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RESEARCH ARTICLE

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Diet of the North American river otter (*Lontra canadensis*) in North Carolina using 2 methods

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Abstract

River otters (*Lontra canadensis*) are key predators in North Carolina's aquatic systems, but they are often seen as competitors by anglers and fish hatcheries. River otter diets typically consist of fish and crayfish, but also include occasional herpetofauna, mammals, and birds. While standard diet studies focus on identification of prey through manual examination of stomach contents and feces, metabarcoding DNA analysis has become more popular to determine the presence or frequency of species that are often missed, misidentified, or underestimated. We collected river otter carcasses from licensed trappers and fur dealers across North Carolina from the 2009–10 trapping season through the 2015–16 season. We conducted necropsies and analyzed the stomach contents using standard observational methods and metabarcoding DNA analysis. We manually examined 522 river otter stomachs, of which 377 contained prey items. Decapods (crustaceans) were identified in 41% of stomachs and made up similar percentages within each Furbearer Management Unit (FMU). The order Perciformes composed the majority (62%) of fish prey across all stomach samples. Coastal Plain river otters primarily consumed crustaceans (50%) and fish (40%). Piedmont and Mountain river otters consumed fish (32% and 42%, respectively) most often followed by crustaceans (62% and 50%, respectively). Prey selection was similar between the sexes. Out of 368 samples,

metabarcoding DNA examination was able to reliably match 164 prey items to species, 5 classes, 18 orders, 25 families, and 42 genera. Fishes made up 33% of the identifications, particularly Perciformes (13%), Cypriniformes (7%), and Siluriformes (5%). Twelve percent of identifications was made up by Amphibia, split evenly by Anura and Urodela. No birds or reptiles were detected in the Mountain or Piedmont FMUs, and no mammals were detected in the Coastal Plain or Mountain FMU. Overall, river otters in North Carolina consume a large variety of prey that varied regionally. The manual examination provided identifications that were not provided by the DNA examination (i.e., crayfish, brown snakes), while the DNA examination provided a more accurate identification of the broad array of prey items. To understand the composition of annual river otter diets we encourage managers to expand research to evaluate river otter diets year-round and incorporate additional non-invasive methods (e.g., scat surveys) throughout the year.

KEYWORDS
diet, DNA, *Lontra canadensis*, metabarcoding, river otter

Diet is an important ecological component for apex predators like the North American river otter (*Lontra canadensis*; hereafter river otter). River otters are key predators in aquatic systems (Melquist and Dronkert 1987), and prey upon a diverse group of fish, reptiles, amphibians, mollusks, and crayfish species (Anderson and Woolf 1987, Crait and Ben-David 2006, Cote et al. 2008, Barding and Lacki 2012, Feltrop 2016). Additionally, river otters will feed on mammals and birds (Toweill 1974, Crimmins et al. 2009, Penland and Black 2009, Wengeler et al. 2010, Barding and Lacki 2012). River otters follow optimal foraging theory (Thompson and Stelle 2014) and select large slow-moving prey when it is available. Further, river otter diets reflect the availability of prey across the landscape and seasons (Stenson et al. 1984, Taylor et al. 2003, Roberts et al. 2008, Penland and Black 2009, Stearns and Serfass 2010).

While otter species serve a vital role in aquatic systems (Hammerschlag et al. 2019), they are often seen as competition by anglers, commercial fishermen, and fish hatcheries (Johnson 1982, Václavíková et al. 2011, Barbieri et al. 2012, Rosas-Ribeiro et al. 2012). Trout fishermen have long complained about river otter predation (Lagler and Ostenson 1942, Knudsen and Hale 1968, Serfass et al. 2014) even though, with the exception of lake ecosystems (Crait and Ben-David 2006, Wengeler et al. 2010), studies have confirmed that trout play a minor role in river otter diets (Lagler and Ostenson 1942, Ryder 1955, Knudsen and Hale 1968, Toweill 1974).

