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Bone appétit: DNA metabarcoding as a non-lethal alternative to morphological dietary assessment in Atlantic bonefish (*Albula vulpes*)

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Abstract Traditional approaches to dietary assessment in fish necessitate the collection of stomach contents through either gastric lavage or lethal sampling. The Atlantic bonefish (*Albula vulpes*) is an economically important sportfish in the western central Atlantic region for which a minimally invasive, non-lethal alternative to morphological dietary assessment would be extremely useful. Here, we compare dietary DNA metabarcoding from cloacal swabs of 16 *A. vulpes* to dietary composition data obtained using traditional morphological classification techniques and metabarcoding of homogenized stomach contents. Further, we compare the performance of two commonly used barcoding genes (18S rRNA and COI) at inferring *A. vulpes* diet composition. We found that detection of taxa and the resolution of taxonomic annotation varied between markers, suggesting a multi-marker approach is likely to provide the most complete results. Importantly however, the number of dietary OTUs identified and the taxonomic composition of the core diet were not significantly different between molecular markers. Dietary compositions identified using metabarcoding approaches differed in both diversity and composition from matched dietary data obtained from morphologically analyzed stomach contents; however, the same core prey classes were identified using both methods, suggesting that metabarcoding does indeed offer a viable alternative to morphological dietary assessment. Importantly, dietary compositions identified by metabarcoding of cloacal swabs did not differ significantly from those identified by metabarcoding of stomach contents. Metabarcoding of minimally invasive cloacal swabs should be considered for dietary studies of bonefish and other fish species for which invasive or lethal sampling is problematic.

Keywords Dietary metabarcoding · DNA · Fish conservation · *Albula vulpes* · Bonefish · 18S · COI · Recreational fisheries

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Introduction

Bonefish (*Albula* spp.) are members of a diverse genus of benthivorous marine fishes inhabiting tropical and sub-tropical regions around the world (Colborn et al. 2001; Pickett et al. 2020). Bonefish primarily forage in near-shore shallow "flats" and are physiologically adapted to high-speed burst swimming (Murchie et al. 2013), making them a prized sportfish (Adams 2017). The recreational catch-and-release fishery for Atlantic bonefish (*Albula vulpes*) contributes significantly to the economies of several countries within their Caribbean range (Fedler 2013, 2019; Palomo and Perez 2021). Recently, populations of *A. vulpes* in Florida experienced marked population declines, with corresponding declines in numbers and sizes of bonefish caught (Santos et al. 2017; Kroloff et al. 2019; Rehage et al. 2019; Boucek et al. 2022). Mounting concerns over the sustainability of the bonefish population in Florida and elsewhere have led to increased efforts to understand their ecological requirements (Adams and Cooke 2015; Adams 2017; Brownscombe et al. 2019). Assessing dietary composition is critical for such efforts (Griffin et al. 2019).

Traditional methods for dietary assessment in fish require either invasive methods (e.g., gastric lavage; Foster 1977) or lethal sampling (Griffin et al. 2019), followed by analysis of the morphology of stomach contents. These efforts are labor-intensive and require experts in the morphology and taxonomy of prey items consumed (Nagareda and Shenker 2008; Jud et al. 2011). Furthermore, data generated by these methods often lack taxonomic resolution, with lists of prey items typically assignable to order or family, rather than genus or species (Nagareda and Shenker 2008; Jud et al. 2011; Griffin et al. 2019). In addition, although gastric lavage is considered non-lethal, the process can increase mortality (Barbour et al. 2012). This is likely true for bonefish, which are susceptible to stress from handling (Danylchuk et al. 2007a, b). One alternative method is the analysis of stable isotopes in fish tissues to assess dietary composition. Even though these methods do not necessitate lethal sampling in all cases, the taxonomic resolution of the inferred dietary components is also poor (e.g., Jepsen and Winemiller 2002; Araújo et al. 2007).

Dietary metabarcoding is emerging as a potential alternative to morphological dietary assessment (e.g., Jakubavičiute et al. 2017; Casey et al. 2019; Waraniak et al. 2019). Most commonly, dietary metabarcoding approaches rely on fecal samples, which can be collected non-invasively or minimally invasively, from which DNA can be extracted and sequenced (Ingala et al. 2021; Lu et al. 2021; Snider et al. 2021). Sequences are then assigned to taxa by comparison to publicly available databases (Ingala et al. 2021; Lu et al. 2021; Snider et al. 2021). Dietary metabarcoding approaches recover equal or greater diversity of prey/forage items and allow the assignment of prey or forage items to a finer taxonomic level than traditional approaches (e.g., Nichols et al. 2016). Dietary metabarcoding also does not rely on the expertise of morphological taxonomists, and it is amenable to high-throughput processing, thus facilitating studies of large numbers of individuals (Nichols et al. 2016). Dietary metabarcoding is also likely to enable the detection of taxa which are rendered physically unidentifiable during digestion.

