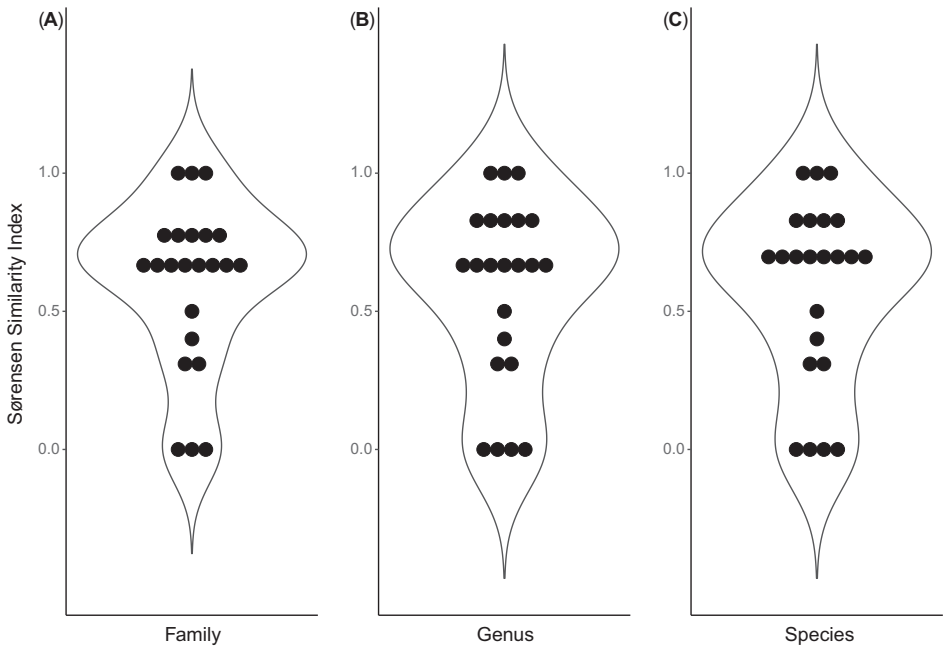

FIGURE 5 | The similarity of prey found in each sample type (i.e., cloacal swabs and stomach slurries) for each individual shark ($n = 23$) using the Sørenson similarity index. All 23 sharks (A) compared to family, (B) compared to genus, and (C) compared to species. Each point depicts the degree of similarity between fDNA and stDNA of the same individual. If the index was 0 the taxonomic composition of the two samples was completely different and if the index was 1 the taxonomic composition of two samples was identical. When prey was analyzed at the family level the mean overlap was 0.60 ± 0.30, and at genus and species level it was 0.58 ± 0.33 and 0.57 ± 0.33 respectively.