Standard river otter diet studies focus on identifying prey through the remaining hard parts in either scat (Gallant et al. 2007) or stomach contents (Hyslop 1980). While hard parts are identified with the help of manuals and keys, they often overestimate the volume of some species in the overall diet (Klare et al. 2011). Conversely, soft prey items are usually underestimated in diet analyses because they are easily digestible (Mizukami et al. 2005, Osaki et al. 2019). Metabarcoding DNA analysis has increased in prevalence due to the availability of affordable high-throughput sequencing and has come into regular use to detect environmental DNA of species communities (Taberlet et al. 2018) and to more accurately determine the diet of various species (Valentini et al. 2009). Our

objective was to establish baseline diet parameters of river otters in North Carolina and determine the feasibility and benefits of metabarcoding DNA analysis on river otter stomach contents.

STUDY AREA

We conducted our study across North Carolina. For management purposes, the North Carolina Wildlife Resources Commission (NCWRC) divides the state into 3 Furbearer Management Units (FMUs; Mountain, Piedmont, and Coastal Plain) which followed physiographic regions and county boundaries (Figure 1). However, because river otters are semiaquatic, their territories are linear and tend to correspond to river-basin geographic features (Melquist and Hornocker 1983, Melquist and Dronkert 1987, Reid et al. 1994, Sauer et al. 1999, Blundell et al. 2001). Therefore, we focused our study on the 14 river basins that occur throughout North Carolina (Figure 1) which include cold water systems in the Mountains and Piedmont and warm water systems throughout the state. Within those river basins are 17 terrestrial and 11 wetland communities including bogs, black and brown water systems, freshwater tidal wetlands, pocosins, swamps, natural lakes, reservoirs, impoundments, and saltwater estuaries (North Carolina Wildlife Resources Commission 2015).

MATERIALS AND METHODS

General and necropsy methods

We collected river otter carcasses from licensed trappers and wildlife damage control agents (WDCA) during the regulated trapping seasons beginning 1 November 2009 and ending 29 February 2016. We did not collect any carcasses during the November 2013–February 2014 trapping season. Trappers and WDCA provided general location data (county) for all river otter samples and we used ArcGIS to assign each sample to a specific FMU. We froze the carcasses until necropsy.

Manual examination data analysis

We emptied the stomach contents into a collecting tray. We identified whole prey items using the naked eye and 40X dissection microscopes. We used dichotomous keys (Chesapeake Bay Multispecies Monitoring and

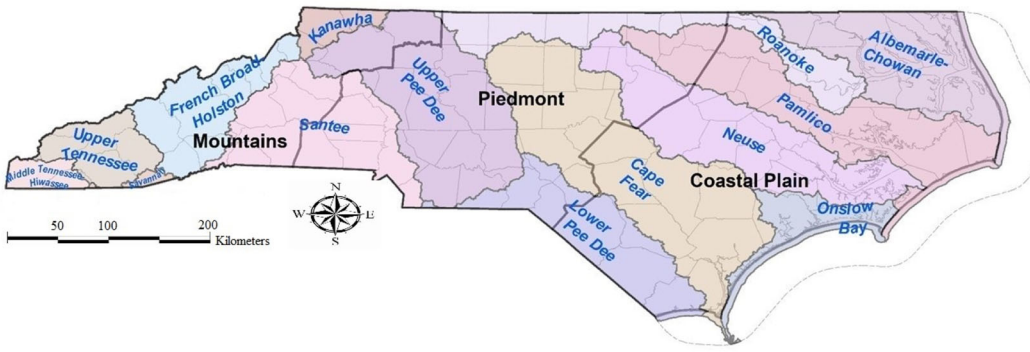

FIGURE 1 Furbearer Management Units (FMU) and river basins of North Carolina, USA, from 2009–2016.

Assessment Program and Northeast Area Monitoring and Assessment Program 2013, 2020; Moore and Spence 1974; Roest 1991; Daniels 1996; Cooper 2004*a*, *b*, *c*, *d*, e, *f*) and identification guides (Martof et al. 1980, Webster et al. 1985, Page and Burr 1991, Conant and Stebbins 1999, Reid 2006, Beane et al. 2010) to identify scales, exoskeletons, and other parts. We identified prey items to the lowest possible taxonomic classification. After the identification process, all stomach contents and all liquid were homogenized with an immersion blender. We extracted 5 mL samples for metabarcoding DNA analysis. No fixative was used and all samples were frozen until DNA processing. We excluded empty stomachs from the data analysis. Frequency of manual examined prey items and DNA extracted from prey items were analyzed across order, FMU, sex, and age using analysis of variance (ANOVA). We set alpha at 0.05 for all analyses.