Here, we investigate the suitability of dietary metabarcoding using cloacal swabs as a non-lethal alternative to morphological dietary analyses in *A. vulpes*. We compare the dietary composition profiles of 16 fish inferred using traditional morphological analysis to profiles of the same fish inferred using metabarcoding of both homogenized stomach contents and cloacal swabs. Further, we compare the suitability of two commonly used molecular markers for dietary metabarcoding studies in *A. vulpes*.

Methods

Bonefish capture and sampling

A total of 16 bonefish were collected from Biscayne Bay, the Upper Florida Keys, Lower Florida Keys, and sites west of Key West (see Campbell et al. 2022, for more detailed descriptions of sampling locations). Individual data on sampled fish are presented in Table S1. Bonefish were captured using fly fishing techniques and traditional hook and line angling techniques (Brownscombe et al. 2013). Immediately following capture, a fine tipped polyurethane swab (MWE Medical Wire, Corsham, UK) was inserted into the cloaca of each fish to obtain fecal material. Cloacal swabs were placed into 2-ml cryovials containing 1ml of RNAlater (Sigma Aldrich, St. Louis, USA). Fish were euthanized with an overdose of the

anesthetic MS-222. Fish and collected swabs were placed on ice for no more than 6 h and then stored in – 20 °C freezers until processing.

Stomach contents analysis

Bonefish were thawed in an insulated cooler for 6 h prior to dissection, to retain the morphology of prey items. Stomachs were removed intact using sterile disposable standard scalpels. To avoid cross contamination, workstations were sanitized using 70% isopropyl alcohol between bonefish and a fresh scalpel blade and gloves were used for each fish. Stomachs were removed intact and placed in a sterile 7-ounce Whirl-Pak (Stamford, USA) and stored at –20 °C.

Morphological stomach content analysis followed methods described by Nagareda and Shenker (2008) and Jud et al. (2011). Briefly, prey items were enumerated, weighed, and identified to the lowest possible taxonomic level. Following analysis, the stomach contents of each fish were stored in 70% ethanol in individual 50-ml sterile tubes.

DNA extraction and library generation

DNA extraction from homogenized stomach contents

Stomach contents were transferred from ethanol into individual petri dishes and residual ethanol was allowed to evaporate for four hours. Large items (e.g., bone and shell fragments) were disrupted using sterilized surgical scissors and then the stomach contents of each fish were placed into 2-ml Qiagen PowerBead tubes containing 2.38-mm metal beads (Qiagen, Hilden, Germany) and 500 µl of sterile phosphate buffered saline (1–5 tubes per fish). Stomach contents were then homogenized in a tissue lysing machine (Biospec Products, Bartlesville, USA) at 2000 Hz for 5 min. Following initial homogenisation, replicate tubes per fish were pooled into 5-ml sterile microcentrifuge tubes and mixed by vortexing for 30 s. DNA was then extracted from homogenized stomach contents using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and using the recommended weight of 0.2 g of starting material.

DNA extraction from cloacal swabs

DNA was extracted from cloacal swabs using a Qiagen DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The tip of each swab was first removed from RNAlater and placed into a Qiagen PowerBead tube containing 0.2-mm diameter glass beads (Qiagen, Hilden, Germany) and 360 µl of lysis buffer (kit-supplied buffer ATL). Tubes were then homogenized at 2000 Hz for 2 min to disrupt the swab, then 40 µl of kit-supplied proteinase K was added. Tubes were then incubated at 56 °C for 30 min before 400 ul of buffer AL and 400 µl of molecular grade ethanol were added to each tube. Lysates were mixed by vortexing for 10 s then loaded into extraction spin columns. Extractions then proceeded according to the manufacturer's instructions. Extraction negative controls were processed in parallel but with no starting material. Subsequently, DNA was purified to remove inhibitors using the Zymo Research (Irvine, USA) Clean and Concentrator kit, following manufacturer's instructions.

Molecular marker and primer selection

We evaluated the performance of two molecular markers often used for detection of a broad range of potential bonefish prey and forage items, based on prior morphological studies of *A. vulpes* diet (Crabtree et al. 1998; Griffin et al. 2019). The markers chosen were an approximately 400 bp portion of the V4 region of the 18s ribosomal RNA gene (18S) and an approximately 300 bp region of the mitochondrially encoded cytochrome c oxidase subunit 1 gene (COI). Candidate primer sets were selected from the literature and compared in silico to sequences of known bonefish prey taxa available in NCBI databases. The COI primer set selected was Ill_B_F (5' – CCIGAYATR GCITTYCCICG – 3'; Shokralla et al. 2015) and ArR5 (5' – GTRATIGCICCIGCIARIACIGG -3'; Gibson et al. 2014). The 18S primer set selected was E527F (5' – CYG CGGTAATTCCAGCTC -3') and E1009R (5' – AYG GTATCTRATCRTCTTYG -3'), both from Comeau et al. (2011). We modified the reverse 18S primer to capture a wider range of arthropod taxa (E1009R_BF_Mod; 5'-GGTATCTRATCRYCTTYG -3').

