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Gutsy genetics: identification of digested piscine prey items in the stomach contents of sympatric native and introduced warmwater catfishes via DNA barcoding

Robert Aguilar · Matthew B. Ogburn · Amy C. Driskell · Lee A. Weigt · Mary C. Groves · Anson H. Hines

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Abstract A major focus of ecology is understanding trophic relationships and energy flows in natural systems, associated food web dynamics and changes in food webs due to introduced species. Predator-prey interactions are often assessed by examining stomach contents. However, partially digested remains may be difficult to accurately identify by traditional visual analysis. Here we evaluate the effectiveness of DNA barcoding to identify digested piscine prey remains in invasive Blue Catfish *Ictalurus furcatus*, non-native, but established Channel Catfish *Ictalurus punctatus* and native White Catfish *Ameiurus catus* from Chesapeake Bay, USA. Stomach contents were examined and piscine prey items were scored as lightly digested, moderately digested or severely digested. A 652 base pair region of the cytochrome c oxidase subunit I (*COI-5P*) mitochondrial DNA gene was sequenced for each prey item. Edited barcode sequences were compared to locally-caught and validated reference sequences in BOLD (Barcode of Life Database). A large majority of prey items were sufficiently digested to limit morphological identification (9.4 % to species and an additional 12.1 % to family). However, overall barcoding success was high (90.3 %) with little difference among the digestion classifications. Combining morphological and genetic identifications, we classified 91.6 % of fish prey items to species. Twenty-three fish species were identified, including species undergoing active restoration efforts (e.g., *Alosa* spp.) and commercially important species, e.g., Striped Bass *Morone saxatilis*, White Perch *Morone americana*, American Eel *Anguilla rostrata* and Menhaden *Brevoortia tyrannus*. We found DNA barcoding highly successful at identifying all but the most heavily degraded prey items and to be an efficient and effective method for obtaining diet information to strengthen the resolution of trophic analyses including diet comparisons among sympatric native and non-native predators.

Keywords Blue catfish · *Ictalurus furcatus* · Predation · Clupeidae · Chesapeake Bay · Genetic analysis

R. Aguilar (✉) · M. B. Ogburn · A. H. Hines
Smithsonian Environmental Research Center, PO Box 28, Edgewater, MD 21037, USA
e-mail: aguilarr@si.edu

A. C. Driskell · L. A. Weigt
Laboratories of Analytical Biology, National Museum of Natural History, Smithsonian Institution, 10th and Constitution Ave, Washington, D.C., USA

M. C. Groves
Maryland Department of Natural Resources, Inland Fisheries, Cedarville Fish Hatchery, Route 4, Box 106E, Brandywine, MD 20613, USA

Introduction

Trophic relationships such as predator-prey interactions are a major focus in ecological research, as they are critical in understanding food web dynamics and energy flow through ecosystems (Beauchamp et al. 2007). Introduced species have the potential to disrupt trophic relationships and alter ecosystem function (Fritts and Rodda 1998; Charles and Dukes 2007), and determining

the scale of impacts is critical to resource managers. Trophic relationships are generally assessed by examining the partially digested remains of consumers (Hyslop 1980). However, the ability to visually identity prey generally decreases as digestion progresses, particularly in fishes (Carreon-Martinez et al. 2011), which can constitute a significant loss of information and affect the basic interpretation of study results (Hyslop 1980; Chandler 1998; Eggleton and Schramm 2004; Schloesser et al. 2011). Differential degradation rates (Hyslop 1980), seasonal changes in digestion rates (Legler et al. 2010), morphological similarity of important prey species (e.g., Centrarchidae, Clupeidae, Cyprinidae, Moronidae) and rarity of some prey species of conservation and management concern highlight the need for sensitive diet analyses that go beyond traditional morphological techniques to maximize the proportion of prey items that can be identified to species in diet studies.

DNA barcoding is a promising technique in ecological studies that utilizes a short standardized genetic sequence which can act as a proxy for species (Hebert et al. 2003; Joly et al. 2014; Kress et al. 2015). DNA barcoding has been used in a variety of studies including trophic dynamics (Valdez-Moreno et al. 2012; Wirta et al. 2014; Moran et al. 2015), environmental forensics (Dalton and Kotze 2011; Handy et al. 2011; Gonçalves et al. 2015), identifying cryptic diversity and invasive species (Hebert et al. 2004; Conway et al. 2014; Bariche et al. 2015), evaluating ecosystem and evolutionary diversity (Ward et al. 2005; Baldwin et al. 2011; Weigt et al. 2012a; Leray and Knowlton 2015) and exploring phylogenetic relationships (Nagy et al. 2012; Baeza and Fuentes 2013; Betancur-R et al. 2013). While there are a number of markers available (e.g., 16sRNA, 18sRNA, matK, rbcL, etc.), one of the most commonly used with fishes (and other animals) is a ~ 650 base pair (bp) region in the mitochondrial cytochrome c oxidase 1 (*COI*) gene (Weigt et al. 2012b). Species identifications are made by comparison with archived sequences stored in reference databases, such as BOLD (Barcode of Life Database; Ratnasingham and Hebert 2007) using the BOLD Identification System (IDS) and GenBank (National Center for Biotechnology Information [NCBI]) using BLAST (Basic Local Alignment Sequence Tool; Altschul et al. 1990). The use of DNA barcoding to classify unidentified digested remains has increased in recent years and has successfully identified items from the gut contents of deepwater sharks (Barnett et al. 2010; Dunn et al. 2010); Laurentian Great Lake fish predators (Carreon-Martinez et al. 2011); invasive lionfish *Pterois* sp. (Valdez-Moreno et al. 2012; Cote et al. 2013; Rocha et al. 2015); North Pacific groundfish (Paquin et al. 2014); African pterygophagous (fin eating; Arroyave and Stiassny 2014) and lepidophagous (scale eating; Boileau et al. 2015) fishes; introduced Largemouth Bass *Micropterus salmoides* (Jo et al. 2014); North American warmwater catfish (Moran et al. 2015); juvenile herbivorous Sandy Spinefoot *Siganus fuscescens* (Chelsky Budarf et al. 2011); gray seals *Halichoerus grypus* and harbor porpoises *Phocoena phocoena* (Méheust et al. 2015) and stranded Humboldt squid *Dosidicus gigas* (Braid et al. 2012). However, few studies have examined the efficacy of DNA barcoding in relation to digestive state of fish prey remains (Carreon-Martinez et al. 2011; Moran et al. 2015).