DNA laboratory methods

Stomach contents were purified by vortexing 200 µl of each sample with 1 ml InhibitEX Buffer (Qiagen, Venlo, Netherlands) for two minutes, and then centrifuging for one minute to pellet the impurities. The supernatant was further processed for DNA extraction using a DNeasy blood and tissue kit (Qiagen, Venlo, Netherlands). Starting with 600 µl of the supernatant, we added 600 µl Buffer AL and lysed with 25 µl Proteinase K at 70°C for 10 minutes in 2-ml microcentrifuge tubes. Following incubation, the lysate was homogenized with 600-µl EtOH, and DNA was collected by centrifuging the entire mixture through the spin column in three 600-µl increments at 13,500 rpm for one minute each. The remaining extraction protocol followed the standard kit guidelines, with the exception that DNA was eluted in 150-µl volume of elution buffer.

We amplified the 16 S rRNA gene using the V16S (Vences et al. 2016) primer pair. We tested these primers on river otter stomach samples, river otter tissue, and tissue from 35 fish and mussel species native to the United States and ran the PCR to verify broad taxonomic coverage. Initial amplification of all samples was performed in a 25-µl reaction volume using 5 µl of KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 10.75-µl nuclease-free water, 0.75-µl dNTPs, 0.5-µl KAPA HiFi DNA polymerase, and 5 µl of template. Every sample contained 1.5 µl (5 µM) of the V16S primer mix. The thermal cycle profile contained an initial denaturation at 98°C for 3 minutes, followed by 17 cycles involving denaturation at 98°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds, and a final extension 72°C for 5 minutes.

The PCR product served as a template for a second PCR to add internal identifying tags. The components in this reaction were the same, except that instead of adding the unlabeled V16S primers across every sample, we added 1.5 µl (5 µM) of each forward (A-H) and reverse (1-12) 16S fusion-indexed primers in combination across each 96-well plate using a TaggiMatrix spreadsheet (Glenn et al. 2019). We ran this reaction for 17 more cycles using the same thermal profile and checked the products on a gel. We pooled 5 µl of the tagged amplicons from each sample into 1.5-ml microcentrifuge tubes by plate with final volumes of 480 µl each. The pooled containers were purified using Speedbeads (ThermoFisher-Scientific, Waltham, MA, USA) in a ratio of PCR product volume to beads of 1:1.5 and resuspended in 100 µl of TE. We checked the purified products for the correct fragments size and primer dimer removal on a gel.

We used the pooled and purified products as a template in a third PCR to add iTru adapter sequences. This round involved a 50-µl reaction volume containing 10-µl 5X Kapa Hifi Buffer, 1.5-µl dNTPs, 1-µl Kapa HiFi DNA polymerase, 10-µl template, and different combinations of 5-µl (5 µM) iTru5 and iTru7 primers. The thermal cycling profile had an initial denaturation at 98°C for 2 minutes, followed by 7 cycles of denaturation at 98°C for 20 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 30 seconds, and a final extension 72°C for 5 minutes. We ran each sample from the previous PCR in three replicate reactions, each with a different iTru5/iTru7 index. All three reactions were quantified with Qubit and pooled in equimolar concentrations before sequencing. We submitted libraries and primers for paired-end sequencing on an Illumina MiSeq platform at the University of Kentucky HealthCare Core Genomics Facility.

DNA data analyses

Reads were demultiplexed using python program Mr. Demuxy v1.2.2 (https://pypi.org/project/Mr_Demuxy/) to separate pooled samples with combinatorial barcodes. We imported the demultiplexed dataset as a fastq to Qiime 2 (Bolyen et al. 2019) for all further analyses. We performed quality control using DADA2 (Callahan et al. 2016) to trim primers and adapters as well as to denoise and dereplicate paired-end sequences. We assembled a custom-made reference database that included all available sequences of vertebrates, crayfish, freshwater mussels, and oyster species within the study area and that had 16 S rRNA sequences and taxonomy information from Genbank (Benson et al. 2013). We extracted target regions from the reference sequences by trimming according to each universal primer set. The trimmed reference reads were used to train a Naive Bayes classifier to match their corresponding taxonomic classification. Then, we applied the trained classifier to our dataset to determine taxon representation within each stomach sample. After classification, prey species were assigned to each sample if sequences were present in all three PCR replicates and if the combined number of reads across replicates exceeded 242 reads. The threshold of 242 reads was selected because it represented the highest number of reads that represented a clearly erroneous classification, which in this case was the identification of Siren salamander (Sirenidae) sequences in a Mountain sample.