Polymerase chain reaction (PCR) was used to amplify 18S and COI in 25-ul reactions. Each reaction contained 12 ul of Qiagen HiFi high fidelity taq mastermix (Qiagen, Hilden, Germany), 0.5 µl of each

primer at 10 uM concentration, 10 µl of molecular grade water, and 2 µl of template DNA. Three replicate reactions were performed for each fish and for each amplicon. The reaction conditions for both molecular markers were as follows: initial denaturation step of 95 °C for 15 min; 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 54 °C for 1 min, and elongation at 72 °C for 1 min; a final elongation step of 10 min at 72 °C. Triplicate amplicons were electrophoresed on a 1% agarose gel and visualized under ultraviolet light, then the three amplicons for each PCR target and anatomic site were pooled for each sampled fish. Any failed PCRs, likely due to pipetting error, were repeated until 3 successful replicates of each amplicon were obtained for each fish. Negative controls were included with each PCR run using molecular grade water instead of template DNA. All negative controls performed as expected, producing no bands upon electrophoresis, and were therefore excluded from sequencing analyses. Pooled PCR products were purified using AMPure beads (Beckman Coulter, Brea, USA) following the manufacturer's instructions. To ensure adequate removal of residual PCR primers, a 0.8:1 ratio of AMPure beads to pooled PCR product was used. Purified PCR products were submitted to the University of Wisconsin-Madison Biotechnology Center for paired-end sequencing on an Illumina MiSeq platform using V3 chemistry and TruSeq adapters.

Sequence analyses

To minimize artefacts of PCR amplification and sequencing, primer and adapter sequences were first removed using cutadapt v3.5 (Martin 2011). Reads were then processed using USEARCH v11 (Edgar 2010), with default parameters. Paired reads were then merged, and non-merged reads were removed. Unique remaining sequences were identified and the abundance of each was recorded. Sequences were then clustered into operational taxonomic units (OTU) at a 97% similarity level, and chimeric sequences were discarded using the UCHIME2 function of USEARCH (Edgar et al. 2011). Following OTU clustering, USEARCH was again used to produce an OTU abundance table for all fish. To investigate the impact of OTU clustering thresholds on the success of taxonomic annotations, we also generated a non-clustered dataset of amplicon sequence variants (ASVs) for both 18S and COI datasets with USEARCH. Taxonomic assignment of representative OTU sequences and ASV sequence datasets was performed using the silva 18S database (v128, Quast et al. 2013) and the IDTAXA algorithm of the DECIPHER R package (Murali et al. 2018), and the COI Barcode of Life Database (BOLD) using BOLDigger (Buchner and Leese 2020).

Taxonomic annotations and OTU/ASV tables for COI, 18S, and morphological datasets were input into phyloseq (McMurdie and Holmes 2013). Because read abundance data in dietary metabarcoding can be difficult to interpret (Deagle et al. 2019), we converted our molecular OTU/ASV tables to a binary presence/absence matrix of each prey item in each fish. To ensure consistency between datasets, our morphological data were similarly converted to presence/absence. To allow for comparisons between sampling methods and molecular markers, we agglomerated annotations to the class level and summed the occurrences of each OTU/ASV from a given class. For 18S, we removed OTUs/ASVs likely corresponding to *A. vulpes* by discarding all OTUs/ASVs corresponding to the Actinopterygii (ray-finned fishes). Although bonefish prey upon other fishes (Crabtree et al. 1998; Griffin et al. 2019), this approach was necessary because no OTU/ASV identified as Actinopterygii could be annotated beyond the taxonomic level of class using 18S and available databases. For COI, OTUs/ASVs were specifically annotated as *A. vulpes* and were therefore removed from the dataset, and other OTUs/ASVs annotated as Actinopterygii or lower were retained. Phyloseq objects for each dietary assessment method were then combined into a single representative object.

Statistical analyses

Annotation success

To assess the influence of molecular marker on detected dietary composition, we merged cloacal swab and homogenized stomach content OTU counts for each fish. Amplicon sequence variant tables for each marker were treated separately but identically.