Catfishes in the tidal freshwater and brackish zones of estuaries such as Chesapeake Bay provide a model system for testing the efficacy of DNA barcoding for comparing predation of sympatric native and non-native species on a diverse prey assemblage. White Catfish *Ameiurus catus* are native to Chesapeake Bay and have historically been a major component of the commercial and recreational Chesapeake Bay catfish fishery (Murdy et al. 1997). Channel Catfish *Ictalurus punctatus*, native to the Laurentian Great Lakes-St. Lawrence and Mississippi-Gulf of Mexico drainages, were widely stocked into Chesapeake Bay from the late 1890s to the early 1900s by the United States Fish Commission (USFC; Worth 1893) and Virginia Fish Commission (VCF; Jenkins and Burkhead 1993) and are now common and widespread in tidal-fresh and low salinity areas. Blue Catfish *Ictalurus furcatus*, native to Mississippi-Gulf of Mexico drainages (Ross 2001), were purposefully introduced in several Virginia tidal rivers in the mid-1970s to enhance sport and commercial fisheries (Greenlee and Lim 2011). Blue Catfish spread to other Virginia and Maryland Chesapeake Bay tributaries and raised concerns that increasing population sizes could negatively impact native fish populations (Schloesser et al. 2011). Blue catfish are North America's largest ictalurid catfish (Graham 1999) and are now established in many areas that historically have not possessed such a large year-round resident generalist predator. Previous diet (Chandler 1998; Moran et al. 2015; Robert Aguilar, unpublished; Mary Groves, unpublished) and stable isotope studies (MacAvoy et al. 2000, 2009) have suggested that Blue Catfish prey on anadromous herrings (*Alosa* spp.) and other species of management concern. Moreover, the dramatic increase in Blue Catfish abundance coincided with declines

of White Catfish in several Virginia Chesapeake Bay tributaries, indicating that blue catfish may be outcompeting their native ally (Tuckey and Fabrizio 2010; Schloesser et al. 2011). Comparative diet analyses of these three species are ongoing and were the source for prey items in the present study (Robert Aguilar, unpublished).

The use of DNA barcoding for species identification of unknown specimens is predicated on the establishment of large and robust reference databases of verified sequences. A lack of vouchered sequences (Ekrem et al. 2007; Valdez-Moreno et al. 2012; Weigt et al. 2012a) or incorrectly identified sequences will severely decrease the usefulness of reference databases. Furthermore, it is important to sequence an adequate number of individuals from across a species' range to capture possible genetic variation, including undocumented cryptic diversity (Weigt et al. 2012b). There is an ongoing effort to catalogue and sequence global biodiversity, including fishes (Ward et al. 2005, 2009; Hubert et al. 2008; Weigt et al. 2012a). As of December 2015, there were publicly available *COI-5P* sequences for nearly 12,000 fish species in BOLD (BOLD 2015). While there is generally good coverage (i.e., at least one sequence per species) of western Atlantic fishes, until recently there were very few fish barcode sequences from Chesapeake Bay or the mid-Atlantic US. In 2011, the Chesapeake Bay Barcode Initiative (CBBI) began creating complete *COI-5P* barcode libraries for fish and macro-invertebrates of Chesapeake Bay. The CBBI database that was used for identifications in the present study (which will become publicly available upon publication), also includes photographic, tissue and specimen vouchers that were deposited into the Smithsonian National Museum of Natural History fish collection, and contains more than two-thirds of Chesapeake Bay fishes to date (Robert Aguilar, unpublished). In the present study, we assessed the effectiveness of DNA barcoding in the identification of digested piscine prey remains collected from a diet analysis of sympatric native and introduced catfishes using locally caught and verified DNA sequences vouchered as part of the Chesapeake Bay Barcode Initiative.

Methods

Sampling sites and field collections

Low frequency (15 Hz) electrofishing was used to collect Blue Catfish, White Catfish and Channel Catfish from tidal freshwater areas within Maryland in Patuxent River and Marshyhope Creek during summer and fall 2012 and 2013, Sassafras River during summer and fall 2012 and Swan Creek during summer and fall 2013 (Fig. 1). After capture, all Blue Catfish and the majority of White Catfish and Channel Catfish were transported to the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland for dissection. Upon arrival, all fish were immediately measured, weighed and stomachs were removed from all Blue Catfish (n = 319) and White Catfish (n = 261) and the majority of Channel Catfish (n = 420). Stomach contents were rinsed with RO water to remove digestive enzymes, excess chyme and particulate matter through a 500 μm sieve and stored at 0 °C for up to several months. The stomach contents of 319 Blue Catfish, 261 White Catfish and 56 Channel Catfish were then thawed and all prey items were identified to the lowest taxonomic level. Seventy-six piscine prey items containing tissue (i.e., no scales, otoliths, skeletal bones, cartilage, etc.) from 58 individual catfish were selected for DNA analysis (which included 6 fish eggs) and either processed immediately or re-frozen for later analysis. An additional 79 piscine prey items obtained from 55 Blue Catfish caught in Potomac River and Swan Creek during 2012 and 2013 (Fig. 1) using similar fish capture and dissection protocols were provided by the Maryland Department of Natural Resources (MD DNR) for a total of 155 piscine prey items from 113 catfish. Each non-egg piscine prey item was scored according to degree of digestion: 1) Lightly Digested, easily identified with scales/skin mostly intact; 2) Moderately Digested, retaining most morphological characteristics, such as fins and/or skull and possibly viscera, skin/scales absent or barely present; and 3) Severely Digested, head, fins skin/scales absent, often pieces of tissue encasing spinal column or loose tissue. Each prey item represents a single individual of prey.