RESULTS

We evaluated river otter diet by manual and DNA comparison across North Carolina, by order, sex, and FMU (Figures 2–7). Additionally, we provided manual and DNA data across North Carolina by class, family, genus, species, FMU, and river basin (Tables S1-S14, available in Supporting Information).

Manual examination

We manually examined the contents of 522 (325 male, 197 female) river otter stomachs. Of these 28% (n = 145, 98 male, 47 female) of river otter stomachs were empty and were excluded from future analyses (Figures 2–7). Of the 377 nonempty stomachs, 11% (n = 43, 27 male, 16 female) contained items unidentifiable to order but we manually

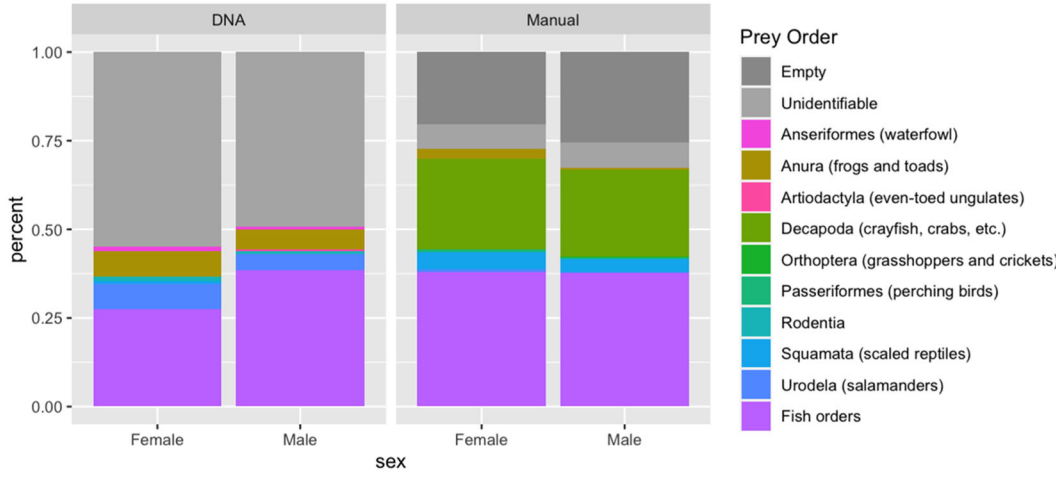

FIGURE 2 Frequency of manually-examined prey items and DNA extracted from prey items of river otters by sex during trapping season (1 November–28/29 February) in North Carolina, USA, from 2009–2016.

