Juvenile Striped Bass consume diverse prey in Chesapeake Bay tributaries

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Abstract

Objective: Anadromous Striped Bass *Morone saxatilis* are dominant predators in estuaries and coastal areas along the U.S. Atlantic coast, with the potential to exert top-down control on prey populations. Although Striped Bass diets have been studied previously, spatiotemporal patterns of diet across ontogeny remain poorly understood, especially for young fish in shallow nursery habitats.

Methods: We collected and examined gut contents from adult, juvenile, and young-of-year (age-0) Striped Bass from nine rivers across the Maryland and Virginia portions of Chesapeake Bay during summer and fall 2018. We compared the use of traditional morphological inspection and new amplicon-based next-generation sequencing methods for identifying gut contents.

Result: Striped Bass in shallow tributary habitats of Chesapeake Bay had diverse diets that varied strongly with ontogeny and salinity zone. In particular, the diet of age-0 Striped Bass varied greatly from those of juveniles and adults when age-0 fish foraged in freshwater habitats. Although our results on prey consumed aligned with previous surveys, we identified additional taxa as important prey for these young fish, including dipteran insects, Banded Killifish *Fundulus diaphanus*, Inland Silverside *Menidia beryllina*, bay barnacle *Amphibalanus improvisus*, and grass shrimp *Palaemon* spp. Comparison of methodologies indicated that 40% of prey by weight could not be identified with morphological analysis, while 76% of mitochondrial cytochrome oxidase I sequences could be assigned binomial names, allowing for high-resolution taxonomic comparisons.

Conclusion: This study adds to the growing body of evidence that amplicon-based next-generation sequencing methods are far superior to traditional morphological analyses of gut contents for fine-scale taxonomic resolution of prey.

KEYWORDS
dietary DNA, forage habitat, juveniles, metabarcode, season, young of year

INTRODUCTION

Anadromous Striped Bass *Morone saxatilis* are dominant predators in estuaries and coastal areas of the U.S. Atlantic coast (Hartman and Brandt 1995), with varied ecological interactions across ontogeny that can have a domino effect within local food webs. Spawning in Chesapeake Bay occurs in spring within tidal freshwater areas of tributaries and the head of the bay; most young fish move to brackish areas by the end of the early juvenile period, but some may remain resident in tidal freshwater areas of tributaries (Conroy et al. 2015; Secor et al. 2020). As they mature, Striped Bass undergo partial migration, with most individuals larger than 80 cm total length (TL) migrating from Chesapeake Bay to the continental shelf and as far north as New England, although some adults remain residents of the bay (Secor et al. 2020). Population genetic data indicate that Striped Bass spawning in Chesapeake Bay consist of a single stock that comprises about 80% of the coastwide population (Gauthier et al. 2013), suggesting that trophic dynamics and growth in the bay are important at a regional scale.

Although Striped Bass diets have been studied previously, spatiotemporal patterns of diet across ontogeny remain only partially understood, particularly for young fish in the tributaries. After the yolk sac is depleted and feeding begins, Striped Bass transition from consuming freshwater prey to brackish species (Markle and Grant 1970; Boynton et al. 1981; Shideler and Houde 2013) as they grow and disperse through different salinity regimes. Ontogenetic shifts in diet continue throughout the juvenile stage, with piscivory becoming increasingly common once the fish exceed 50 mm TL (Conroy et al. 2015). Diets of larger Striped Bass (150–500 mm TL) in the main stem of Chesapeake Bay have been primarily studied, and the top-five prey categories by weight are Bay Anchovy *Anchoa mitchilli* (22.2%), mysids (14.2%), polychaete worms (13.7%), Atlantic Menhaden *Brevoortia tyrannus* (8.7%), and unidentified material and fish (12.5%; Buchheister and Latour 2015; Ihde et al. 2015; Bonzek et al. 2022). Seasonal shifts in diet are also observed, with invertebrates dominating the diet in spring and fish dominating the diet in fall (Ihde et al. 2015). Despite the extensive data available for some areas, there remains relatively little information on Striped Bass diet composition and variability in the shallow foraging habitats within Chesapeake Bay tributaries that serve as critical nurseries for young-of-year (age-0) and juvenile Striped Bass.

Foraging habitat quality likely varies in space and time due to multiple, often co-dependent factors, including water temperature and oxygen conditions, salinity, prey availability and composition, and the quality of biogenic and abiotic habitat structure (Hartman and Brandt 1995; Costantini et al. 2008). Additionally, tributary habitats in Chesapeake Bay vary substantially by benthic habitat, shoreline habitat,

Impact statement

Genetic methods provided increased resolution of species eaten by Striped Bass as these fish age and move through different salinities in the Chesapeake Bay, while adding important prey species to the current list for Striped Bass.

and watershed land use, with local to regional effects on fish and benthic invertebrate community abundance and structure (Kornis et al. 2017a, 2017b). The shoreline of Chesapeake Bay has changed over time, with an increasing extent of hardened shorelines as human populations have increased in the watershed (Gittman et al. 2015). Hardened shorelines tend to support a high biomass of fish and crustaceans due to deeper waters facilitating the occurrence of planktivore and benthivore–piscivore species (Kornis et al. 2017a). The quality of tributary nursery and forage habitats is likely to vary seasonally and spatially for young Striped Bass and could be an important driver of growth.

One major challenge with diet studies of small fish is the difficulty of visually identifying prey items from gut contents, as the low resolution of prey identification leads to a substantial loss of information in diet studies (Hyslop 1980; Carreon-Martinez et al. 2011; Aguilar et al. 2016). Use of DNA barcoding with the mitochondrial cytochrome oxidase I (COI) gene is one technique that substantially increases the resolution of prey identification across a wide range of taxa (Roslin and Majaneva 2016). This technique can also be applied to a wide range of taxa for gut content identification to better understand trophic linkages and interactions within coastal systems (Sousa et al. 2016; Siegenthaler et al. 2019; Traugott et al. 2021). For example, in a recent study of Blue Catfish *Ictalurus furcatus* diets in Chesapeake Bay, the use of this genetic marker increased the fraction of individual fish prey items that were identifiable to species from 9.4% to 90.3% (Aguilar et al. 2016). This method can also be used to assess the prevalence of parasites for both the predator (Cabodevilla et al. 2022) and the prey (Berry et al. 2015). Furthermore, amplicon-based high-throughput sequencing (also referred to as "metabarcoding") is a similar technique that can efficiently quantify diet diversity from discrete stomach samples (Pompanon et al. 2012; Leray and Knowlton 2015). With this method, multiple genes can be targeted, allowing for varying levels of taxonomic resolution and coverage across the potential prey species. For instance, the mitochondrial COI gene offers higher taxonomic resolution for most metazoan species but not for nonmetazoans, whereas the small subunit (18S) gene of the ribosomal gene complex (ribosomal

DNA) offers wider taxonomic coverage for both metazoan and protistan species but lower taxonomic resolution for metazoan species (Pompanon et al. 2012). Metabarcoding of gut contents has led to novel discoveries, particularly in aquatic systems, where direct observation of prey items is often impossible, and also has led to adoption of the term "dietary DNA" to describe approaches for the genetic characterization of prey (de Sousa et al. 2019).

In this study, we assessed the variation in the diet of Striped Bass across multiple tributaries of Chesapeake Bay. More specifically, gut contents from Striped Bass collected from multiple locations within nine rivers in Maryland and Virginia were assessed using metabarcoding, and a subset of gut contents from young-of-year (YOY [age-0]) fish was assessed using standard visual examination. Our goals were to examine the prevalence, richness, and spatiotemporal variation in diet across (1) ontogeny, (2) rivers (within and among), (3) salinity regimes, and (4) seasons.

METHODS

Sample collection

Striped Bass of varying sizes were collected across multiple months from several rivers in the Chesapeake Bay system using a variety of sampling techniques. Age-0 Striped Bass were collected in Maryland and Virginia during July, August, and September 2018 by the Maryland Department of Natural Resources (DNR) Juvenile Striped Bass Survey (upper Chesapeake Bay [hereafter, "Upper Bay"] and the Choptank, Nanticoke, Patuxent, and Potomac rivers; Durell and Weedon 2020) and by the Virginia Institute of Marine Science (VIMS) Juvenile Striped Bass Seine Survey (Rappahannock, York, and James rivers; Buchanan et al. 2022; Figure 1). These surveys were designed to cover the major habitats used by age-0 Striped Bass, including the salinity gradients from north to south in Chesapeake Bay as well as within each tributary. Both surveys followed the same protocol for deployment of a 30.5- × 1.24-m bagless seine consisting of 6.4-mm bar mesh. Additional age-0 fish were collected in 2018 by the Smithsonian Environmental Research Center (SERC) Rhode River Summer Seine Survey, which samples 13 sites in the Rhode River (Figure 1) from June to August, employing a 16.0- × 1.24-m bagless seine with 7.0-mm bar mesh that was swept parallel to the shoreline for 33.3 m (Hines et al. 1987). Sampling occurred during the day, when Striped Bass generally exhibit the greatest stomach fullness (Rudershausen and Loesch 2000; Howe and Juanes 2001; Muffelman 2006).