Firstly, we compared the annotation success between OTU and ASV datasets for each molecular marker. The annotation completeness (number of OTUs/ASVs) assigned to a given taxonomic level

was compared using a chi-squared test of contingency tables. In light of no detrimental impact of OTU clustering on annotation success being detected (see results), we proceeded with further analyses using only the clustered OTU dataset, because the use of ASVs would likely introduce bias during our class level agglomeration procedures due to variation in genetic diversity within taxa.

Dietary diversity

To allow for maximally accurate comparisons of dietary assemblages between sampling methods and between molecular markers, we discarded molecular OTUs likely to represent incidental detections (phytoplankton, nematodes, fungi, etc.) or gastrointestinal parasites (trematodes, etc.). See Table S3 for relevant classifications. Due to non-normality of response distribution, we compared the number of OTUs present for each marker per fish using a paired Wilcoxon test.

To examine differences in detected dietary diversity between morphological and molecular methods, we combined COI and 18S data for each sampled fish to maximize taxonomic resolution. We then compared the number of OTUs recovered among morphological analyses of stomach contents, molecular analyses of homogenized stomach contents, and cloacal swabbing using a Friedman test and subsequent pairwise paired Wilcoxon test with Benjamini Hochberg correction.

Dietary composition

To assess whether different methods and/or choice of molecular marker yielded different dietary compositions, we computed a slightly modified version of the metric of importance described in Griffin et al. (2019). As per Griffin et al. (2019), we used O (frequency of occurrence of a given class) multiplied by N (count/occurrence of a given class as percentage of the counts/occurrences of the whole dataset). This importance metric can be interpreted as a measure of taxonomic dominance but is hitherto referred to as importance for consistency. This importance metric was computed for each dietary marker based on combined swabbing and homogenized stomach contents datasets and for each sampling method using our full, multi-marker, dataset. We compared the importance values of each individual prey class between each molecular method using a paired Wilcoxon test. We compared the importance values of each prey class between each sampling method using a paired Friedman test, and subsequent pair-wise paired Wilcoxon tests with Benjamini-Hochberg correction.

Statistical tests were implemented using the R computing environment (v4.0.5, R Core Team 2020). Figures were also produced in R, using the packages ggplot2 (Wickham 2016) and ggven (Gao et al. 2021).

Results

Sample collection and sequencing

Amplicon libraries were successfully generated for all 16 fish for both 18S and COI. Following trimming and quality filtering, the 18S database consisted of 2.9 million reads with a median length of 392 base pairs (range 1–536) and the COI data set consisted of 2.3 million sequencing reads with a median length of 303 base pairs (range 1–529). Taxa identified using each sampling method and molecular marker are presented in Table S3.

Comparison of molecular markers

A total of 330 and 392 unique OTUs were detected in the initial 18S and COI datasets, respectively. Following annotation, 161 and 139 of these OTUs could not be classified to phylum in their respective datasets and were therefore removed from subsequent analyses. Similarly, poorly classified OTUs misassigned to the classes Mammalia and Aves were removed from both datasets. Additionally, all bacterial OTUs (192) in our COI dataset were excluded from further analysis. The final dataset contained 156 and 53 OTUs for 18S and COI, respectively (Table S2). Annotation was significantly more complete for the COI OTU versus the 18S OTU dataset ($X2 = 33.6$, $df = 4$, $p = < 0.001$).

A total of 856 and 865 unique ASVs were detected in the initial 18S and COI datasets respectively. Following annotation, 386 and 383 of these OTUs could not be classified to phylum in the 18S and COI datasets, respectively, and were therefore removed from subsequent analyses. Similarly, all bacterial ASVs (384) in our COI dataset were excluded from further analysis. The final ASV dataset contained 438 and 98 ASVs for 18S and COI, respectively (Table S2). Annotation success was not significantly different

between OTU and ASV datasets for either molecular marker (18S, X2 = 7.9, df = 5, $p = 0.16$; COI, X2 = 10.2, df = 5, $p = 0.07$).

Operational taxonomic units belonged to 36 and 12 unique classes in the 18S and COI datasets, respectively. Eight classes were shared between the two datasets, and the remainder were exclusive to one dataset (Fig. 1). Shared classes were Malacostraca, Polychaeta, Insecta, Holothuroidea, Ostracoda, Gastropoda, Arachnida, and Ophiuroidea. Shared classes accounted for 13% and 31% of the total generated sequencing reads in our 18S and COI datasets, respectively. Most additional classes present in the 18S dataset were planktonic algae, fungi, or trematode/nematode worms; however, the class Bivalvia was also represented in the 18S dataset (Table S3). Classes unique to the COI dataset also represented planktonic algae as well as annelid worms. As mentioned previously, due to our ability to identify and omit bonefish sequences, we were able to retain OTUs identified to other marine fishes within the class in our COI dataset. Some OTU assignments were likely due to gaps and low resolution in existing DNA sequence databases (e.g., insects and arachnids), and others likely represent parasites either of bonefish or their prey (e.g., trematodes).