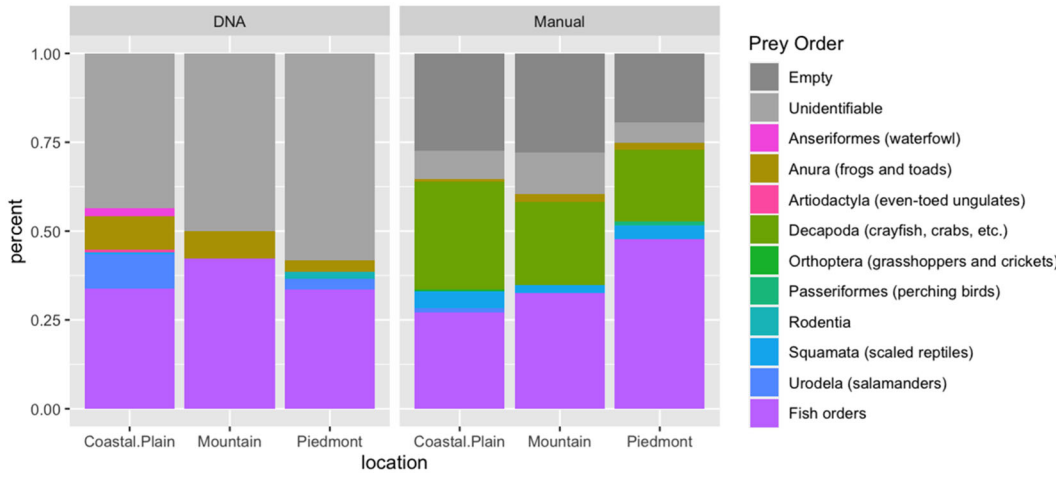

FIGURE 3 Frequency of manually-examined prey items and DNA extracted from prey items of river otters by Furbearer Management Units (FMU) during trapping season (1 November–28/29 February) in North Carolina, USA, from 2009 to 2016.

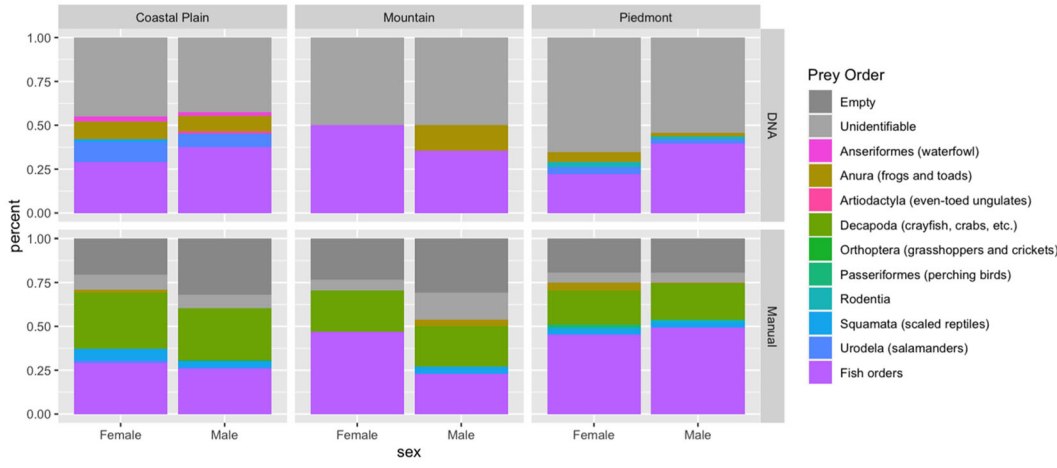

FIGURE 4 Frequency of manually-examined prey items and DNA extracted from prey items of river otters by sex and Furbearer Management Units (FMU) during trapping season (1 November–28/29 February) in North Carolina, USA, from 2009 to 2016.

identified prey from 23 biological orders across North Carolina. Decapods (e.g., crayfish, crabs, shrimp) were identified in 41% ($n = 153$, 93 male, 60 female) of stomachs and made up similar percentages within each FMU (Figures 2–4). The order Perciformes (perch-like fishes), including the families Centrarchidae (sunfish) and Percidae (perch), composed the majority (62%) of fish prey. Cypriniformes (carps and minnows) were present in 14% of stomachs, and Squamata (snakes and lizards) were present in 7% (Tables 5–7).

Orders consumed by otters differed by FMU. Cypriniformes ($F = 5.15_{x,374}$, $P = 0.0062$) were mostly consumed in the Piedmont FMU, while Decapoda ($F = 6.18_{x,374}$, $P = 0.0023$) was mostly consumed in the Piedmont and Coastal Plain FMUs (Figures 5–7). Lepisosteiformes ($F = 7.61_{x,374}$, $P = 0.0006$) was only consumed in the Mountain FMU, while Mugiliformes ($F = 6.20_{x,374}$, $P = 0.0022$) were only consumed in the Coastal Plain FMU (Figures 5–7).