Juvenile Striped Bass (~1–4 years old) were collected by gill nets to permit comparisons of the diet between fish from tributary foraging habitats (this study) and fish
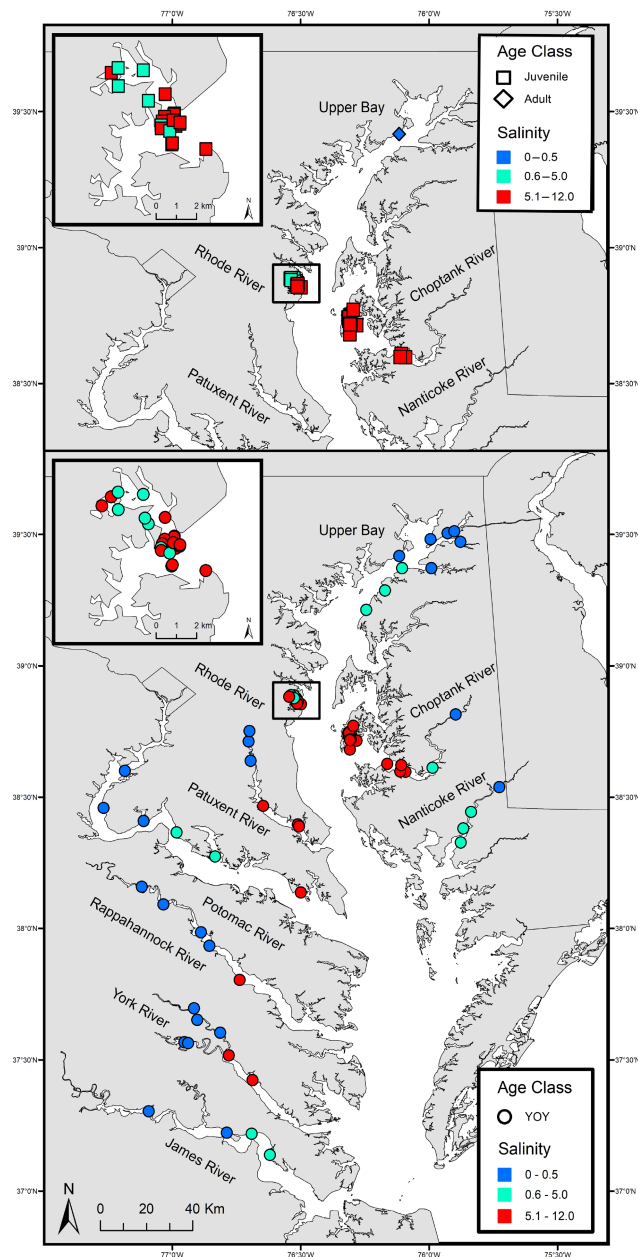

FIGURE 1 Map of the sampling locations within Chesapeake Bay, showing the locations where adult (top panel), juvenile (top panel), and age-0 (young-of-year [YOY]; bottom panel) Striped Bass were caught and the relative salinity ranges (practical salinity units) across the rivers where sampling occurred.

caught by the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP) survey (Bonzek et al. 2022) in the main-stem Chesapeake Bay. Distinct features (e.g., points and inlets) within each tributary were used to segment the tributary for sampling. The Rhode and West rivers were separated into seven segments, and the Choptank River was separated into 11 segments. Sampling effort was stratified by segment, with 2–10 samples/segment in each of three seasons: early summer (June/July), late summer (August/September),

and fall (October/November). The number of samples per segment in each season was not standardized because our goal was to capture enough fish per tributary and season for the diet analysis, so more samples were taken at times and locations with lower catches. Gill nets (set for 30 min during daytime) were used to collect samples. This sampling gear was selected to avoid the use of bait and to prevent the Striped Bass from feeding on other species captured in the net, as is possible with trawls, pound nets, and haul seines. In early summer, we used two 1.2-m-high gill nets and one 1.8-m-high gill net with two 3-m panels of 5.0-, 7.6-, 10.2-, and 15.2-cm stretch mesh (6 m total of each mesh size). In late summer and fall, we fished with three gill nets that were 1.8 m high and 30.5 m long, with four 7.6-m panels. Fish were immediately placed in an ice slurry and transported to SERC for euthanasia by immersion in a 400-mg/L dose of clove oil in accordance with SERC Animal Care and Use Proposal 2018-0510. All samples were then stored frozen until processing for gut contents. No regurgitation of gut contents was observed. Finally, adult Striped Bass from the Upper Bay were obtained from the Maryland DNR Adult Spawning Stock Survey (Table 1; Figure 1); these fish were incidental gill-net mortalities. For all collections, age-class was estimated by size, with age-0 fish ranging from 44 to 133 mm TL, juveniles ranging from 200 to 480 mm TL, and adults being larger than 680 mm TL. We acknowledge that male Striped Bass can mature within the range of 200–480 mm TL; however, we did not determine sex for fish smaller than 480 mm TL.

Fish were measured, weighed, and dissected to obtain stomachs for diet analysis. Fish from Maryland were assigned a unique identification number, measured for TL (mm), and weighed (wet weight, g) prior to dissection. Fish from Virginia were measured for fork length and TL, but weight data were unattainable due to the manner of processing used for those fish. Stomachs were individually labeled and stored in a −20°C freezer for later processing.

Metabarcoding of stomach contents

Stomach contents were thawed, weighed (wet weight, g), and homogenized using a Fisherbrand 150 handheld homogenizer motor. A total of 0.25 g of homogenate was used for DNA extraction unless the total stomach contents weighed less than 0.25 g, in which case the entire contents were used. After overnight digestion with proteinase K, we extracted genomic DNA using a Qiagen DNeasy Blood and Tissue Spin Column Kit, with slight modifications to the protocol to ensure maximum DNA elution. See the Appendix for additional details.

The primers mlCOlintF (Leray et al. 2013) and jgH-CO2198R (Geller et al. 2013) were used to amplify and sequence an approximately 310-base-pair (bp) fragment of the mitochondrial COI gene. The primers 3NDeukF and V4eukR2 (Bråte et al. 2010) were used to amplify and sequence an approximately 450-bp fragment of the 18S gene. For both primer sets, primers containing both partial Nextera indices added to the 5′ ends and degenerate base pairs (0, 1, 2, and 3 bp) were added in equal concentration across samples. All polymerase chain reactions (PCRs) were conducted in triplicate, and the triplicate

TABLE 1 Numbers of young-of-year (age-0), juvenile, and adult Striped Bass collected from Chesapeake Bay tributaries and the upper Chesapeake Bay (Upper Bay) and successfully processed for morphological, small subunit ribosomal DNA (18S) metabarcode, and mitochondrial cytochrome oxidase I (COI) metabarcode data. Note that the same fish were processed for both the COI and 18S metabarcoding, but different samples were used for morphological analyses.

Data set and life stage	Upper Bay	Rhode River	Choptank River	Patuxent River	Nanticoke River	Potomac River	Rappahannock River	York River	James River
Morphology									
Age 0	5	10	4	5	5	5	5	5	5
Juveniles									
Adults									
18S									
Age 0	15	22	22	15	15	15	10	10	10
Juveniles		30	26						
Adults	2								
COI									
Age 0	15	12	20	14	14	12	10	9	5
Juveniles		30	25						
Adults	2								

PCR amplicons were then pooled for each sample based on gel band intensity. All PCR thermocycler protocols are provided in the Appendix. All of the samples amplified with the 18S PCRs, whereas 12.5% of the samples ($n = 24$) did not amplify with any of the COI PCRs and were subsequently removed from further processing for that gene. We used dual indexing with Nextera adapters employing a unique combination for each sample to bioinformatically separate the samples based on unique tag combinations. Samples were then bead cleaned to remove small fragments and quantified (see the Appendix for details). Samples were pooled based on equimolar concentrations into two separate libraries (COI and 18S, respectively). The final pooled libraries were sequenced using a MiSeq v3 600 Reagent Kit (Illumina) on an Illumina MiSeq platform at the Laboratories of Analytical Biology (Smithsonian National Museum of Natural History). Raw sequence data were deposited in the Sequence Read Archive under BioProject PRJNA981150.