Dietary diversity and composition

A median of 7.5 (range 1–20) and 9 (range 3–16) dietary OTUs per fish were identified in the COI and 18S datasets, respectively (Fig. 2A). A paired Wilcoxon-test showed that per fish, the number of OTUs detected for each molecular marker was not significantly different between the two datasets (V = 102.5, $p = 0.08$).

In both molecular datasets, Malacostraca (crabs, shrimp, and diverse other crustacean taxa) was identified as the most important prey class (Fig. 3). The rank relative importance of all other prey classes varied (Fig. 3, Fig. S1, Table 1); however, the relative importance rankings of each class were not statistically significantly different between molecular markers (V = 69, $p = 0.64$).

Comparison of metabarcoding to morphological analysis

Nine uniquely identifiable prey items were morphologically identified. Several of these prey items were not confidently classifiable below the level of order and three were not identifiable below the level of class (Table S3). The prey items identified belonged to the classes Malacostraca, Gastropoda, Bivaliva, Actinopterygii, and Ophiuroidea (Fig. 3). A median of 1 prey item (range 0–3) was identified per fish in our morphological dataset (Fig. 2B).

Combining both molecular markers, a median of 8.5 (range 3–29) dietary OTUs were identified in homogenized stomach contents, and a median of 5 (range 1–12) dietary OTUs were identified in cloacal swab samples (Fig. 2B). The number of prey items/OTUs identified per fish varied significantly between

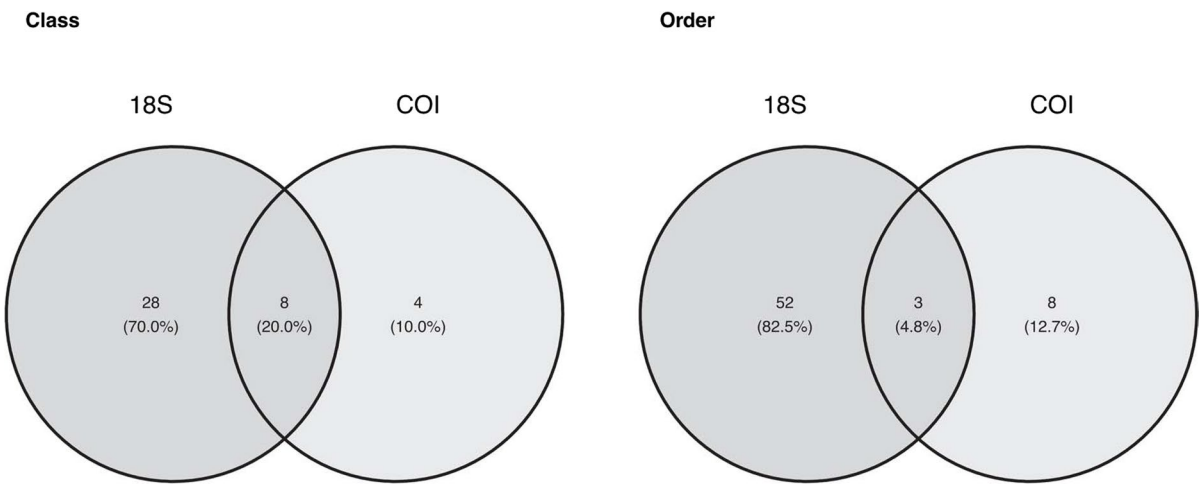

Fig. 1 Venn diagram of taxonomic classes and orders shared between molecular marker datasets