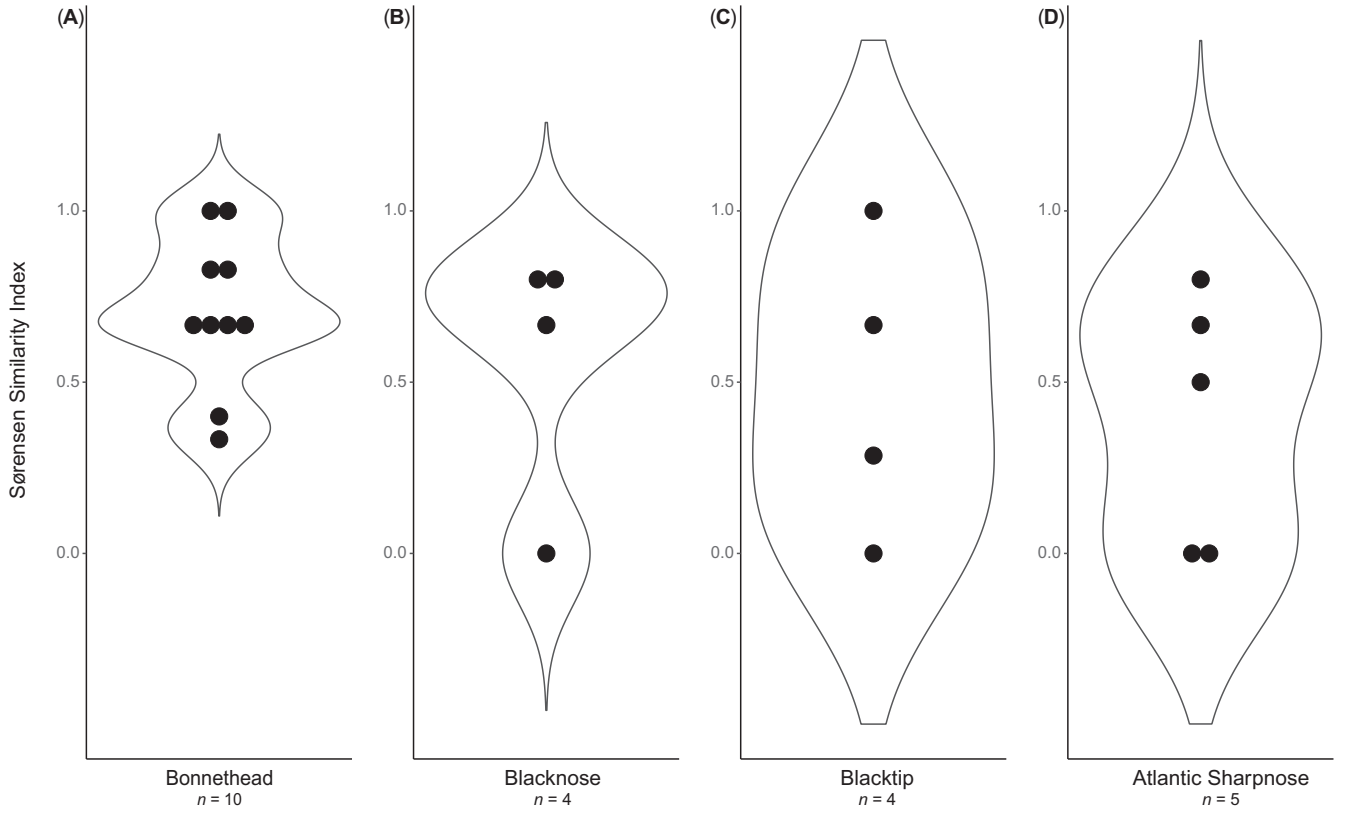

FIGURE 6 | The similarity of prey found in each sample type (i.e., cloacal swabs and stomach slurries) for each individual shark ($n = 23$) using the Sørenson similarity index. (A) Bonnetheads ($n = 10$), (B) blacknose ($n = 4$), (C) blacktip ($n = 4$), and (D) Atlantic sharpnose ($n = 5$) all compared prey indentified at the genus level. Each point depicts the degree of similarity between fDNA and stDNA of the same individual. If the index was 0, the taxonomic composition of the two samples was completely different, and if the index was 1, the taxonomic composition of the two samples was identical. When prey was analyzed at the genus level for only bonnetheads, the mean overlap was 0.71 ± 0.22, for blacktips the mean was 0.49 ± 0.44, for blacknoses the mean was 0.57 ± 0.38, and for Atlantic sharpnoses the mean was 0.39 ± 0.37.

and cloaca longer than the period of gastric evacuation and excretion; therefore, our sampling most likely captured multiple recent foraging events from each individual shark (Figures 1, 2, 5, 7, and 8). In future studies, the combination of stomach slurry and cloacal swabs could enhance the number of prey taxa identified; however, further research is needed to develop a less invasive method than gastric lavage for collecting stomach slurry. Prey rarefaction curves (Figure 4) also show that none of the sample sizes sufficiently captured the full richness of the diet of these four shark species since none of the curves reached a plateau. So, in future studies a greater sample size would be needed to capture the complete diet.