Bioinformatics

Primer sequences were removed using cutadapt (Martin 2011). Sequences were quality trimmed and merged, chimeras were removed, and small sequences were removed using the DADA2 package (Callahan et al. 2016) in R (R Core Team 2020; see the Appendix for additional details). Taxonomy was assigned to amplicon sequence variants (ASVs) using the PR2 database version 4.12.0 (Guillou et al. 2013) for the 18S sequences and using the COI library from the Chesapeake Bay Barcode Initiative (CBBI; Ogburn et al., BioProjects PRJNA396533 and PRJNA498040) or the National Center for Biotechnology Information (NCBI) for the COI sequences. To refine the taxonomy further, we generated a neighbor-joining consensus tree and assessed how sequences were grouped in clades (see the Appendix for additional details). Taxa were also binned by other taxonomic categories (i.e., major group, E11 prey group, E10 prey group, and fine prey group) based on Ihde et al. (2015). Parasitic taxa were identified based on taxonomic assignments at the appropriate taxonomic level. For example, both Rohde (2005) and Roberts and Janovy (2005) were used to determine major taxonomic rankings for parasites. If orders or classes were known to contain only parasitic taxa, then all ASVs in those orders or classes were included. For orders or classes with both parasitic and free-living taxa, we conducted literature searches to assess which genera were or were not parasitic, with free-living being the default designation if no other information could be found. For the 18S data, review of the taxonomic assignments showed that many metazoan species-level assignments were likely inaccurate. Thus, we considered only higher level classifications (e.g., phylum or kingdom) to characterize the diets.

Due to the interest in examining interspecific diversity within diets rather than the intraspecific diversity provided by ASV data, ASVs with identical taxonomic identifications in the COI data set were merged based on the full scientific name using the tax_glom command in the phyloseq package (McMurdie and Holmes 2013) in R. Alpha diversity using the Chao1 (Chao 1984) and Shannon (1948) distance indices and taxonomic bar plots were calculated in the phyloseq package. Additionally, we used the vegan package (Oksanen et al. 2014) in R to conduct a permutational analysis of variance (PERMANOVA) using the Jaccard similarity index and 1000 iterations to determine whether gut contents varied across salinity regimes. We also examined the beta dispersion significance to determine the influence of the spread of objects from the centroid. For all statistical analyses, we used an α of 0.05 to determine significance.

To confirm the identities of two potentially nonnative species found in the stomachs of Striped Bass, we created alignments of our sequences and those from the NCBI nucleotide database for those species and an outgroup from the same taxonomic family. Alignments were made in Geneious Prime using the Clustal Omega plug-in (Sievers et al. 2011) with default parameters. Neighbor-joining trees were generated in Geneious Prime using the alignments with 1000 bootstrap replicates.

Morphological analysis of stomach contents

Morphological gut content analysis was conducted on a subset of age-0 Striped Bass for comparison with the results of genetic metabarcoding. Each stomach was defrosted and weighed before and after contents were removed, allowing us to obtain the total wet weight of stomach contents. Stomach contents were sorted under a dissecting microscope and identified to the lowest possible taxonomic level based on morphology. The number of prey items within each taxonomic group was counted, and the wet weight of each group was obtained to calculate the percent contribution of each taxonomic group by weight.

RESULTS

In total, across all tributaries and months, we collected or obtained 1302 Striped Bass, including age-0, juvenile, and adult individuals. Samples analyzed in this work were part of a broader sampling effort that included samples used for other assessments of Striped Bass ecology; thus,

the findings reported here represent a subset of the total Striped Bass collected.

Metabarcoding of stomach contents: COI

A subset of 192 stomachs (adults: $n=2$; juveniles: $n=56$; age 0: $n=134$) was selected for COI metabarcode analysis (Table 1). The juvenile and age-0 samples chosen were selected to reflect the spatial and seasonal distribution of samples obtained (Table S1 available in the Supplemental Material separately online). Because all of the adults had empty stomachs, these became critical negative controls for determining which sequences were only from Striped Bass and not from stomach contents. We successfully amplified and sequenced the COI gene from 168 of the 192 stomachs. In total, 2,839,914 raw reads were generated from 176 samples (including 8 negative extraction controls); this total was reduced to 1,448,402 reads after initial filtering, merging, and chimera removal. With the removal of (1) negative control samples, (2) Striped Bass sequences, (3) bacterial sequences, and (4) adult empty stomachs, 874,060 sequences remained in 844 ASVs. We merged ASVs that were identified as the same species to generate operational taxonomic units (OTUs) that would more accurately portray species-level richness; after the merging of ASVs, the data set contained 223 OTUs.

Within the major prey groupings (e.g., crustaceans, fish, mollusks, polychaetes, and other worms), nearly all COI sequences were identified to genus or species with confidence, and 76% of COI sequences were assigned binomial names. An important example is the fishes, for which all sequences were confidently identified to the species level ($n=15$), including the Alewife *Alosa pseudoharengus*, Bay Anchovy, Atlantic Menhaden, Striped Blenny *Chasmodes bosquianus*, Gizzard Shad *Dorosoma cepedianum*, Tessellated Darter *Etheostoma olmstedi*, Banded Killifish *Fundulus diaphanus*, Mummichog *Fundulus heteroclitus*, Striped Killifish *Fundulus majalis*, Atlantic Stingray *Hypanus sabinus*, Redear Sunfish *Lepomis microlophus*, Inland Silverside *Menidia beryllina*, Atlantic Silverside *Menidia menidia*, White Perch *Morone americana*, and Cownose Ray *Rhinoptera bonasus*. Insects comprised the one notable exception to our high classification success, as they were predominantly identified to only the family or genus level due to the dearth of COI reference sequences with fine-scale taxonomy available in reference databases. Across both Striped Bass life stages (age 0 and juvenile), crustaceans were the most prevalent (using the presence of ASVs in gut contents) prey group (39%; Figure 2A), followed by worms, miscellaneous items, and insects, which were overwhelmingly dipterans. Crustaceans (42%) were the most prevalent prey detected in age-0 Striped Bass, followed by miscellaneous taxa (18%), insects (18%), and worms (14%; Figure 3A). For juvenile fish, the most prevalent prey items were worms (40%), followed by crustaceans (28%), and finfish (17%; Figure 3B). Crustaceans were represented by the greatest number of reads: 45% of sequences from age-0 Striped Bass stomachs were identified as crustaceans, with amphipods, mysids, and isopods being the most abundant crustaceans. The most prevalent prey after crustaceans were insects (28%) and worms (12%). In contrast, 33% of the sequences from juvenile fish stomachs were from worms, while 27% of sequences were from finfish and another 27% of sequences were from crustaceans.

The most abundant prey (based on the total number of sequences from the COI metabarcode data set) observed in age-0 Striped Bass stomachs were the mysid shrimp *Americamysis bigelowi*, the isopod *Cyathura polita*, the amphipod *Apocorophium lacustre*, dipteran larvae (mainly chironomids), and Bay Anchovy (Table 2). The most abundant prey (based on the total number of sequences from the COI metabarcode data set) found in juvenile stomachs were Atlantic Menhaden, *C. polita*, the polychaete *Heteromastus filiformis*, and soft-shell clam *Mya arenaria*.

Overall, the majority of prey that were genetically identified from the stomachs of age-0 and juvenile Striped Bass were species previously noted to occur in Chesapeake Bay. The two notable exceptions were the amphipod *Grandidierella japonica* (Figure S1 available in the Supplemental Material separately online) and the polychaete *Marenzelleria bastropi* (Figure S2), which to our knowledge have not been reported previously from Chesapeake Bay. Six unique ASVs identified as *G. japonica* were recovered from seven age-0 Striped Bass and one juvenile Striped Bass collected from mesohaline sites (6.09–8.78 practical salinity units [psu]) in the Choptank, Patuxent, and Potomac rivers. We also recovered 10 unique ASVs identified as *M. bastropi* from five age-0 Striped Bass collected from tidal freshwater sites (0.0–0.2 psu) in the James, Rappahannock, and York (Mattaponi and Pamunkey River tributaries) rivers as well as the Upper Bay (Elk River).