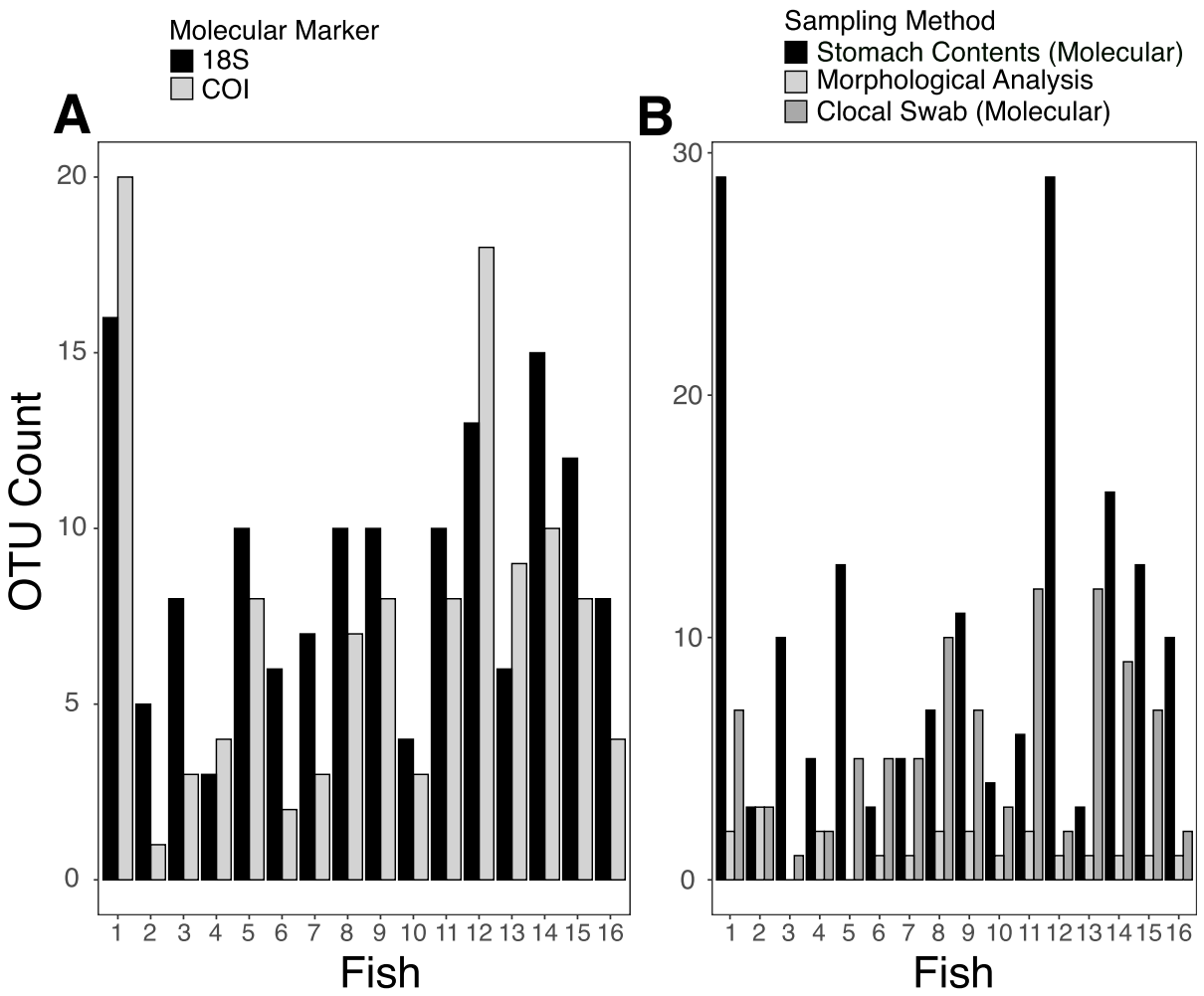

Fig. 2 Number of dietary OTUs observed per sampled fish in (**A**) datasets of merged cloacal swab and homogenized stomach contents based on each molecular marker, and (**B**) multi-marker dataset based on each molecular sampling method, and traditional morphological stomach contents analyses

sampling methods (FX2 = 23.2, df = 1, $p = < 0.01$). Pairwise comparisons showed this difference to be due to the greater number of OTUs detected with either molecular method than with morphological analysis (Swab, FV = 95, $p = < 0.01$; Homogenized stomach content, V = 120, $p = < 0.01$). Differences in OTU count between molecular methods were not significantly different (V = 81, $p = 0.07$).

Across all sampling methods, Malacostraca was again the most important prey class, with the rank order of importance for other prey classes varying between sampling methods (Fig. 3, Fig. S2, Table 1). The relative importance of each prey class differed significantly between sampling methods (FX2 = 10.4, df = 2, $p = < 0.01$). Notably, the importance of prey classes did not differ significantly between cloacal swabs and homogenized stomach contents (V = 83, $p = 0.22$). However, results obtained using molecular methods differed significantly from those obtained using morphological analyses, again due to the greater number of OTUs detected with either molecular method than with morphological analysis (Swab, V = 10, $p = 0.04$; Homogenized stomach content, V = 108, $p = < 0.01$).