The Atlantic sharpnose, blacktip, and blacknose sharks had similar, taxonomically broad prey results (Figure 7; Data S3). These three shark species are more generalist predators (Drymon et al. 2012; Ford 2012; Matich et al. 2021) so the difference in the taxonomic composition between the two sample types (fDNA and stDNA) for each individual was greater (Figures 6 and 7). In contrast, the bonnethead had a much more specialized diet that consisted mostly of Atlantic blue crab (Figures 1 and 2) therefore there was a greater chance that the meal found in the stDNA was comprised of the same species as the previous meal(s) found in the fDNA (Figures 5 and 6). This suggests the variation in taxonomic composition between sample types from the same individual was most likely due to the timing of sample collection and the inability to differentiate specific foraging bouts (i.e., meals) rather than differences between the two sampling techniques. Additionally, the sample size for Atlantic sharpnose, blacknose, and blacktip sharks was smaller, potentially creating bias due to the bonnetheads having a more specialized diet.

Many shark dietary studies rely on morphological stomach contents identification and are limited by the amount of empty stomachs found (Hoffmayer and Parsons 2003; Bush and Holland 2002; Joyce 2002; Hernández-Aguilar et al. 2016; Plumlee and Wells 2016; Huveneers et al. 2007). Forty two percent of the 48 collected stomachs were empty, 46% of the 48 stomachs had no prey that could be morphologically identified, and 42% of the 48 stomach slurries produced no results while only 10% of the 48 cloacal swabs did not produce results. Whether the stomachs were intended to be dissected and morphologically identified or metabarcoded, our results show that the cloacal swabs produced a higher success rate in terms of producing diet data and that diet can be assessed even when the stomach is empty.

Only a small portion of the fish and crustacean species that are present in the sampling area (www.fishbase.org, www.sealifebase.org) were sequenced within the fDNA and stDNA, suggesting that eDNA contamination in the cloacal swabs was minimal if present. In total our samples sequenced 22 species