The highest alpha diversity of prey in the stomachs of age-0 and juvenile Striped Bass was observed in the southernmost rivers, the York and James rivers (Figure S3). Sequences from arthropods (primarily crustaceans) were the most abundant in age-0 stomachs across all rivers and salinities (Figure 4). Within the arthropod sequences, those from insects comprised a larger portion of the sequences only at the 0.0–0.5-psu sites in the Upper Bay and the Rappahannock and York rivers as well as at the 0.6–5.0-psu sites in the Upper Bay and the Potomac River. This variation in prey across tributaries was most likely attributable to the variation in salinity across the rivers (Figure 5; see Figure 1 for how salinities changed across tributaries) and was also observed in analyses of the proportion of sequences across salinities,
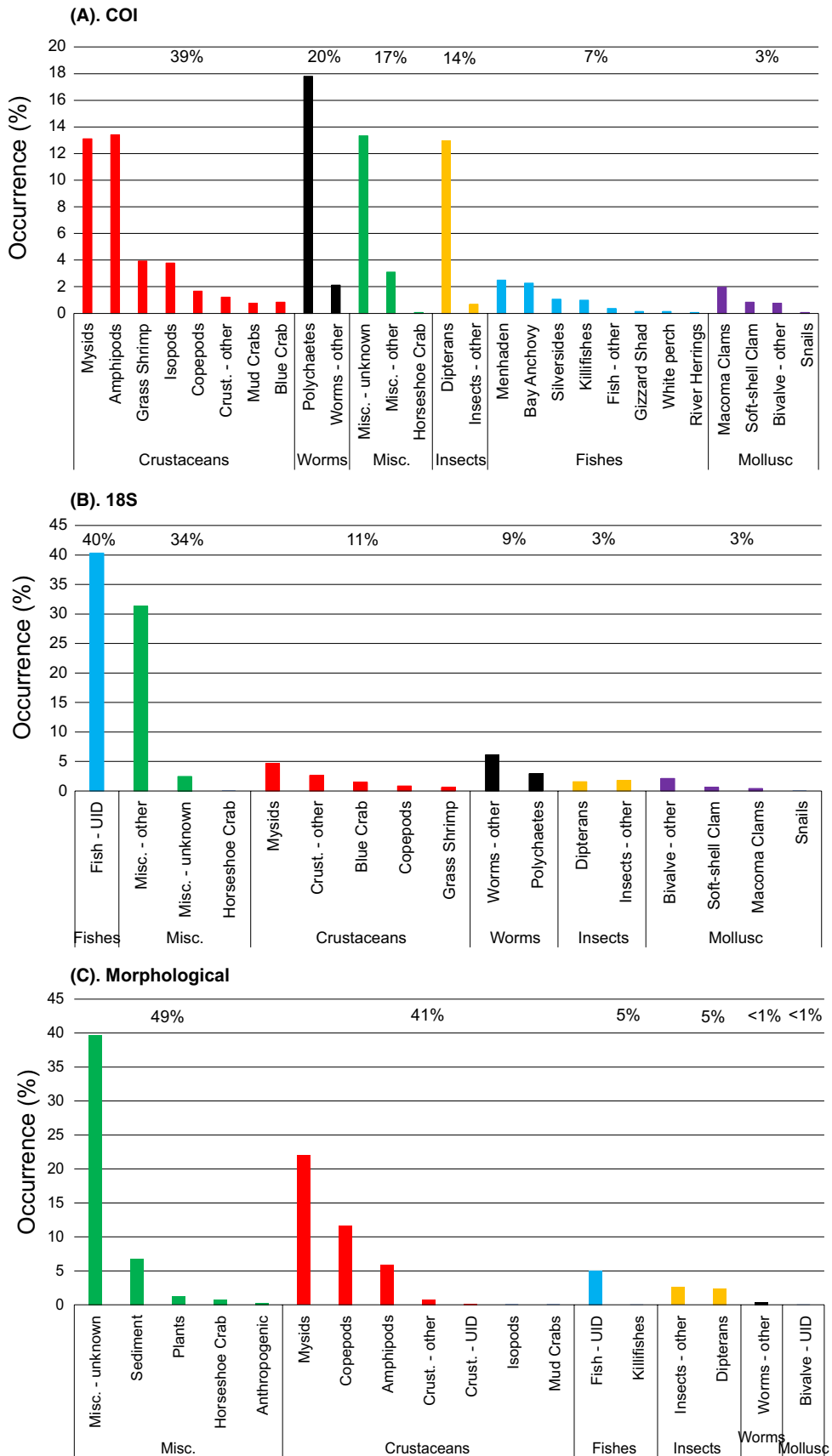
FIGURE 2 Comparison of the proportion of major prey classes identified in the gut contents of Striped Bass based on the (A) mitochondrial cytochrome oxidase I (COI) metabarcode data set, (B) small subunit ribosomal DNA (18S) metabarcode data set, and (C) morphological data set. For this analysis, all Striped Bass samples regardless of age or collection site were pooled together. Crust., crustaceans; Misc., miscellaneous; UID, unidentified.
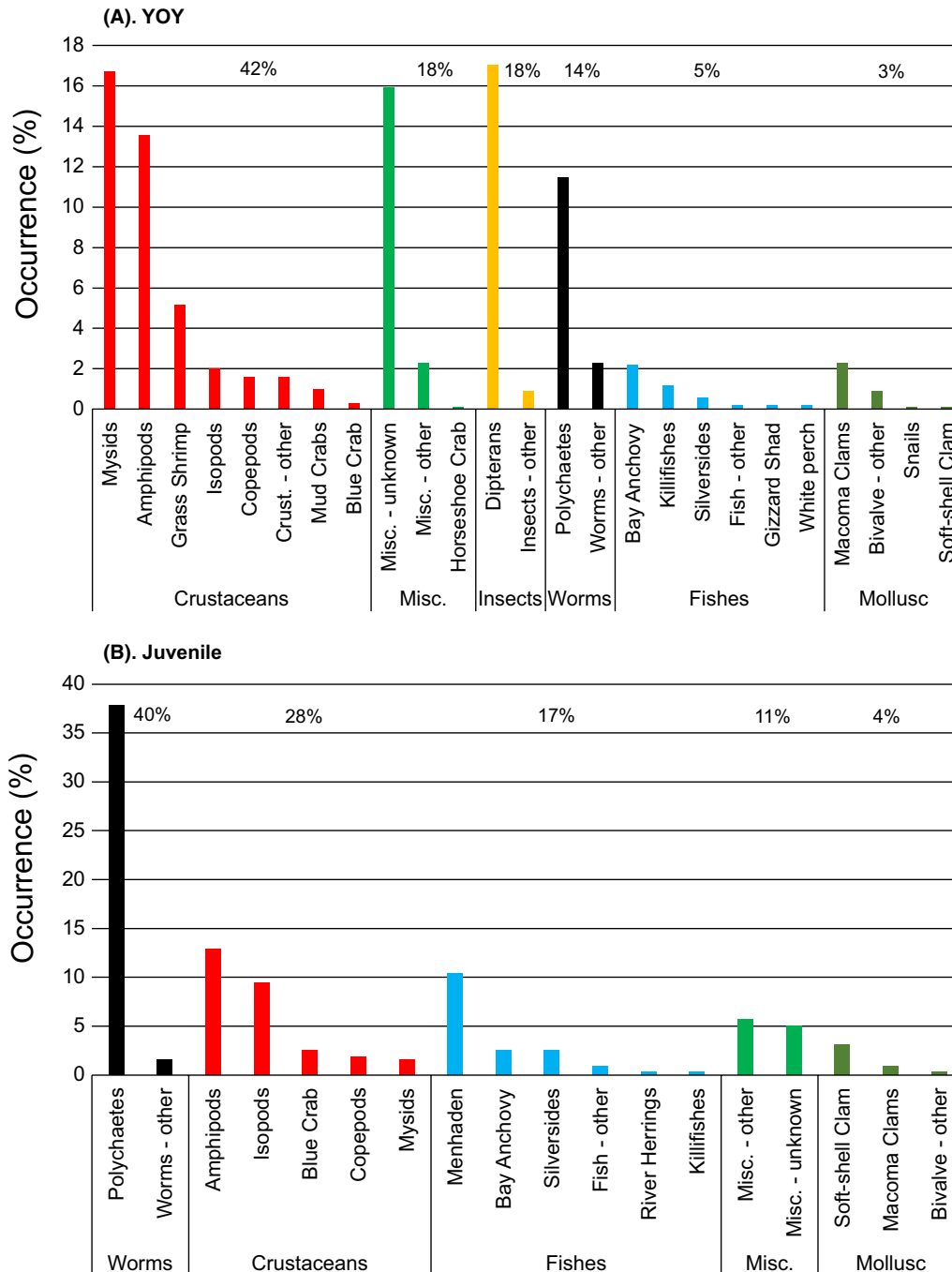
FIGURE 3 Percentages of each major prey group (based on the total number of sequences per grouping from the total of all sequences) found in the gut contents of Striped Bass via metabarcoding of the mitochondrial cytochrome oxidase I gene for (A) age 0 (young of year [YOY]) and (B) juveniles. Crust., crustaceans; Misc., miscellaneous.

with 52% of the sequences from 0.0 to 0.5 psu identified as dipterans, followed by Bay Anchovy (10%) and isopod (11%) sequences. In the 0.6–5.0-psu zone, sequences from mysids (28%), amphipods (21%), and polychaetes (11%) were most dominant. Most of the sequences generated from stomach contents of fish collected within the highest salinity regime (5.1–12.0 psu) were from amphipods (34%), polychaetes (23%), and mysids (10%; Figure 5). Seasonal analyses showed that crustaceans were the dominant prey items in

early and late summer for both age-0 fish (early summer: 56%; late summer: 44%) and juveniles (early summer: 32%; late summer: 32%; Figure 6). For age-0 Striped Bass, worms (15%) and insects (19%) appeared to be more dominant prey in early summer, which increased to more insects (29%) and fishes (from 6% to 11%) and a lesser representation of worms (11%) in late summer (Figure 6A). For juveniles, worms (45%) and crustaceans (32%) dominated the diet in early summer and then shifted with an increase in fish (from 2%

TABLE 2 Comparison of the most abundant prey items (based on the total number of sequences) for juvenile and age-0 Striped Bass. Those listed as key taxa were represented by over 10,000 reads in our data set, while those listed as additional key taxa were represented by over 5200 reads for age-0 individuals or over 1000 reads for juveniles.