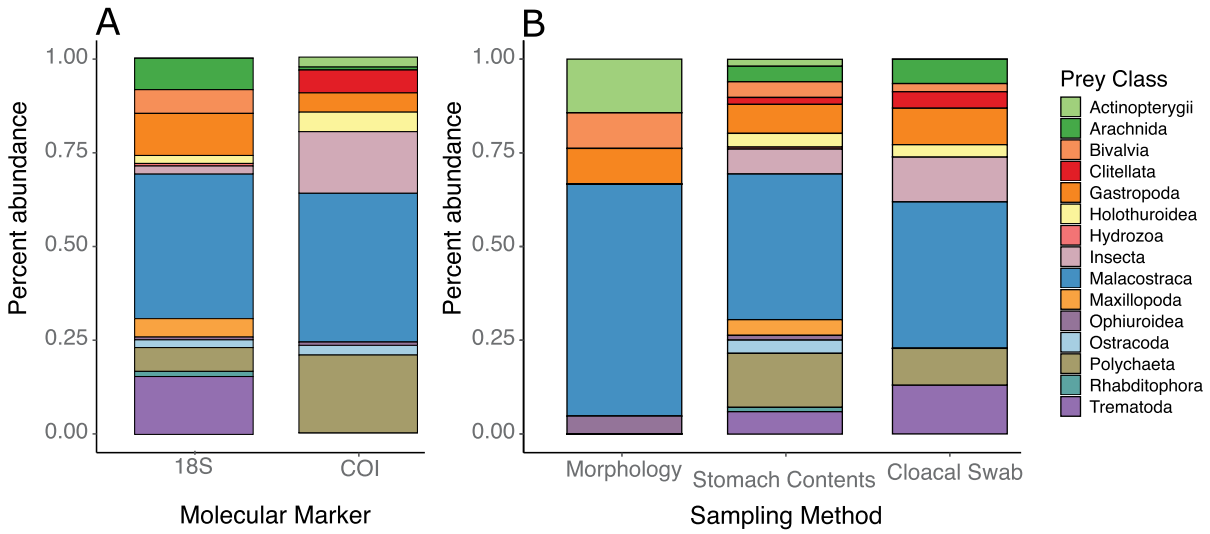

Fig. 3 Proportional occurrence of OTUs belonging to each likely dietary prey class in (**A**) datasets of merged cloacal swab and homogenized stomach contents based on each molecular marker, and (**B**) multi-marker dataset based on each molecular sampling method, and traditional morphological stomach contents analyses

Discussion

Our results suggest that non-invasive dietary metabarcoding provides a viable alternative to traditional dietary assessment in *A. vulpes*. We found that metabarcoding of cloacal swabs returned a dietary composition with significantly increased richness compared to traditional morphological analyses. Despite both methods identifying the same prey class (Malacostraca) as the primary component of bonefish diet, the importance of other dietary items was significantly different between metabarcoding of cloacal swabs and traditional morphological analyses. Greater dietary richness assessed using molecular methods than using morphological methods is consistent with prior work (e.g.. Nichols et al. 2016). Importantly, we found no significant difference in dietary richness or composition between cloacal metabarcoding and the

Table 1 Relative importance value of each dietary prey class. COI and 18S report importance of classes in dataset of merged sampling methods for each molecular marker. Morphology, cloacal swab, and stomach contents report importance of each class in multi-marker dataset for each sampling method

Class	COI	18S	Morphology	Cloacal swab	Stomach contents
Malacostraca	1824.14	2115.38	804.76	1408.70	2529.94
Gastropoda	31.03	179.02	19.05	88.04	101.20
Rhabditophora	0.00	2.80	0.00	0.00	2.40
Maxillopoda	0.00	34.27	0.00	0.00	29.34
Arachnida	0.86	100.70	0.00	39.13	29.34
Ostracoda	7.76	6.29	0.00	0.00	21.56
Trematoda	0.00	338.46	0.00	156.52	59.88
Hydrozoa	0.00	0.70	0.00	0.00	0.60
Bivalvia	0.00	56.64	19.05	4.35	29.34
Ophiuroidea	0.86	0.70	4.76	0.00	2.40
Polychaeta	496.55	56.64	0.00	88.04	344.91
Actinopterygii	7.76	0.00	42.86	0.00	5.39
Insecta	311.21	6.29	0.00	131.52	72.46
Holothuroidea	31.03	6.29	0.00	9.78	21.56
Clitellata	42.24	0.00	0.00	17.39	5.39

metabarcoding of homogenized stomach contents. Dietary assessment using metabarcoding of cloacal swabs, which are minimally invasive, therefore offers an attractive substitute for assessments based on more proximal sections of the gastrointestinal tract, which can only be accessed invasively. Because characterizing the diets of economically and socially important fish is necessary for informed conservation and management (Adams 2017; Griffin et al. 2019), this result represents a significant expansion of the available tool kit for these purposes.

Our results also highlight the importance of careful consideration of molecular markers. The 18S dataset recovered OTUs belonging to many more classes than did the COI dataset. Importantly, only eight taxonomic classes were shared between the molecular marker datasets whereas 28 were unique to the 18S dataset and only 4 were unique to the COI dataset. Many of the discrepant OTUs represent various classes of planktonic organisms, likely representing incidental ingestion. Such differences were also evident among OTUs representing prey. For example, the class Bivalvia, which was detected using morphological analysis, was present in the 18S dataset but not in the COI dataset. Conversely, the COI dataset returned a higher number of OTU belonging to the classes Clitelatta, Insecta, and Polychaeta. These differences are likely due to the representation of a given class in each molecular reference database (Guo et al. 2015; Giebner et al. 2020).