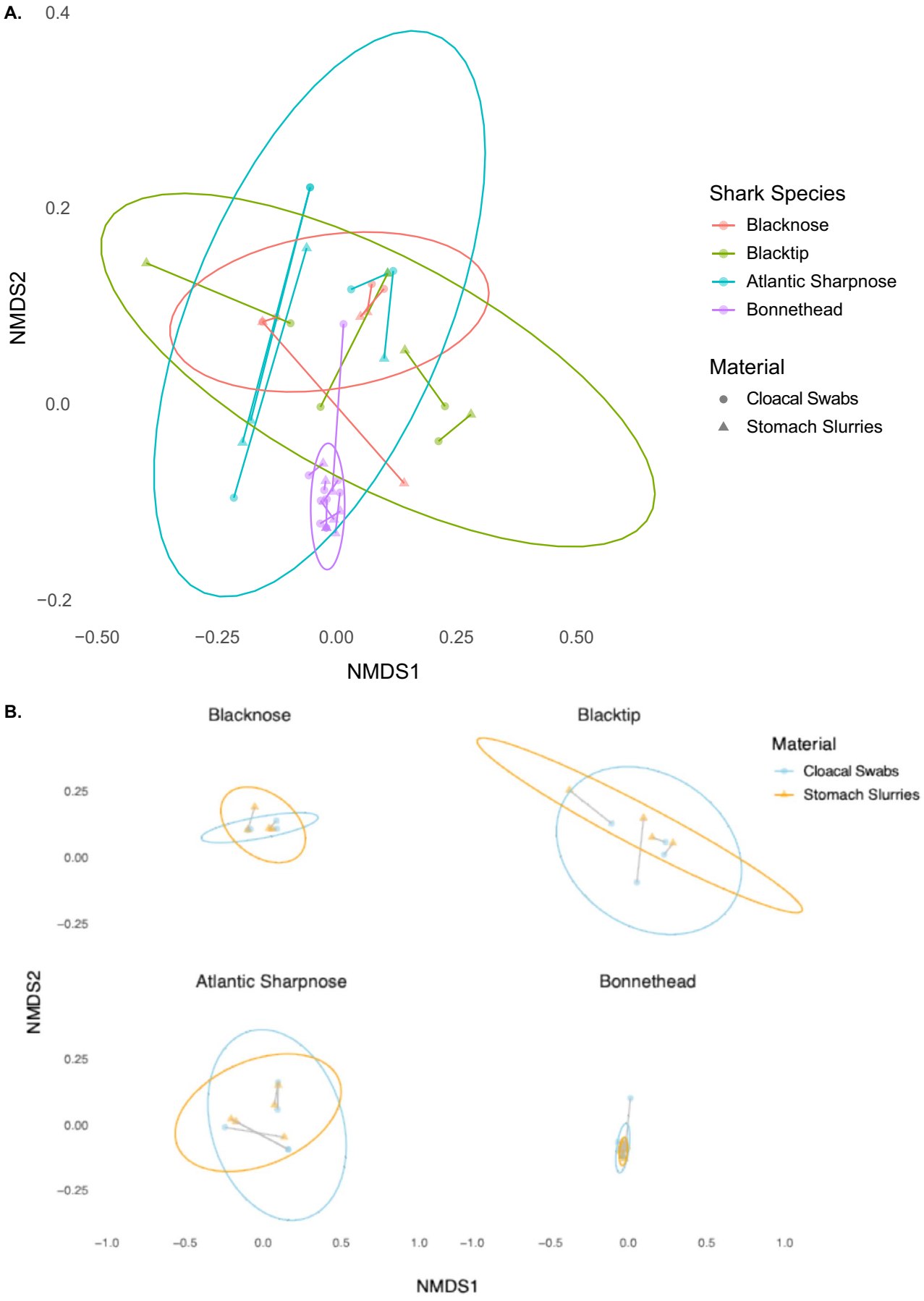

FIGURE 7 | Legend on next page.

FIGURE 7 | Nonmetric multidimensional scaling (NMDS) plots based on Jensen Shannon distance of relative prey read abundance (compositional transformation in *phyloseq*) of prey composition by shark species and material. Material type is denoted by shape: cloacal swabs (fDNA) are circles and stomach slurries (stDNA) are triangles. Degree of overlap in diet composition is represented by the line that connects the paired samples (i.e., fDNA and stDNA from the same individual shark). The shorter the line (i.e., the closer the paired points), the more similar the composition. Figure contains all individual sharks ($n = 23$) and all samples (fDNA $n = 23$, stDNA $n = 23$). (A) Shark species is represented by color and prey genus driving patterns of dietary differentiation. (B) Material type is represented by color and prey genus driving patterns of dietary differentiation.