Juvenile	Age 0
Key taxa	
Atlantic Menhaden *Brevoortia tyrannus*	Mysid shrimp *Americamysis bigelowi*
Isopod *Cyathura polita*	Isopod *Cyathura polita*
Polychaete *Heteromastus filiformis*	Amphipod *Apocorophium lacustre*
Soft-shell clam *Mya arenaria*	Diptera
Polychaete *Marenzelleria neglecta*	Bay Anchovy *Anchoa mitchilli*
Amphipod *Leptocheirus plumulosus*	Polychaete *Marenzelleria neglecta*
	Polychaete *Laeonereis culveri*
	Amphipod *Leptocheirus plumulosus*
	Banded Killifish *Fundulus diaphanus*
	Harris mud crab *Rhithropanopeus harrisii*
Additional key taxa	
Clam worm *Alitta succinea*	Bay barnacle *Amphibalanus improvisus*
Blue crab *Callinectes sapidus*	Giant mayfly *Hexagenia limbata*
Atlantic Silverside *Menidia menidia*	Polychaete *Heteromastus filiformis*
Polychaete *Laeonereis culveri*	Amphipod *Gammarus tigrinus*
Striped Blenny *Chasmodes bosquianus*	Inland Silverside *Menidia beryllina*
Bay Anchovy *Anchoa mitchilli*	Amphipod *Platorchestia platensis*
Mummichog *Fundulus heteroclitus*	Daggerblade grass shrimp *Palaemon pugio*
Inland Silverside *Menidia beryllina*	Amphipod *Grandidierella japonica*
Isopod *Erichsonella attenuata*	

to 79%) from early summer to fall (Figure 6B). Note that the sample size in fall, however, was low ($n = 15$).

The principal coordinates analysis of gut contents from age-0 and juvenile Striped Bass across salinities showed little to no differentiation within the 0.0–0.5-psu zone (Figure S4). However, differential grouping of samples by tributary occurred at higher salinity regimes, as samples within a river were more similar to each other than to samples from other rivers (Figure S4). The variation in gut contents across salinity regimes (PERMANOVA: df = 2, sum of squares = 1.9, mean square = 0.98, $F_{model} = 2.21$, $R^2 = 0.027$, $p = 0.001$) and across rivers (PERMANOVA: df = 8, sum of squares = 6.5, mean square = 0.81, $F_{model} = 1.79$, $R^2 = 0.088$, $p = 0.001$) was significantly different, although neither model explained more than 10% of the variation. In both cases, the significance was not attributed to dispersion ($F_{salinity} = 0.46$, $p_{salinity} = 0.63$; $F_{river} = 1.15$, $p_{river} = 0.33$).

Metabarcoding of stomach contents: 18S

We successfully amplified and sequenced the 18S gene from all 192 Striped Bass stomachs. We obtained 4,555,741 raw reads, and this total was reduced to 2,406,208 reads after initial filtering, merging, and chimera removal. With the removal of negative control samples and the reads found in empty stomachs (most likely from Striped Bass), 1,101,372 reads remained for comparative analyses, resulting in 1148 ASVs. Many of the taxonomic assignments for the 18S data set either were not to the species level or were unlikely to be accurate after further assessment of the assignments. For example, none of the fish identified with this marker represented accurate assignments at the species level and most were not accurate at the genus level. Inaccurate identifications were assigned to the "unidentified fish" prey group, which was the most prevalent group in the diet (40%; Figure 2B). This method identified the largest portion of miscellaneous prey items (30%), including a variety of parasitic taxa, protists, and fungi. Parasitic taxa that were identified included parasites assumed to directly infect Striped Bass, such as acanthocephalans (e.g., *Pomphorhynchus*) and nematodes (e.g., *Philometra*). Additionally, our results also identified internal and external parasites that were likely infecting fish and nonfish prey of Striped Bass (e.g., *Argulus*, *Kudoa*, leeches, and gregarines).

Morphological analysis of stomach contents

Stomach contents from 49 age-0 Striped Bass were identified morphologically. Miscellaneous unidentified material, mysids, copepods, amphipods, unidentified fish, and insects were prevalent in these stomachs (Figure 2C). Insects were abundant in the stomachs of fish collected from freshwater tidal sites, and crustaceans became more abundant among fish collected from increasing salinities and as fish size increased. The percent contribution of different prey taxa varied by tributary (Figure S5). Important examples of prey groups identified using metabarcoding that were confirmed with morphological and genetic approaches included insects, barnacles (larval stages only), mud crabs (larvae and juveniles), and Atlantic horseshoe crab *Limulus polyphemus* larvae. As expected, many species of worms, amphipods, and other small prey items as well as fish were degraded and difficult to identify to species or genus. For example, most fish remains were highly digested, and only a single sample could be identified to a lower classification (*Fundulus* sp.; Figure 2C). In fact, 40%
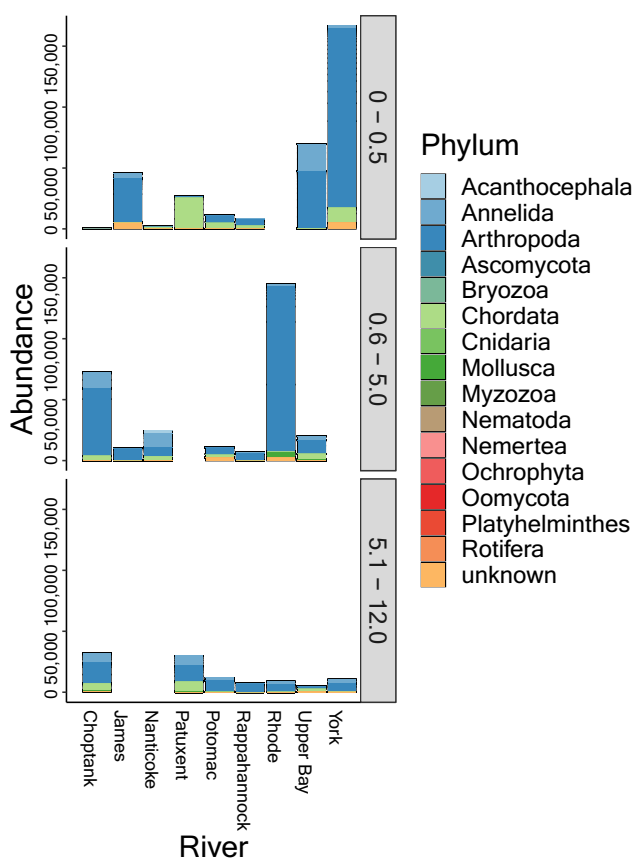
FIGURE 4 Sequence abundance of each phylum detected in the gut contents from age-0 Striped Bass included in the mitochondrial cytochrome oxidase I metabarcode data set across the salinity regimes (0.0–0.5, 0.6–5.0, or 5.1–12.0 practical salinity units) in each river and the upper Chesapeake Bay.

of prey by weight in the stomachs of age-0 Striped Bass could not be identified using morphological analysis. Additional gut content items, such as algae, plants, detritus, and sediment, were also observed (Figure 2C).

DISCUSSION

We compared two approaches for assessing diet—traditional morphological examination and newer metabarcoding methods—to identify gut contents of Striped Bass from tributaries across Chesapeake Bay. Although many of our results aligned with those of previous studies, we found that insects were an important yet underappreciated component of the diet for age-0 Striped Bass. We also identified multiple parasitic taxa, which were also not generally considered in prior studies, as well as two species that were previously unreported in Chesapeake Bay. Our results fill a gap that was present in ongoing surveys within Chesapeake Bay (i.e., ChesMMAP trawl surveys conducted in the main-stem bay), which have not previously integrated diets between tributary and main-stem bay efforts, and they provide key information for management of early life stages of the ecologically and economically important Striped Bass.