Based on the potential for non-detection of important dietary taxa (see above), our results support the concurrent use of both 18S and COI. Such a multiple marker approach is likely to detect the broadest taxonomic diversity of dietary items (da Silva et al. 2019). If a multi-marker approach is not possible, then the overall objectives of the study should be carefully considered to determine the most appropriate molecular marker to use. For example, 18S is known to perform well in the detection of certain eukaryotic parasites (Gogarten et al. 2020). Alternatively, although COI does not provide the same breadth of taxonomic coverage as 18S, the resolution to which classified taxa are annotated using the Barcode of Life Database is often significantly higher (Wu et al. 2015), as was the case in this study. Therefore, if an accurate classification of primary dietary prey items to genus or species level is the aim of a study, then COI would be the most appropriate individual marker.

Several classes of potential prey that were identified by both of our molecular markers are either unknown or unlikely occurrences in the diet of *Albula vulpes*. For example, the class Insecta was present in both 18S and COI datasets; however, these OTUs were poorly annotated, suggesting possible misassignment due to database gaps. True marine insects are rare, but several intertidal insect taxa do occur within the range encompassed by this study (Cheng 1976). Alternatively, marine insects are known to parasitize other taxa consumed by *A. vulpes* (Cheng 1976). Similarly, the class Arachnida was also detected using both molecular markers. Marine arachnids are also rare, but some true marine spiders are known to inhabit intertidal zones (Döbel et al. 1990). There is also debate regarding the phylogenetic position of horseshoe crabs (Xiphosura: Limulidae) in relation to arachnids (Nong et al. 2021), such that misassignment due to DNA sequence similarity and database gaps is a possibility. The class Clitellata are segmented worms best known from terrestrial habitats. However, the class also contains marine Tubificid worms found in sediments and mangrove habitats in Florida, along with the Hirudinea, (leeches) which include several taxa that reside in marine environments in Florida and the Gulf of Mexico (Sawyer et al. 1975).

Due to limited total sample size and geographic coverage, our results should be interpreted as promising but preliminary. We also made informed choices about analytical methods that could have affected our findings. For example, following comparison with de-noized amplicon sequence variants constructed from the same dataset, we settled on a 97% similarity threshold to create OTUs. The suitability of such similarity thresholds for OTU clustering is a matter of constant debate and can be taxon-specific (Xiong and Zhan 2018). Future refinements of our methods could improve the accuracy of OTU clustering by optimizing similarity thresholds. Additionally, we chose our primer sets to maximize the extent of *A. vulpes* diet that would be captured in our molecular analyses, due to the documented dietary breadth of bonefish (Crabtree et al. 1998; Griffin et al. 2019). However, primer set breadth and taxonomic resolution are inversely associated, such that our data likely underestimated true dietary diversity (Hajibabaei et al. 2019) compared to a comprehensive suite of taxonomically specific primers.

Overall, our results show that cloacal swab metabarcoding provides an attractive way to collect data

on the diet of *A. vulpes* without sacrificing fish. Non-lethal sampling is essential for a species such as A. *vulpes* that support an important catch and release fishery and is listed as Near Threatened on the IUCN red list due to habitat loss and, in some locations, overharvest (Adams et al. 2014). Non-lethal dietary data will be useful not only for understanding bonefish nutritional ecology, but also for conservation and management. For example, bonefish habitat quality could be assessed using a combination of cloacal swab metabarcoding and environmental assessment, to identify areas of high or low value with respect to bonefish diet, or to track habitat change (e.g., habitat degradation) effects. Similarly, high-resolution data on bonefish diet from large numbers of wild fish could help define the formulation of diets for bonefish in captive settings, where accurately mimicking natural diets may be an important consideration. We hope that the methods of minimally invasive dietary metabarcoding described herein expand the range of research questions that can be answered for *A. vulpes* and other species, ultimately building a more comprehensive picture of bonefish dietary ecology than has heretofore been possible.

Data availability Raw sequence data associated with this work are available from the sequencing read archive under BioProject PRJNA874874.

Declarations

All bonefish handling and processing techniques follow the approved Florida International University protocol IACUC-21-058 and permit numbers: SAL-22-2100-SRP, BISC-2021-SCI-0010, EVER-2021-SCI-0025.

Competing interests Aaron Adams is a Guest Editor of this special issue, but he was not involved in the peer review of this article and had no access to information regarding its peer review. The authors declare no competing interests.

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