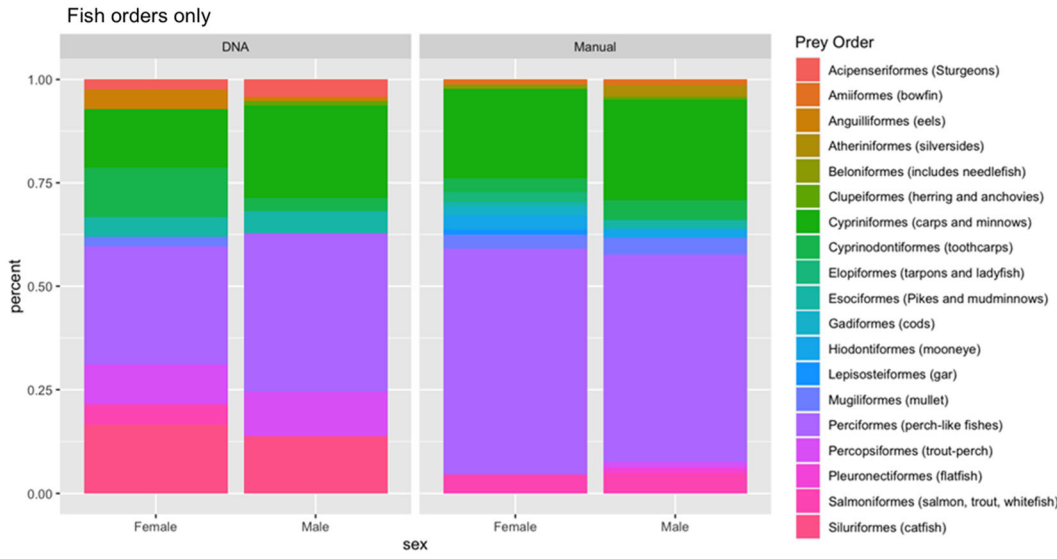

FIGURE 5 Frequency of manually-examined fish orders and DNA extracted from fish orders of river otters by sex during trapping season (1 November–28/29 February) in North Carolina, USA, from 2009 to 2016.

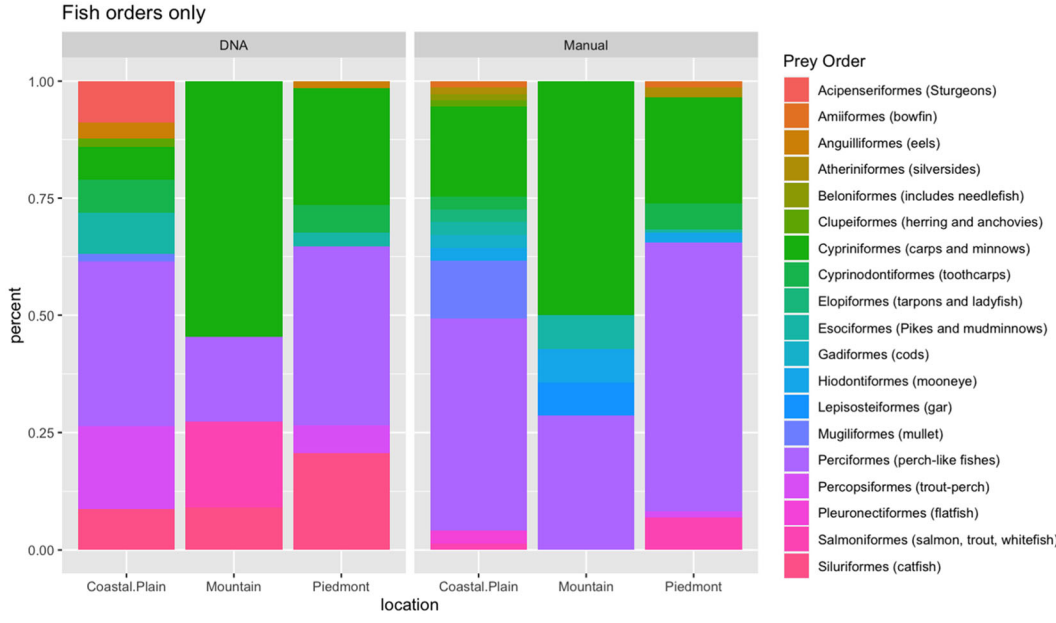

FIGURE 6 Frequency of manually-examined fish orders and DNA extracted from fish orders of river otters by Furbearer Management Units (FMU) during trapping season (1 November–28/29 February) in North Carolina, USA, from 2009 to 2016.

Perciformes ($F = 13.24_{x,374}$, $P < 0.0001$) and Salmoniformes ($F = 3.78_{x,374}$, $P = 0.0237$) were consumed more in the Piedmont FMU than in other FMUs, and Salmoniformes were not consumed in the Mountain FMU (Figures 5–7. The only significant difference by sex was that more members of the order Anura (frogs and toads) were consumed by females ($F = 4.25_{x,347.5}$, $P = 0.0406$) in the Piedmont FMU ($F = 5.76_{x,247}$, $P = 0.0171$; Figures 2–4).