Sources of variation

Striped Bass foraging in tributary habitats of Chesapeake Bay during summer and fall 2018 had diverse diets that varied strongly by ontogeny, salinity zone, and tributary. Like observations in prior studies (Cooper et al. 1998; Rudershausen and Loesch 2000), age-0 Striped Bass diets were dominated by small crustaceans (amphipods, mysids, and isopods), insects (mainly dipterans), polychaete worms, and fishes. Like Boynton et al. (1981), we found that insects were extremely important prey for age-0 fish. In contrast, we found that juvenile Striped Bass diets were comprised of polychaete worms, fishes, small or medium crustaceans (isopods, amphipods, and the blue crab), and bivalves (especially the soft-shell clam), which is also consistent with prior studies (Harding and Mann 2003; Buchheister and Latour 2015). Interestingly, the diets of age-0 Striped Bass included in the study by Ihde et al. (2015) varied geographically: age-0 fish that were caught in main-stem bay sites in Maryland primarily consumed small crustaceans (amphipods, mysids, and isopods), fish, and insects, while those caught in Virginia primarily consumed fish, with lesser contributions from crustaceans and insects. In our study, salinity appeared to be the primary driver of variation in the diet of age-0 Striped Bass, with insects being the dominant prey item in freshwater tidal zones and small crustaceans, polychaete
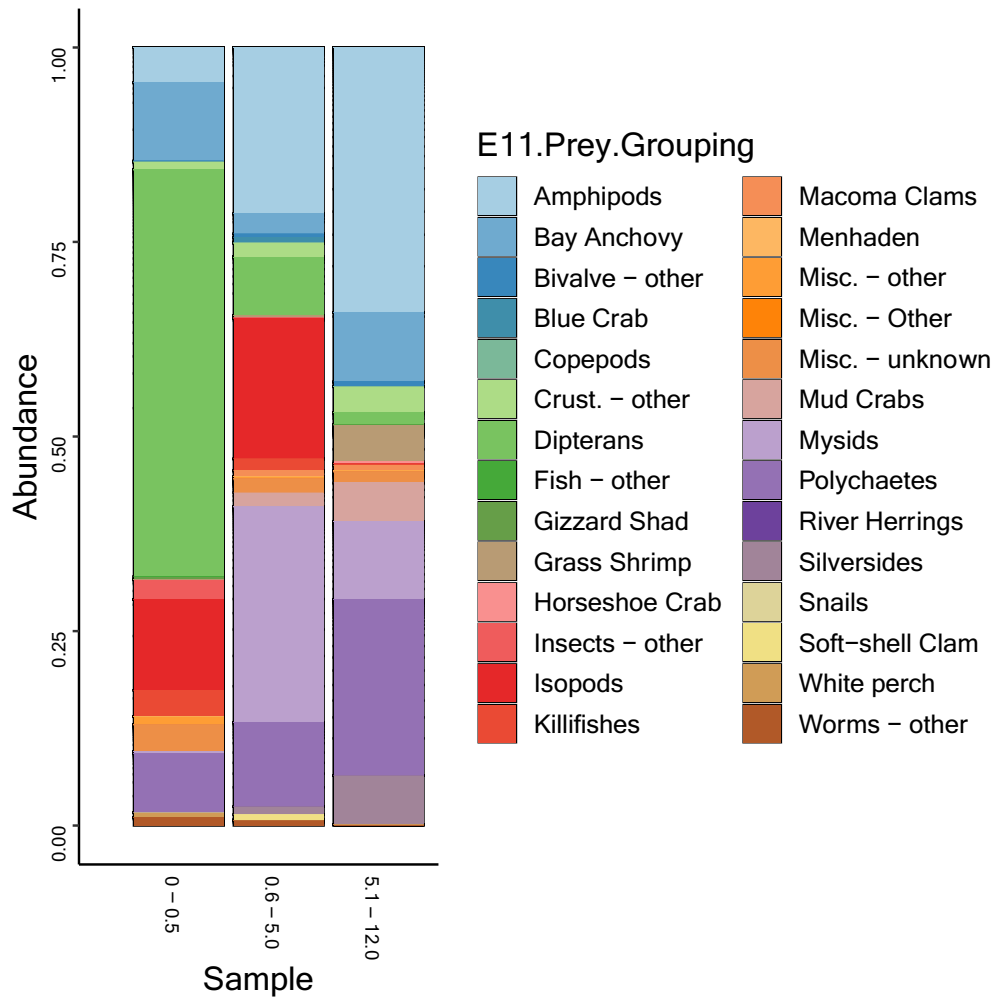
FIGURE 5 Major taxonomic prey groupings detected in the gut contents from age-0 Striped Bass included in the mitochondrial cytochrome oxidase I metabarcode data set across the three salinity regimes (0.0–0.5, 0.6–5.0, and 5.1–12.0 practical salinity units) sampled. Crust., crustaceans; Misc., miscellaneous.

worms, and fish making up most of the diet in oligohaline and mesohaline zones. These broad patterns of ontogenetic shifts in forage taxa (Griffin and Margraf 2003; Overton et al. 2009; Buchheister and Latour 2015) and a shift in age-0 diets with salinity (Markle and Grant 1970; Boynton et al. 1981) are generally consistent with prior observations and indicate that age-0 and juvenile Striped Bass are actively feeding from the water column as well as from the benthos. This study leveraged an existing long-term sampling program with a small number of fixed sites in each tributary, which made it difficult to evaluate the relative importance of salinity and tributary for influencing diets. This is particularly important given that salinities across tributaries can change with the volume of precipitation from year to year, with potential subsequent yet unknown impacts on the distribution of potential prey for age-0 Striped Bass in these portions of the tributaries. Further studies that are designed specifically to answer this question are needed to tease apart

differences in diet due to salinity and tributary-scale factors (e.g., habitat type, habitat area and connectivity, and shoreline development).

The important taxa in age-0 Striped Bass diets highlight the value of connected riparian habitats and nearshore benthic habitats for supporting age-0 Striped Bass. Insects were the most abundant prey item in freshwater tidal environments. Many of the insect prey were mayflies, dipterans/chironomids, and other insect species with aquatic phases, but we could not determine the species identification for most of them due to a lack of representation of these groups in publicly available genetic databases. Banded Killifish, Inland Silverside, bay barnacle *Amphibalanus improvisus*, and grass shrimp *Palaemon* spp. were also important diet components (Table 2) in our study, although they had not been identified previously from diet data as important forage (Buchheister and Latour 2015; Ihde et al. 2015). Some species, such as the bay barnacle, were likely not listed as key taxa by Ihde
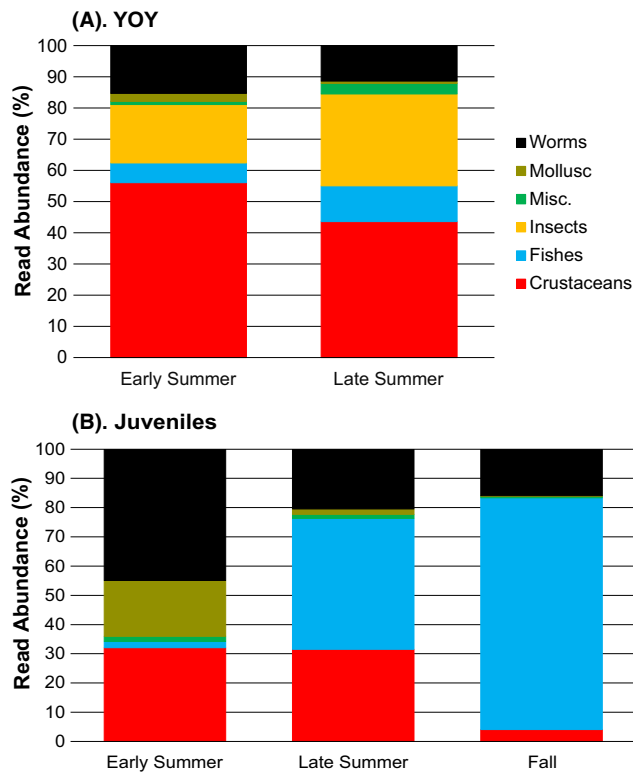
FIGURE 6 Major taxonomic prey groupings based on mitochondrial cytochrome oxidase I metabarcode data across (A) age-0 (young-of-year [YOY]) and (B) juvenile Striped Bass across each of the three seasons in which samples were collected. Forty-eight age-0 and juvenile Striped Bass were collected in early summer, 103 age-0 and juvenile fish were collected in late summer, and only 15 juveniles were collected in fall. For this analysis, Striped Bass samples across these ages were pooled together across locations and salinities. Misc., miscellaneous.

et al. (2015) or Buchheister and Latour (2015) because they may only serve as prey for age-0 Striped Bass (i.e., as planktonic larvae, as noted in our morphological data set) in tidal tributaries, which were not the focus of those reports. Many of these species are negatively impacted by human activities: for example, insects are declining at a global scale (Wagner et al. 2021), and killifishes, silversides, and grass shrimp decline in abundance with shoreline hardening (Kornis et al. 2017b). Addressing these impacts through shoreline restoration or other actions may represent an opportunity for ecosystem-based management of Striped Bass.

For juvenile Striped Bass in our study, the most abundant prey items were polychaete worms, Atlantic Menhaden, isopods, the soft-shell clam, and the blue crab, whereas in main-stem samples from Maryland, the five most abundant prey were Bay Anchovy, mysids, polychaete worms, Atlantic Menhaden, and amphipods. Atlantic Silverside, Inland Silverside, and Mummichog were identified by regional experts (Ihde et al. 2015) as likely

important prey that were not represented in the gut content data from ChesMMAP samples. We showed that Atlantic and Inland silversides, Mummichog, and Striped Blenny are indeed important prey of juvenile Striped Bass. Similarly, Kellogg et al. (2019) and Rudershausen and Loesch (2000) observed a smaller proportion of fish prey in Striped Bass diets from the Choptank and James rivers compared to Striped Bass diets from the main-stem bay, including an abundance of polychaetes, small crustaceans (mysids and copepods), blue crabs, and Striped Blenny in diets of Striped Bass collected in the tributary habitats.