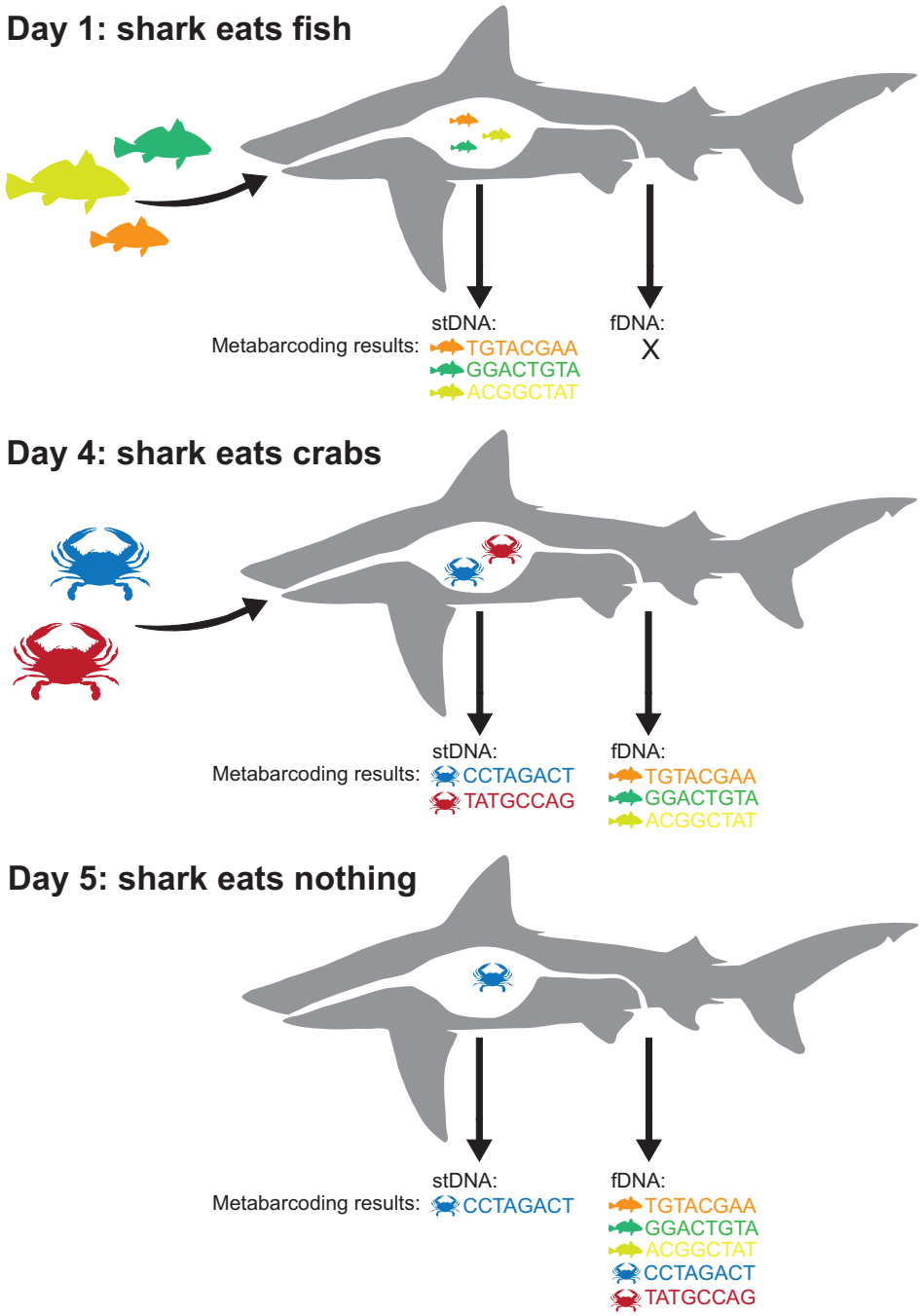

FIGURE 8 | Illustration exemplifying how the timing of foraging bouts and rates of digestion can influence diet reconstruction based on stomach slurry DNA (stDNA) and fecal DNA (fDNA). Based on these timing contingencies, for samples collected simultaneously, the two techniques might not necessarily provide the same results within an individual shark. The different colors of fishes and crabs represent different species.

of fishes and crustaceans and all prey species present in both sample types can be found off the coast of North Carolina (www.fishbase.org, www.sealifebase.org). The UNC IMS shark survey has been conducting trawl surveys to assess the biodiversity of Onslow Bay since 1972 and has estimated the presence of over 100 different species in the past decade. Our samples only sequenced a fraction of the fish and crustacean species found on the survey. Also, the most common prey species for each shark

varied (e.g., bonnetheads mainly consumed Atlantic blue crabs while the Atlantic sharpnose had a high proportion of silver seatrout DNA (*Cynoscion nothus*)) (Figures 1 and 2) and our results were consistent with previous dietary studies conducted on the four species of sharks (Branham et al. 2022; Hoffmayer and Parsons 2003; Ford 2012). Additionally, the stDNA had no direct contact with the seawater and there was no statistical difference in taxonomic diversity and richness between sample types. Therefore, our results suggest that if eDNA contamination on the cloacal swabs occurred, it was minimal. For future studies using cloacal swabs, we recommend collecting a negative control by swabbing the exterior of the cloaca after it has been wiped with ethanol and allowed to dry.