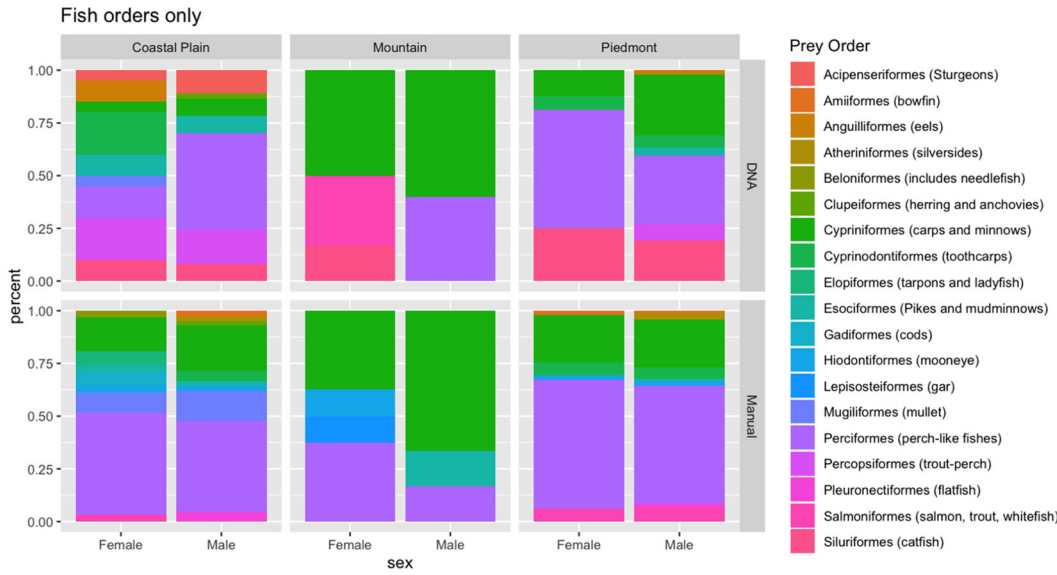

FIGURE 7 Frequency of manually- examined fish orders and DNA extracted from fish orders of river otters by sex and Furbearer Management Units (FMU) during trapping season (1 November–28/29 February) in North Carolina, USA, from 2009 to 2016.

DNA examination

We identified a total of 320,245 reads to species other than river otter across 368 samples and were able to match prey species to 164 samples representing 198,508 total reads. We were unable to identify prey items in 204 specimens based on the minimum threshold of 242 DNA reads. We identified prey from 17 orders and the statewide analysis revealed only one significant difference in prey selection between males and females; males selected fish at a higher level than females (males 37%, females 25%, $F = 6.38_{x,366}$, $P = 0.0120$; Figures 5–7). When examined by FMU, however, the difference was specific to the Piedmont FMU ($F = 6.15_{x190}$, $P = 0.0140$) with 20% of females consuming fish as opposed to 37% of males.

When separated by order no eels (Anguilliformes), pikes (Esociformes), trout-perch (Percopsiformes), or salamanders (Urodela) were observed in the Mountain FMU (Figures 5–7). Sturgeons (Acipenseriformes), waterfowl (Anseriformes), mullet (Mugiliformes), and scaled reptiles (Squamata) were only consumed in the Coastal Plain. Rodents (Rodentia) were only observed in the Piedmont FMU. While frogs and toads (Anura) were present in the diet for each FMU, they were more common (11%) in the Coastal Plain river otters ($F = 3.29_{x,365}$, $P = 0.0385$). Carps and minnows (Cypriniformes) were present in all three FMUs as well but were significantly more common (24%) in the Mountain FMU ($F = 8.16_{x,365}$, $P = 0.0003$). Salmonids (Salmoniformes) were only detected in the Mountain FMU, and while salamanders (Urodela) were detected in both the Coastal Plain (11%) and Piedmont FMUs (3%), they were far more common in the Coastal Plain ($F = 5.15_{x,365}$, $P = 0.0062$; Figures 5–7).