Prior to DNA analysis, piscine prey items were thawed (if needed) and rinsed with RO water. A small piece (~25 mm^3, but sometimes less in heavily degraded samples) of tissue was excised using sanitized forceps and a #10 scalpel upon a sanitized surface (340 mm × 240 mm plastic board). Whenever possible, interior portions (i.e., not directly exposed to digestion) of muscle tissue that did not appear degraded were preferentially selected. Tissue samples were placed in a sterile 0.75 ml micocentrifuge tube containing 150 μl of TD-M2 extraction buffer (Autogen) and frozen until DNA amplification and sequencing. As a limited control, five muscle tissue samples

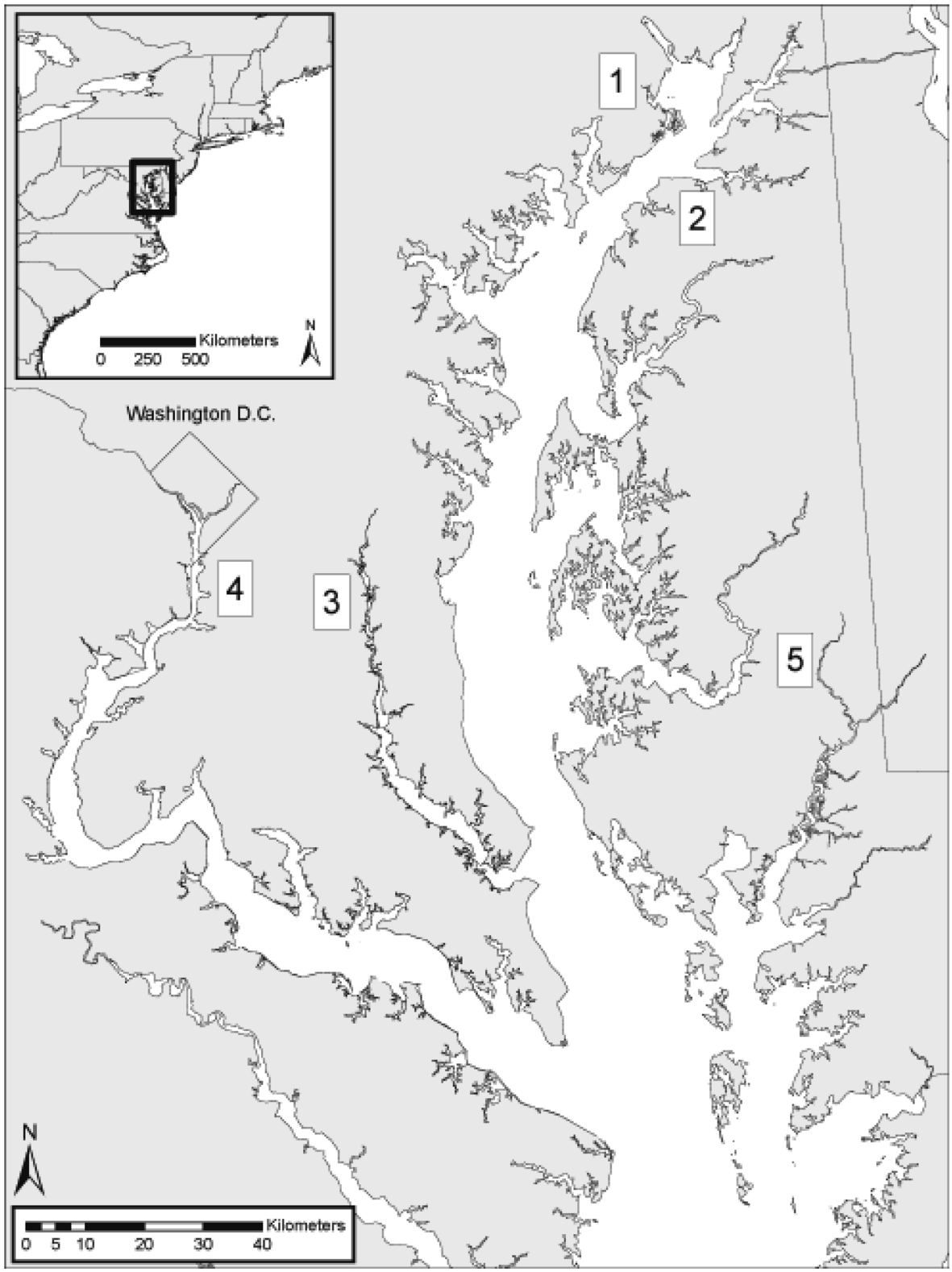

Fig. 1 Map of Chesapeake Bay indicating capture locations of catfish: 1) Swan Creek; 2) Sassafras River; 3) upper Patuxent River; 4) upper Potomac River; 5) Marshyhope Creek. Inset represents location of Chesapeake Bay along the eastern United States

of wild-caught Windowpane *Scophthalmus aquosus* (a high salinity estuarine fish unlikely to occur in tidal fresh areas) were randomly processed using the same protocols as gut content prey items.

To avoid contamination, all tools and work surfaces were sanitized prior to tissue processing by exposure to a 10 % bleach solution for at least 10 min, followed by a thorough rinsing with RO water and drying. Each prey item was handled with its own sanitized forceps and scalpel, and was processed on an unused area of the sanitized workspace. That is, each forceps and scalpel only handled a single prey item and was sanitized before any additional uses. Furthermore, after all the unused portions (the number of which varied by size of prey items) of the work surface were used or all samples on-hand were processed it was sanitized before any additional uses.