The specificity afforded by metabarcoding enabled detection of two species (one nonnative and one assumed native) that we believe had not been reported previously from Chesapeake Bay. The gammaridean amphipod *G. japonica* is native to the northwestern Pacific but has been introduced in the eastern Pacific (British Colombia to northern Mexico), Hawaii, eastern Atlantic/Mediterranean, Australia, and most recently the northwestern Atlantic (Fofonoff et al. 2018; Trott et al. 2020). Trott et al. (2020) reported *G. japonica* for the first time along the U.S. East Coast at multiple locations in the Gulf of Maine and Long Island Sound (Connecticut). The sequences presented here likely represent the first record of *G. japonica* in Chesapeake Bay or the wider U.S. mid-Atlantic region. Although *G. japonica* is often encountered in polyhaline coastal areas, this species possesses a wide salinity tolerance and has been collected in salinities as low as 5 psu (Fofonoff et al. 2018). Targeted sampling and morphological examinations of collected amphipods are required to confirm the introduction of *G. japonica* in Chesapeake Bay. Nevertheless, our genetic data strongly suggest that *G. japonica* is present and likely has become established in several tributaries of the upper Chesapeake Bay. Our data on the polychaete *M. bastropi* likely represent the first report of this species outside of the type locality of Currituck Sound, North Carolina, and suggests that it is more widely distributed in lower salinity areas in the region. The lack of previous Chesapeake Bay records for *G. japonica* and *M. bastropi* probably reflects limited sampling of benthic and epifaunal communities, the superficial resemblance of *G. japonica* to the corophiid amphipod *A. lacustre* (which is extremely common in Chesapeake Bay and prevalent in the genetic data presented here), and the resemblance of *M. bastropi* to other related species of *Marenzelleria*.

Metabarcoding for diet analyses: Advances and limitations

Sequence-based tools for identifying prey items, such as metabarcoding (i.e., amplifying and sequencing a locus for all the contents in a single sample), allow for low-level

taxonomic identification (in most cases) while also decreasing the cost per sample through the ability to pool samples together. Thus, one can cost effectively assess the diet contents of many animals with little a priori knowledge of the available prey. For example, metabarcoding provided species-level identification for taxonomic groups such as amphipods (e.g., *Ameroculodes edwardsi*, *A. lacustre*, *Gammarus daiberi*, *Gammarus mucronatus*, *Gammarus tigrinus*, *G. japonica*, *Leptocheirus plumulosus*, and *Platorchestia platensis*), isopods (*C. polita* and *Erichsonella attenuata*), and polychaete worms (e.g., clam worm *Alitta succinea*, *Eteone heteropoda*, *H. filiformis*, *Laeonereis culveri*, *Marenzelleria neglecta*, and *Streblospio benedicti*), which are often difficult to identify to the species level using morphological methods. Finally, due to the high sensitivity of the method, there is the possibility of amplifying nondetectable organisms associated with the prey of prey found in the gut.

In addition to identifying prey items, the sensitivity of metabarcoding provides potential insight into the intestinal parasites of the predator and those of the prey. For example, sequences from the acanthocephalan genus *Pomphorhynchus* and the nematode genus *Philometra* were detected, which have been reported as important parasites of Striped Bass (Paperna and Zwerner 1976). Additionally, multiple other parasites were detected that were not intestinal parasites and thus were more likely to be parasites of prey. For example, we detected sequences from myxozoans *Kudoa* spp., which primarily infect the musculature of finfish, their final host, although some species require an invertebrate intermediate host (Moran et al. 1999). We also detected fish lice (e.g., *Argulus* spp.), which are obligate ectoparasites of teleosts (Walker et al. 2004), and gregarines (apicomplexan alveolates), which are most often parasites of invertebrates (Rohde 2005). Further exploration of the potential importance of these taxa in the broader food web dynamics is warranted, as these organisms, which are likely accidentally consumed along with target prey, may be useful in tracking trophic interactions across food webs (Carroll et al. 2019; Zamora-Terol et al. 2020).

Although this level of sensitivity can be helpful for determining some trophic interactions, it also has the potential to lead to confounding results when the prey of prey species or entirely unexpected species are identified in gut contents. For example, our results indicated that COI sequences for Atlantic Stingray and Cownose Ray were amplified from the stomach contents of juvenile Striped Bass ($n = 1$ for each ray species) collected in the Rhode River. These two elasmobranch species are known to occur in the Rhode River but are unlikely prey items for age-0 or juvenile Striped Bass, as these potential prey are typically much larger than the predator. Thus, it is more likely that these amplifications were the result of (1) direct scavenging, (2)

amplification of gut contents from scavenging secondary prey, or (3) incidental swallowing of sloughed secretions from the water column or in or on other prey species. Thus, the high sensitivity of these methods can highlight many aspects of trophic interactions within ecosystems while also leading to additional questions regarding interpretation of results when the desired outcome is to assess only the prey of a single species. One way to handle these data is to carefully consider the ecology of the predator in question, relying on experts who have studied the predator, including its general ecology and diet, to point out potential discrepancies in diet results that yield further research questions for future studies.

As with any technique, there are limitations to data that are generated via metabarcoding. First, the ability to assign taxonomy to sequence data depends on the availability of reliable reference sequences (which require taxonomic expertise to assemble) for the taxa that are present in the samples. For this study, the CBBI generated COI markers for a large majority of the finfish and macroinvertebrates in Chesapeake Bay, so we had a sequence database from which most of the available prey items could be identified. Importantly, these sequences are linked to photographs, tissue biorepository samples, and voucher specimens that can be revisited if questions about identifications arise. Second, estimating prey abundance in the diet is problematic, as many of the loci used for metabarcoding studies are multi-copy in the genome, often making abundance estimation difficult if not impossible (Deagle et al. 2019). Instead, relative abundance or prevalence likely offers more accurate estimates of prey utilization by a certain population or species, which is how we handled analyses in this study. Third, there is the potential for swamping of the sequence data by the DNA of the predator itself. In this case, we chose not to use a blocking primer to reduce the abundance of Striped Bass sequences. Because Striped Bass are piscivorous, development and optimization of a blocking primer that would reduce Striped Bass DNA but not inhibit the amplification of any prey items was unlikely to succeed and would have been extremely time consuming. Rather, we opted to aim for more reads per sample to fully capture the available prey despite the predator amplification.

Comparison of methodologies

Our results indicated that genetic methods can substantially improve taxonomic identifications in diet studies, particularly when supported by a robust local genetic barcode database. For age-0 Striped Bass, low-level taxonomic identification from morphological analysis of stomach contents was not possible with 40% of the prey by weight.

Similarly, the 18S marker used in this study was unable to accurately identify many important prey groups, such as finfish. Specifically, use of the 18S marker was hampered by both the lack of reference sequences from our local species pool (most sequences were only identified to the infraclass Teleostei, and the sequences that were assigned a binomial name or to genus were mostly unknown from the Atlantic) and a poor ability to discriminate between closely related species. For example, a 354-mm TL Striped Bass collected from the mesohaline Rhode River possessed a sequence identified as the Cownose Ray (a species that is common throughout the bay) based on COI, but the closest match using 18S was the Yellow Stingray *Urobatis jamaicensis*, a species that is found primarily in the tropical western Atlantic. Another juvenile Striped Bass (356 mm TL) from the Rhode River possessed sequences of Atlantic Menhaden (a highly abundant species in the bay) based on COI, whereas the use of 18S returned numerous high-percentage matches from several western Pacific/Indo-Pacific endemics with no previous Chesapeake Bay records, including two clupeids (Bony Bream *Nematalosa erebi* and Reeves Shad *Tenualosa reevesii*) and two perch species (Spangled Grunter *Leiopotherapon unicolor* and Macquarie Perch *Macquaria australasica*). In contrast, the 18S gene provided the widest breadth of taxonomic diversity within the gut contents, identifying organisms that are not commonly considered in trophic interactions because they are likely not the primary prey target, such as diatoms and parasites. Due to the limitations of our morphological and 18S data sets in identifying the organisms comprising Striped Bass diets, we focused our more detailed analyses on the COI metabarcode results, which were able to assign binomial names to 76% of the sequences obtained. The success of assigning binomial names is likely due to the regional COI database (CBBI), which had previously been generated for most taxa in Chesapeake Bay. Further highlighting the overall success of our COI metabarcoding efforts and the strength of the CBBI barcode library (in addition to the high identification rate) was that none of the unknown COI ASVs/OTUs was likely included in our major prey groups (e.g., fish, crustaceans, mollusks, etc.). Examinations of the Basic Local Alignment Search Tool results and consensus tree suggested that these sequences represented other taxonomic lineages distinct from the major taxa identified.