DISCUSSION

We noted a large difference in the species identified between manual examination and DNA metabarcoding. The manual examination identified more species including the presence of invertebrates, particularly crayfish, that DNA analysis did not reveal (but note that a metabarcoding primer set targeted toward decapods would likely amplify

crayfish). Also, manual identification provided for the identification of species such as brown snakes, which provided reads in the DNA analysis, but the reads were less than our filtering threshold. We note, however that soft-bodied species were more common in the metabarcoding results. While amphibians were detected with both methods, there were a higher percentage of detections with metabarcoding. Two groups of scaleless fishes, catfish and sturgeon, were only detected using our genetic evaluation. Furthermore, DNA analysis provided better precision in prey classification and based on spatial appropriateness, better accuracy. Therefore, our results indicated that manual and genetic approaches are complementary and that a complete inventory of prey items would likely require both, especially for a generalist predator.

Our manual examination of river otter stomachs confirmed both vertebrate and invertebrate prey, with vertebrates, particularly fish, being the most common by presence. Also, crustaceans were common and considered a staple in the diet of North Carolina river otters. However, the manual examination was only able to reliably identify prey items to family, and in some cases to order, which was mostly done by examination of hard parts such as exoskeletons and scales, though some soft-bodied species such as frogs and salamanders were still intact enough to identify visually based on markings. While most of the identifications seem to be spatially appropriate, we did identify prey from the families Pomatomidae (bluefish), Serranidae (sea basses and groupers), and Sparidae (porgies or sea bream) in the Piedmont FMU. While it is possible that trappers could have used these species as bait for their trap sets, it is more likely that the lack of complete and spatially-specific dichotomous keys, particularly in the Piedmont and Mountain FMUs, led to misidentification.

Our DNA examination failed to identify invertebrates but was able to identify vertebrates to species. The V16S marker we used was designed for vertebrates, however, we tested the 208 primers on DNA extracts from representative mussels and were able to amplify the mussel species in those tests, which is why we used V16S as a single marker. However, it is likely that in a vertebrate and invertebrate species mix (such as stomach contents), vertebrates are preferentially sequenced to invertebrates. Therefore, future diet studies with otters should include both vertebrate and decapod primer pairs. The other notable difference in broad taxonomic representation between manual and genetic examination was the lack of reptiles in the metabarcoding results (only a single detection). Future studies would be useful to determine if the V16S marker does not amplify reptiles effectively or if the lack of reptiles is due to low DNA yield from scales in stomach contents. Centrarchidae (sunfishes) was the major prey family identified, but Aphredoderidae (pirate perches), Catostomidae (suckers), Cyprinidae (minnows and carps), and Ictaluridae (common catfishes) were all important fish families. In addition, Ranidae (true frogs) and Sirenidae (sirens) were important prey items, particularly in the Coastal Plain (both) and Piedmont (Ranidae only) FMUs. We identified some smaller fish such as killifish (Fundulidae) and mosquitofish (Poecilidae) which could have been the prey of a larger fish that was captured by the river otter. The identifications are all spatially appropriate, with species such as sheepshead minnows and mummichogs limited to the Coastal Plain FMU and orangefin madtom limited to the Piedmont FMU.

We confirmed that river otters in North Carolina consume a large range of prey, but mostly concentrate on aquatic species such as fish, crayfish, and amphibians. Fish from the order Perciformes, particularly sunfish, were selected at higher levels than other fish species. Fish most commonly consumed included largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*), and redear sunfish (*Lepomis microlophus*). While some anglers have expressed concern about river otter consumption of trout species, we did not identify any fish of the family Salmonidae by manual examination, and we only identified trout species (brook trout) through DNA analyses in two river otters from the Mountain FMU. Although we conducted our study over a 7-year period during January and February, to fully understand the annual composition of river otter diets we encourage managers to expand this research to evaluate river otter diets year-round and incorporate additional noninvasive methods (e.g., scat surveys) throughout the year.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

All methods involving the handling and capture of live animals in this study were approved by the North Carolina State University Institutional Animal Care and Use Committee (protocol number 15-171-O). Methods involving deceased animals were overseen by North Carolina State University and NCWRC personnel.

DATA AVAILABILITY STATEMENT

The data that support the results of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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