DNA amplification and sequencing

DNA was extracted using the Autogen Prep 965 phenol-chloroform automated extractor. The target 652 bp region of the 5′ end of the cytochrome oxidase subunit 1 gene (*COI*) was amplified using primers FISHCO1LBC and FISHCO1HBC (Baldwin et al. 2009). The PCR reaction mix was comprised of 2.0 mM $MgCl_2$, 0.3 μM of each primer, 0.5 mM dNTPs and 5 units of Biolase DNA polymerase (Bioline). PCR cycling conditions were as follows: 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 45 s at 72 °C. PCR products were purified with ExoSAP-IT (Affymetrix). Sequencing was performed using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems).

Electropherograms were processed using Sequencher 5.0.1 (Gene Code Corporation). Each was subjected to the same, very conservative, trimming parameters (trim until the first and last 25 bp contain fewer than three ambiguities, and trim until the first and last 10 bp contain fewer than three bases with a Phred score below 30). Only trimmed fragments greater than 500 bp in length and with overall "confidence" above 90 % (as calculated by Sequencher) were used to construct the final sequences. Passing sequences were visually examined for errors.

Data analysis

To ensure accurate species identifications, DNA sequences obtained from prey items were compared with verified CBBI sequences (as well as other achieved sequences) in BOLD (Ratnasingham and Hebert 2007). To avoid false positives, species identifications were determined with similarity percentages >99 % (Cote et al. 2013) and placement within the BIN (Barcode Index Number) system (Ratnasingham and Hebert 2013). Identifications were also assessed using BLAST (Altschul et al. 1990) based on high % Identification, % Query and Max Scores (Moran et al. 2015; Rocha et al. 2015).

Conditional exact logistic regression analysis was used to determine if digestion classification was significantly related to barcoding success (omitting fish eggs from the model). This analysis was chosen over traditional unconditional asymptotic methods because our data possessed low cell counts (i.e., few sequencing failures for lightly and moderately digested prey items; Derr 2011). The agency source of prey items (SERC and MD DNR) was originally included in the analysis, but after no significant result was found it was omitted from further analyses. The overwhelming majority of samples were obtained from Blue Catfish and samples from all species were pooled for analysis. All analyses were performed with SAS® v9.2 software (SAS Institute 2008), with an alpha level of 0.05 used for all significance testing.

Results

Most non-egg prey items were digested to the point that visual morphological identification was difficult (28.2 % moderately digested and 59.1 % severely digested, respectively). We were only able to identify 9.4 % (n = 14) of the non-egg prey items to species and an additional 12.1 % (n = 18) to family solely using morphometric analysis. Using DNA barcoding, we assigned 90.3 % (n = 140) of all prey items to species based on similarities to both verified and archived CBBI and publically available *COI-5P* sequences (Fig. 2). Thus, we were able to assign 126 more samples to species (81.2 %) by using DNA barcoding in comparison to visual analysis. In total, we identified 91.6 % (n = 142) of all prey samples to species using morphometric analysis and DNA barcoding in combination. All prey item sequences were assigned to species level (i.e., no unknown sequences or higher-level [genus, family, etc.] assignments). Furthermore, all of our visual identifications were supported by DNA barcoding, with the exception of two

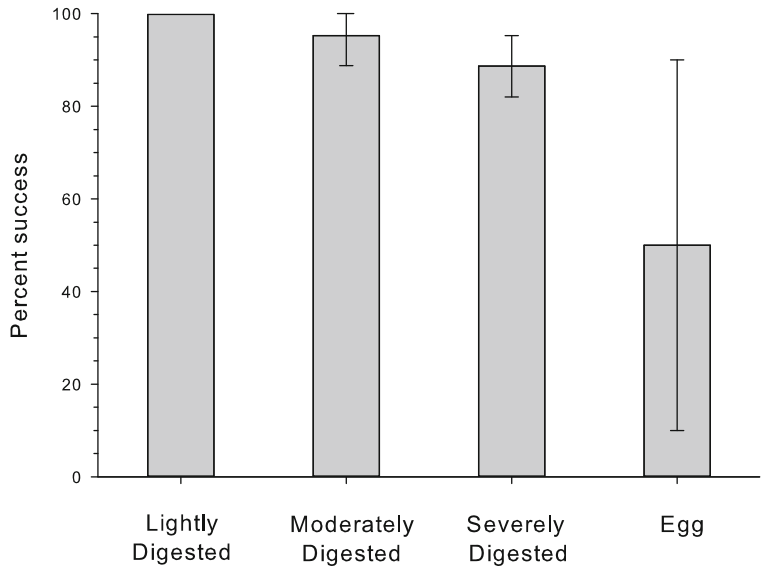

Fig. 2 Percent of prey items successfully identified with DNA barcoding by digestion classification: lightly digested (n = 19), moderately digested (n = 42), severely digested (n = 88) and egg (n = 6). Error bars represent 95 % confidence intervals. Conditional exact logistic regression analysis did not find a significant relationship between barcoding success and digestion classification. Note: Eggs were not included in logistic regression analysis

moderately digested Tessellated Darters *Etheostoma olmstedi* that failed to sequence. DNA barcoding identified a diverse array of fishes (23 species from 11 families; Table 1), which included salt-tolerant freshwater (e.g., centrarchids, cyprinids, ictalurids and Tessellated Darter), estuary-resident (*Fundulus* spp., Bay Anchovy and Hogchoker), euryhaline (Menhaden and Atlantic Croaker) and diadromous (e.g., *Alosa* spp., American Eel, Gizzard Shad and *Morone* spp.) species. Similar to Moran et al. (2015), the species of management concern and commercial interest (e.g., *Alosa* spp., Menhaden, American Eel and Striped Bass) were often heavily degraded and difficult to identify by visual examination. We also identified one bird species, Double-crested Cormorant *Phalacrocorax auritus* from a small amount of loose muscle tissue that appeared to be fish at the time of stomach dissection.