In addition to eDNA contamination, another concern when metabarcoding stDNA and fDNA is secondary predation (i.e., prey consumed by prey or the "Russian doll" effect). Few studies have tested this (but see: de Bruyn et al. 2021; Varennes et al. 2014), but our results suggest that the DNA of the prey's prey was most likely too degraded to be amplified by our primers. If the DNA of the prey's prey was being sequenced, our results would have included more species of lower trophic levels (Data S1). For example, the 20 most common species of prey DNA (Figures 1 and 2) are shown, and the majority of the bonnethead's diet was Atlantic blue crab. The diet of the Atlantic blue crab mainly consists of a variety of mollusks and smaller crustaceans (e.g., *Mya arenaria* and Xanthidae) (Meise and Stehlik 2003; Laughlin 1983), which were not detected in our samples. So, if the DNA of the Atlantic blue crab's prey was also being sequenced in our samples, we would have had more results for mollusks and smaller crustaceans in the diet results for the bonnethead sharks. There is always a small possibility that secondary predation is present, but overall we did not find the expected prey's prey DNA in our results; therefore, secondary predation is likely not a large concern and should not be a deterrent from using this method. However, this is clearly a topic for future research since secondary predation is an issue present in all dietary studies regardless of technique.

Ultimately, both sample types were effectively equivalent in prey taxa detection and can both be used in future studies to describe the diet at the individual and population level. Our results confirm that the non-lethal, minimally invasive method of metabarcoding fDNA from cloacal swabs is an efficient and effective alternative for shark diet reconstruction. The cloacal swabs provided higher prey taxonomic resolution (i.e., at least to genus level with a majority identified to species level) than other methods such as morphological stomach contents identification and stable isotope analysis (Plumlee et al. 2023). The swabs were also able to identify rare prey species that are not consumed as frequently and species that are subject to a faster rate of digestion (Data S1 and S2). When designing future studies, it is important to consider the robustness of the location's genetic library and which primers are suitable for amplifying the specific shark species' prey. Collecting fDNA with cloacal swabs is easier and quicker compared to collecting stomach contents. This method also causes little to no harm to the shark. Sharks play critical functional roles in coastal and open ocean ecosystems, for example, through the regulation of prey populations (Wallach et al. 2015). One-third of shark species are threatened with extinction and designated as "Critically Endangered", "Endangered", or "Vulnerable" by the International Union for Conservation of Nature (IUCN) (Dulvy et al. 2021) with populations continuing to decline. Despite their importance, basic natural history information such as diet, range, and life cycle are largely unknown for many species. A greater understanding of this basic ecological information could improve shark conservation and management through the implementation of more specific ecosystem-based management schemes.

Author Contributions

S.J.R., J.D.P., and E.W. designed the study. S.J.R., J.D.P., and C.C.B. collected the samples. C.C.B. and J.D.P. completed the morphological identification of the stomach contents. S.J.R. and E.W. completed the DNA analysis. S.J.R. and E.W. analyzed the data. The manuscript was written by S.J.R. with help from E.W. and J.F.B. All authors revised the manuscript and gave final approval for publication.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in FigShare at https://figshare.com/s/fa78cef15da8d9e61cc6.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** The number of ASV reads for each prey taxa in each sample. Tables include catch information for each shark and the trophic level, environment, and IUCN status of the prey taxa. The first page is the fDNA results from the cloacal swabs and the second page is the stDNA results from the stomach slurries. **Data S2:** Number of prey items per taxon in each shark stomach. Table also includes the full weight of the stomach contents and the full weight of the identified stomach contents. **Data S3:** The top blast matches for ASVs identified as contaminants in the *MetabaR* workflow. **Data S4:** The (A) MiFish 12_S and (B) Crustacean_16S read depth histograms show the range of sequencing depths for each sample per primer set. The filtering threshold for read depth of 2000 reads per sample is shown by the dashed orange vertical line.