We were unable to make direct comparisons between the morphological and metabarcode data from prey of age-0 Striped Bass because these data sets were not generated from the same subsample of fish. However, we were able to use the same samples to generate both metabarcode data sets. Thus, in addition to the differences between the two markers in the taxonomic resolution of the prey items, there was clear variation in which taxa were most abundant (Figure 2B,C). The observed variation in dominant taxa could be due to several technical sources, such as the nature of the different primer sets to preferentially amplify certain taxa. For example, in the 18S metabarcode data set, fish accounted for 40% of the gut contents, but in the COI metabarcode data set, fish comprised only 7% of the data. Additionally, in the COI metabarcode data set, crustaceans made up 39% of the gut contents, whereas in the 18S metabarcode data set, crustaceans accounted for only 11% of the gut contents. These data further highlight the need to use multiple markers to gain a full breadth of the taxonomic scope and variation when using metabarcoding to identify organisms (Pompanon et al. 2012). The COI gene, however, appears to provide the highest taxonomic resolution. This may be due in large part to the fact that we have a regional COI library for most of the potential prey items, which, as others have noted, can be a major boost to accurate identification of local species (Gold et al. 2021).

Conclusions

Our study provides key diet information for an important predator, filling a knowledge gap regarding the diets of young Striped Bass in tributary habitats. Our results demonstrate that both ontogeny and location are important factors in Striped Bass diet composition and should be considered when identifying foraging habitat and forage taxa for this species. The composition of the diet is also primarily driven by salinity, with Striped Bass consuming different prey through ontogeny, as the growing fish move from freshwater to brackish waters. Our results also highlight the importance of certain prey that were not recognized previously as being primary components of the Striped Bass diet, particularly dipterans and smaller crustaceans, which appear to become less important in the diet as the fish move downriver. Finally, our study adds to the growing body of evidence demonstrating the utility of metabarcoding for understanding diet composition, including some advantages over traditional morphological identification of prey and the importance of using multiple markers to obtain a holistic view of the diet.

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CONFLICT OF INTEREST STATEMENT
The authors have no conflicts of interest to declare.

ETHICS STATEMENT
Fish caught for this study by SERC personnel were handled in accordance with SERC Animal Care and Use Proposal 2018-0510 and collections were made under Maryland permit SCP201814.

DATA AVAILABILITY STATEMENT
Raw sequence data and associated metadata can be found Sequence Read Archive (SRA) under BioProject PRJNA981150.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

APPENDIX: METHODS FOR GENERATING METABARCODE LIBRARIES FOR HIGH-THROUGHPUT SEQUENCING

Stomach contents from Striped Bass were thawed, weighed (wet weight, g), and homogenized using a Fisherbrand 150 handheld homogenizer motor. A total of 0.25 g of homogenate was used for DNA extraction unless the total stomach contents weighed less than 0.25 g, in which case the entire contents were used for DNA extraction. After overnight digestion with proteinase K, we extracted genomic DNA by using a Qiagen DNeasy Blood and Tissue Spin Column Kit following the manufacturer's protocols for animal tissues. For adults and juveniles, we applied 100 µL of 2× AE Buffer (200-µL final elution) with a 10-min incubation at room temperature. For age-0 Striped Bass, we applied 100 µL of AE Buffer twice (200-µL final elution) with a 10-min incubation each time at room temperature. All extractions within the same day included a blank extraction, which served as a negative extraction control for polymerase chain reaction (PCR). Extraction blanks were treated exactly like samples to identify contaminants that may have been introduced during genetic processing.

The primers mlCOIintF (Leray et al. 2013) and jgH-CO2198R (Geller et al. 2013) were used to amplify and sequence an approximately 310-base-pair (bp) fragment of the mitochondrial cytochrome oxidase I (COI) gene. The primers 3NDeukF and V4eukR2 (Bråte et al. 2010) were used to amplify and sequence an approximately 450-bp fragment of the small subunit ribosomal DNA (18S) gene. For both primer sets, primers containing both partial Nextera indices added to the 5′ ends and degenerate base pairs (0, 1, 2, and 3 bp) were added in equal concentration across samples. All PCRs were conducted in triplicate, and triplicate PCR amplicons were then pooled for each sample based on gel band intensity. All of the samples amplified with the 18S PCRs, whereas 12.5% of the samples ($n = 24$) did not amplify with any of the COI PCRs and were subsequently removed from further processing.

We used dual indexing with Nextera adapters employing a unique combination for each sample. Indexing PCR reagents consisted of 12.5 μL of KAPA Ready Mix, 1 μL of each index (i7 or i5), 1 μL of amplicon (pooled product), and 9.5 μL of water, resulting in a final reaction volume of 25 μL. Thermocycling was carried out with an initial denaturation of 95°C for 5 min; followed by 12 cycles of 98°C for 20 s, 60°C for 45 s, and 72°C for 45 s; and a final extension of 72°C for 5 min. To verify that indexing was successful, an aliquot of indexed product and unindexed product were both electrophoresed on agarose gel (2% weight/volume) stained with GelRed and visualized under UV light. The indexed product was purified with AMPure XP Beads (Beckman-Coulter) following the manufacturer's instructions for a 10-μL sample reaction volume and a 1.5× ratio.

The bead-cleaned samples were then quantified using a Qubit Double-Stranded DNA HS Assay Kit (Thermo Fisher Scientific) with a Qubit 2.0 fluorometer following the manufacturer's instructions. Samples were pooled based on equimolar concentrations into two separate libraries (COI and 18S, respectively). The final pooled libraries were sequenced using a MiSeq v3 600 Reagent Kit (Illumina) on an Illumina MiSeq platform at the Laboratories of Analytical Biology (Smithsonian National Museum of Natural History). Raw sequence data were deposited in the Sequence Read Archive under BioProject PRJNA981150.

Bioinformatics

Primer sequences were removed using cutadapt (Martin 2011). Sequences were quality trimmed and merged using the DADA2 package (Callahan et al. 2016) in R (R Core Team 2020). Chimeras were identified and removed using the consensus method in DADA2. Amplicon sequence variants (ASVs) were generated in DADA2. We only retained sequences from 308 to 318 bp for COI and from 378 to 488 bp for 18S based on the expected sizes of the fragments as well as the size distribution of sequences. The 18S ASVs were annotated using the DADA2 package with the PR2 database version 4.12.0 (Guillou et al. 2013). The COI ASVs were first compared using the Basic Local Alignment Search Tool (BLAST; Madden 2002) within Geneious Prime against the COI library from the Chesapeake Bay Barcode Initiative (CBBI; Ogburn et al., BioProjects PRJNA396533 and PRJNA498040), and those that matched with 95% pairwise identity and 90% coverage with database records were annotated. Sequences were also BLASTed against the National Center for Biotechnology Information (NCBI) nucleotide (nt/nr) database using the same thresholds for accepting annotations. If discrepancies existed, then the identification from the CBBI library was given priority. To refine the taxonomy

further, the representative sequences for each marker were aligned using the MAFFT plug-in (Katoh et al. 2002) with default parameters in Geneious Prime. A neighbor-joining consensus tree was generated using the Geneious Tree Builder in Geneious Prime with the Tamura–Nei model and 1000 bootstrap replicates. The tree topology was used to refine taxonomic identities, particularly for those unspecified taxa that were then grouped into different clades. Additionally, the taxonomic identities were verified based on known taxa in Chesapeake Bay. Taxa were also binned by other taxonomic categories based on Ihde et al. (2015). Parasitic taxa were identified based on taxonomic assignments at the appropriate taxonomic level. For example, both Rohde (2005) and Roberts and Janovy (2005) were used to determine major taxonomic rankings for parasites. If orders or classes were known to contain only parasitic taxa, then all ASVs in those orders or classes were included. For orders or classes with both parasitic and free-living taxa, we conducted literature searches to best assess which genera were or were not parasitic, with free-living being the default designation if no other information could be found. For the 18S data, review of the taxonomic assignments showed that many metazoan species-level assignments were likely inaccurate. Thus, we considered only higher level classifications to characterize diets.

Due to the interest in examining interspecific diversity within diets rather than the intraspecific diversity provided by ASV data, ASVs with identical taxonomic identifications in the COI data set were merged based on the full scientific name using the tax_glom command in the phyloseq package (McMurdie and Holmes 2013) in R. Alpha diversity metrics (Chao1 and Shannon) and taxonomic bar plots were calculated in the phyloseq package. Additionally, we used the vegan package (Oksanen et al. 2014) in R to conduct a permutational analysis of variance using 1000 iterations with the Jaccard method to determine whether gut contents varied across salinity regimes. We also examined the beta dispersion significance to determine the influence of the spread of objects from the centroid. For all statistical analyses, we used an α of 0.05 to determine significance.

To confirm the identities of two potentially nonnative species found in the stomachs of Striped Bass, we created alignments of our sequences and those from the NCBI nucleotide database for those species and an outgroup from the same taxonomic family. Alignments were made in Geneious Prime using the Clustal Omega plug-in (Sievers et al. 2011) with default parameters. Neighbor-joining trees were generated in Geneious Prime using the alignments with 1000 bootstrap replicates.