The stomachs of catfish predators with non-egg fish prey typically contained a single individual of fish prey (Table 2). However, 20.9 % percent (n = 23) of these stomachs contained multiple individuals (2 to 5), mostly of the same species. Five of these 23 stomachs contained multiple fish prey items of up to four separate species. Of the 110 catfish stomachs examined that contained non-egg fish prey, 11 contained an item that failed to sequence. The majority of these stomachs (9 of 11) only contained a single prey item, whereas one stomach contained two individuals that failed to sequence and one that successfully sequenced (all were Tessellated Darter), and the other stomach contained one individual that failed to sequence and one that successfully sequenced. Three catfish stomachs contained two fish eggs each with no other fish remains. In one instance both eggs successfully sequenced, in another, one failed and one successfully sequenced, and in the last both eggs failed.

Although sequencing success decreased with increased digestion, conditional exact logistic regression analysis found no significant relationship between barcoding outcome and digestion score (Score statistic =3.56; mid P = 0.1520). However, 83 % (n = 10) of the non-egg samples that failed to sequence were the most heavily degraded and were either extremely small amounts of skin/tissue attached to spinal segments or loose tissue.

Discussion

DNA barcoding was found to dramatically increase the proportion of piscine prey items (including severely degraded tissue) for which species identifications could be made in a comparative diet study of sympatric native and non-native catfishes. The digestion process quickly degrades morphological characteristics of prey (Schooley et al. 2008; Legler et al. 2010; Carreon-Martinez et al. 2011) and in many studies, traditional visual and morphometric analyses of digested remains have not been able to identify all fish prey items to species (Legler et al. 2010; Paquin et al. 2014; Moran et al. 2015). This can present a significant loss of information to researchers and resource managers. In this

Table 1 Number of non-egg prey Items successfully identified by DNA barcoding from the stomach contents of Blue Catfish, White Catfish and Channel Catfish caught in Chesapeake Bay, 2012–2013. GenBank accession numbers of representative reference *COI-5P* sequences generated by the CBBI are listed for each fish prey species. A publically available reference sequence was used to identify the Double-crested Cormorant. Numbers in parenthesis indicate the number of prey items that failed to sequence by catfish species. Note: Three of the 6 fish eggs were identified as Gizzard Shad

Family	Scientific name	Common name	Blue Catfish	White Catfish	Channel Catfish	GenBank Accession
Anguillidae	*Anguilla rostrata*	American Eel	2			KX459333
Engraulidae	*Anchoa mitchilli*	Bay Anchovy	5	5		KX459322
Clupeidae	*Alosa aestivalis*	Blueback Herring	2	4		KX459323
	Alosa pseudoharengus	Alewife	2			KX459332
	Alosa sapidissima	American Shad	1			KX459321
	Brevoortia tyrannus	Menhaden	14			KX459326
	Dorosoma cepedianum	Gizzard Shad	11		1	KX459339
Cyprinidae	*Carassius auratus*	Goldfish	2			KX459330
	Notropis hudsonius	Spottail Shiner	7			KX459337
Ictaluridae	*Ameiurus catus*	White Catfish		1		KX459335
	Ameiurus nebulosus	Brown Bullhead	2			KX459334
	Ictalurus furcatus	Blue Catfish	8		1	KX459336
	Ictalurus punctatus	Channel Catfish	16		1	KX459340
Fundulidae	*Fundulus diaphanus*	Banded Killifish	1			KX459324
	Fundulus heteroclitus	Mummichog			1	KX459328
Moronidae	*Morone americana*	White Perch	17		1	KX459331
	Morone saxatilis	Striped Bass	1			KX459342
Sciaenidae	*Micropogonias undulatus*	Atlantic Croaker		1		KX459327
Centrarchidae	*Lepomis gibbosus*	Pumpkinseed	3		1	KX459341
	Lepomis macrochirus	Bluegill	9			KX459329
	Micropterus salmoides	Largemouth Bass	1			KX459325
Percidae	*Etheostoma olmstedi*	Tessellated Darter	13	1		KX459343
Achiridae	*Trinectes maculatus*	Hogchoker	1			KX459338
Phalacrocoracidae	*Phalacrocorax auritus*	Double-crested Cormorant	1			DQ433077
$N = 12$	$n = 23$		119 (9)	12 (2)	6 (1)	

Table 2 Matrix indicating the number of individual prey items per stomach by prey item species richness (number of prey species per stomach). Data are for 110 catfish predators caught in Chesapeake Bay during 2012–2013 that had non-egg piscine prey in stomach contents. Note: Three catfish stomachs contained two fish eggs each (with no other fish remains)

	Number of prey species				
No. of prey individuals	1	2	3	4	5
1	87	-	-	-	-
2	1	3	-	-	-
3	5			-	-
4	2		1	1	-
5	1				

study, the overwhelming majority of samples were too digested for morphological identification to species (>87 % were either moderately or severely digested). As a result, we were only able to visually classify roughly 10 % of fish prey items to species with high confidence. DNA barcoding success was high across all levels of digestion with 90 % of samples identified to species. Without the use of DNA barcoding, nearly all of the piscine prey items would have been classified as "unidentified fish", which would have resulted in a significantly poorer understanding of catfish predator-prey relationships and the potential impacts of non-native catfishes on Chesapeake Bay food webs.

The broad coverage of the CBBI and other DNA barcode databases insured that there were no ambiguous prey item sequences in this study. That is, all prey items that successfully sequenced were classified to the species level based on high similarities to reference DNA barcodes. The classification success was likely aided by the use of CBBI reference sequences, which were obtained from morphologically verified (and vouchered) fishes caught in Chesapeake Bay. This is important because the ability to identify unknown samples can be hampered by cryptic diversity, haplotype sharing, hybridization, misidentification of reference specimens and lack of matching sequences in reference databases. April et al. (2011) reported that North American freshwater fishes exhibit a high amount of cryptic diversity, largely found within Cyprinidae and Percidae, but also found in possible Chesapeake Bay catfish prey species within Catostomidae, Centrarchidae, Fundulidae, Petromyzontidae, Ictaluridae, Esocidae and Aphredoderidae. Dissimilar lineages (>2 % difference) can also be seen among several euryhaline species with large geographic ranges, such as Menhaden *Brevoortia tyrannus* and Striped Mullet *Mugil cephalus* (BOLD 2015; Robert Aguilar, unpublished). Prior to our CBBI fish collections there were no available *COI-5P* sequences for Hickory Shad *Alosa mediocris* and Rough Silverside *Membras martinica* in BOLD or GenBank. Although neither species was detected in this study, both species are potential prey for catfish in Chesapeake Bay (Moran et al. 2015; Robert Aguilar, personal observation) and if they had been present in our samples it would have been difficult to identify them genetically. Given the large number of available *Alosa* sequences, Hickory Shad could have been assigned to genus with confidence, but Rough Silverside would likely only have been assigned to family because of the lack of any *Membras* spp. sequences (besides those generated by the CBBI) in BOLD or GenBank at the time of analysis. This further demonstrates the need for robust reference barcode databases and continued collections for this effort. This is especially true for benthic invertebrates where there is both much greater biodiversity and substantial gaps in availability of DNA barcode sequences even for many common taxa. To date, the CBBI has uploaded a large number of invertebrate and fish *COI-5P* (and some 16sRNA) sequences for species not previously in BOLD or GenBank, which have been used to identify an invasive cymothoid isopod in Egyptian waters (K. Mohammed-Geba, Menoufia University, unpublished), an unknown caridean shrimp in Chesapeake Bay (Robert Aguilar, unpublished) and Hickory Shad from the stomachs of warm water catfish (Moran et al. 2015). CBBI sequencing efforts are continuing and trophic analyses incorporating CBBI invertebrate barcode sequences are currently underway. Continued development of the CBBI and other barcode databases will ultimately allow for much more detailed diet studies using metabarcoding of complete stomach content samples and other next generation sequencing techniques (Pompanon et al. 2012; de Barba et al. 2014; Berry et al. 2015; Leray et al. 2015).

A detailed comparative diet analysis of Chesapeake Bay catfishes is forthcoming (Robert Aguilar, unpublished), but we can report that catfishes examined in the present study preyed upon a wide variety of fishes (23 species from 11 families), including many commercially and ecologically important species and species of management concern, most notably anadromous herrings (*Alosa* spp.). Along the US Atlantic coast there has been a concerted effort to recover populations of alosines by removing migration barriers (dams, culverts,

etc.) and decreasing fishing mortality (directed and by-catch); however, substantive predation pressure by invasive Blue Catfish could negatively impact the recovery of anadromous alosine populations in Chesapeake Bay tributaries (Schloesser et al. 2011). Furthermore, Blue Catfish have the potential to affect resident fish assemblages, mediated by top-down predatory impacts or interspecific completion among other top-level predators. Blue Catfish are markedly larger than other Chesapeake Bay catfishes and resident predators (Murdy et al. 1997), exceeding 45 kg and 165 cm (Graham 1999), and exhibit a shift to piscivory with increasing size (Schloesser et al. 2011). Interestingly, one of the most numerous prey items of Blue Catfish was Tessellated Darter, a small fish typically associated with running streams and shallow water (Murdy et al. 1997). Other common prey items included soft-bodied, filter feeding fishes in Clupeiformes (Clupeidae and Engraulidae), and White Perch *Morone americana*, centrarchids and Channel Catfish. Although we cannot be completely certain whether the fish prey contained in catfish stomachs resulted from active feeding (i.e., taking live prey), scavenging or even secondary predation (i.e., derived from the stomach contents of prey items), these data suggest that Blue Catfish (and to some extent White Catfish and Channel Catfish) may be feeding at a range of depths and habitats, including shallow margins where smaller fish often seek refuge and in open waters. Nearly all the prey items identified as Channel Catfish were recently hatched yolk-sac larvae ingested by Blue Catfish, indicating Blue Catfish may raid the guarded nests of Channel Catfish. Although universal fish primers were used on suspected fish tissue, we recovered one sequence that matched closely with a bird, Double-crested Cormorant *Phalacrocorax auritus*. This sample consisted of a small amount of loose muscle tissue (which was assumed to be fish at time of dissection) obtained from the stomach of a 666 mm total length Blue Catfish that contained no other bird remains. Thus, we suspect this prey item resulted from scavenging. Blue Catfish are opportunistic feeders and are known to scavenge avian and terrestrial derived carcasses (Eggleton and Schramm 2004) or target wounded or injured fish (Graham 1999). Moreover, a number of Blue Catfish stomachs examined in our broader Chesapeake Bay catfish diet surveys contained scales of fish much too large to be ingested whole, further suggesting the importance of scavenging in catfish diets (Robert Aguilar, unpublished).

This work demonstrated the ability to successfully sequence multiple prey items from the same predator and identified potential cannibalism in all three species. We detected up to four distinct prey species and five separate individuals in a single catfish stomach. Furthermore, the majority of sequence failures in this study occurred from stomachs that did not contain multiple fish prey items. Co-amplification of DNA among prey items, between prey items and predator or from non-target DNA contamination can hinder sequencing success of prey items (Vestheim and Jarman 2008; Leray et al. 2015). In laboratory experiments, Carreon-Martinez et al. (2011) successfully sequenced fish prey items of multiple species from the stomachs of centrarchids that were force-fed known fish prey (two to three different species at time). Moreover, no predator DNA was detected in any of these feeding trials (n = 127). In the present study, we classified a small number of prey items to the same species as predators for all three catfishes. While it is possible this resulted from predator DNA contamination, it likely reflects true cannibalism in Blue Catfish, White Catfish and Channel Catfish. Although contamination may have contributed to sequencing failures, it did not appear to result in false detections. All of the prey items with species level identifications matched with their corresponding DNA barcodes and all five of the Windowpane control samples successfully sequenced in the proper order.

DNA barcoding was extremely successful in identifying fish prey items regardless of degree of digestion. In the present study and other studies of fish predator-prey relationships (Carreon-Martinez et al. 2011; Schloesser et al. 2011; Moran et al. 2015; Rocha et al. 2015), a large portion of the fish prey items were digested beyond the point at which morphological characteristics could reliably be used for species identification. Although there may have been differences in the digestion rank classifications, the barcoding success rates of fish prey items presented in this study were higher than those reported by Carreon-Martinez et al. (2011; ~60–80 % at 1–4 h digestion time and 20 °C); Cote et al. (2013; 70 %) and Moran et al. (2015; 65 %–86 %), particularly for moderately and severely digested samples. These differences could be attributed to inherent properties of predator and/or prey species contained within each study, predator handing/stomach content acquisition, prey item handling or genetic sequencing techniques. Universal fish primers are generally robust and techniques for the DNA barcoding of fresh fish

tissue are well established and highly successful (Ivanova et al. 2007; Weigt et al. 2012a). In general, barcoding success may be most affected by prey item acquisition and laboratory hygiene protocols when accounting for degree of digestion. Valdez-Moreno et al. (2012) noted differences in barcoding success between freshly processed samples and those where whole fish predators were stored in ethanol prior to stomach dissection. In this study, we restricted genetic analyses to samples containing soft tissues (preferentially muscle). However, there appears to be markedly lower DNA barcoding success rates for extremely degraded tissues or in samples mostly consisting of chyme, particulates, bones, etc. (Carreon-Martinez et al. 2011; Robert Aguilar, personal observation). In future gut content DNA barcoding studies, we recommend: 1) acquiring stomach/gut contents as soon as possible and removing any digestive enzymes and chyme; 2) using sanitized tools and work surfaces for each prey item to avoid contamination; 3) preferentially selecting muscle tissue or the least degraded tissue as possible; and 4) conducting genetic analyses on fresh tissue. If genetic analysis of fresh tissues is impractical, prey items should be frozen instead of stored in ethanol, which typically leads to a higher probability of barcoding success in wild-caught fishes (Amy Driskell, personal observation). Moran et al. (2015) also highlight the need for a controlled study assessing the effect of digestion time on catfish prey in relation to environmental conditions and DNA barcoding success, which would reduce uncertainty in the interpretation of DNA barcode diet studies. Additionally, studies evaluating the feasibly of sequencing partly digested fish eggs and investigating methodologies to increase barcoding efficacy of extremely degraded remains are warranted.

In conclusion, we found DNA barcoding highly successful at identifying all but the most heavily degraded prey items and to be an efficient and effective method to strengthen the resolution of trophic analyses in fishes. Our prey item acquisition/handling and laboratory hygiene protocols can provide a framework for future gut content DNA barcoding studies. These data (along with the associated full diet analysis) will increase our understanding of catfish trophic dynamics in Chesapeake Bay and aid in developing management strategies, particularly as it relates to predation of anadromous alosines and competitive interactions between native and non-native catfishes. The CBBI will continue to collect and sequence fish and macro-invertebrates of Chesapeake Bay. To date, we have sequenced over two-thirds of Chesapeake Bay fishes and achieved roughly 80 % coverage for several important invertebrate taxa (e.g., decapods and bivalves; Robert Aguilar, unpublished). The completeness of reference barcode databases becomes increasingly important as metabarcoding (e.g., whole stomach analyses) and eDNA studies become less costly and more prevalent (Leray et al. 2013; Miya et al. 2015).

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

April J, Mayden RL, Hanner RH, Bernatchez L (2011) Genetic calibration of species diversity among North America's freshwater fishes. Proc Natl Acad Sci U S A 108(26): 10602–10607

Arroyave J, Stiassny MLJ (2014) DNA barcoding reveals novel insights into pterygophagy and prey selection in distichodontid fishes (Characiformes: Distichodontidae. Ecol Evol 23(4):4534–4542

Baeza JA, Fuentes MS (2013) Exploring phylogenetic informativeness and nuclear copies of mitochondrial DNA (numts) in three commonly used mitochondrial genes: mitochondrial phylogeny of peppermint, cleaner, and semi-terrestrial shrimps (Caridea: *Lysmata*, *Exhippolysmata*, and *Merguia*). Zoo J Linn Soc-Lond 168:699–722

Baldwin CC, Mounts JH, Smith DG, Weigt LA (2009) Genetic identification and color descriptions of early life-history stages of Belizean *Phaeoptyx* and *Astrapogon* (Teleostei: Apogonidae) with comments on identification of adult *Phaeoptyx*. Zootaxa 2008:1–22

Baldwin CC, Brito BJ, Smith DG, Weigt LA, Escobar-Briones E (2011) Identification of early life-history stages of Caribbean *Apogon* (Perciformes: Apogonidae) through DNA barcoding. Zootaxa 3133:1–36

Bariche M, Torres M, Smith C, Sayar N, Azzurro E, Baker R, Bernardi G (2015) Red Sea fishes in the Mediterranean Sea: a preliminary investigation of a biological invasion using DNA barcoding. J Biogeogr 42:2363–2373

Barnett A, Redd KS, Frusher SD, Stevens JD, Semmens JM (2010) Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. J Exp Mar Biol Ecol 393: 188–192

Beauchamp DA, Wahl DH, Johnson BM (2007) Predator-prey interactions. In: Guy CD, Brown ML (eds) Analysis and interpretation of freshwater fisheries data. American Fisheries Society, Bethesda, Maryland, pp. 765–842

Berry O, Bulman C, Bunce M, Coghlan M, Murray DC, Ward RD (2015) Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. Mar Ecol Prog Ser 540:167–181

Betancur-R R, Broughton RE, Wiley EO, Carpenter K, López JA, Li C, Holcroft NI, Arcila D, Sanciangco M, Cureton II JC, Zhang F, Buser T, Campbell MA, Ballesteros JA, Roa-Varon A, Willis S, Borden WC, Rowley T, Reneau PC, Hough DJ, Lu G, Grande T, Arratia G, Ortí G (2013) The Tree of Life and a New Classification of Bony Fishes. PLoS Curr Tree of Life. Apr 18. Edition 1. doi:10.1371/currents.tol.53ba26640df0ccaee75bb165c8c26288.

Boileau N, Cortesi F, Egger B, Muschick M, Indermaur A, Theis A, Büscher HH, Salzburger W (2015) A complex mode of aggressive mimicry in a scale-eating cichlid fish. Biol Lett 11(9):20150521

BOLD (2015) Barcode of Life Database (BOLD). Available: www. Boldsystems.org/. Accessed 15 December 2015

Braid HE, Deeds J, DeGrasse SL, Wilson JJ, Osborne J, Hanner RH (2012) Preying on commercial fisheries and accumulating paralytic shellfish toxins: a dietary analysis of invasive *Dosidicus gigas* (Cephalopoda Ommastrephidae) stranded in Pacific Canada. Mar Biol 159:25–31

Carreon-Martinez L, Johnson TB, Ludsin SA, Heath DD (2011) Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. J Fish Biol 78:1170–1182

Chandler L (1998) Trophic ecology of native and introduced catfishes in the tidal James River. Virginia Commonwealth University, Richmond Virginia. Master's Thesis, 116p

Charles H, Dukes JS (2007) Impacts of invasive species on ecosystems services. *In:* W. Nentwig (ed.) biological invasions. Ecological Studies 193. Springer, Berlin, Germany. pp. 217–237

Chelsky Budarf A, Burfeind DD, Loh WK, Tibbetts IR (2011) Identification of seagrasses in the gut of a marine herbivorous fish using DNA barcoding and visual inspection. J Fish Biol 79:112–121

Conway KW, Baldwin C, White MD (2014) Cryptic diversity and venom glands in western Atlantic clingfishes of the genus *Acyrtus* (Teleostei: Gobiesocidae). PLoS One 9(5): e97664

Cote IM, Green SJ, Morris JA, Akins JL, Steinke D (2013) Diet richness of indo-Pacific lionfish revealed by DNA barcoding. Mar Ecol Prog Ser 472:249–256

Dalton DI, Kotze A (2011) DNA barcoding as a tool for species identification in three forensic wildlife cases in South Africa. Forensic Sci Int 207:e51–e254

de Barba M, Miquel C, Boyer F, Mercier C, Rioux D, Coissac E, Taberlet P (2014) DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet. Mol Ecol Resour 14:306–323

Derr RE (2011) Performing exact regression with the SAS system. *In*: Proceedings of the 25th Annual SAS Users Group International Conference, April 2000, Cary, NC, USA Paper P254–P225

Dunn MR, Szabo A, McVeagh MS, Smith PJ (2010) The diet of deep water sharks and the benefits of using DNA identification of prey. Deep-Sea Res 57:923–930

Eggleton MA, Schramm HL (2004) Feeding ecology and energetic relationships with habitat of blue catfish *Ictalurus furcatus* and flathead catfish *Pylodictis olivaris* in the lower Mississippi River, USA. Environ Biol Fish 70:107–121

Ekrem T, Willassen E, Stur E (2007) A comprehensive DNA sequence library is essential for identification with DNA barcodes. Mol Phylogenet Evol 43:530–542

Fritts TH, Rodda GH (1998) The role of introduced species in the degradation of island ecosystems: A case history of Guam. Annu Rev Ecol Syst 29:113–140

Gonçalves PFM, Oliveira-Marques AR, Matsumoto TE, Miyaki CY (2015) DNA Barcoding identifies illegal parrot trade. J Hered 106(S1):560–564

Graham K (1999) A review of the biology and management of blue catfish. *In:* Irwin ER, Hubert WA, Rabeni CF, Schramm HL, Coon T (ed.) Catfish 2000: Proceedings of the International Ictalurid Symposium Amer Fish Soc Symp 24 Bethesda, MD pp. 37–49

Greenlee RS, Lim CN (2011) Searching for equilibrium: Population parameters and variable recruitment in introduced Blue Catfish populations in four Virginia tidal river systems. *In:* Michaletz PH, Travnichek VH (ed.) Conservation, Ecology, and Management of Catfish: The Second International Symposium American Fisheries Society, Symposium 77, Bethesda, MD pp. 349–367

Handy SM, Deeds JR, Ivanova NV, Hebert PDN, Hanner RH, Ormos A, Weigt LA, Moore MM, Yancy HF (2011) A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. J AOAC Int 94(1):201–210

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. P R Soc London 270: 313–321

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical kipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci U S A 101(41):14812–14817

Hubert N, Hanner R, Holm E, Mandrak NE Taylor E (2008) Identifying Canadian freshwater fishes through DNA barcodes. PLoS One 3(6): e2490

Hyslop EJ (1980) Stomach contents analysis - a review of methods and their application. J Fish Biol 17:411–429

Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. Mol Ecol Resour 7(4):544–548

Jenkins RE, Burkhead NM (1993) Freshwater fishes of Virginia. American Fisheries Society, Bethesda, Maryland

Jo H, Gim JA, Jeong KS, Kim HS, Joo GJ (2014) Application of DNA barcoding for identification of freshwater carnivorous fish diets: is number of prey items dependent on size class for *Micropterus salmoides*? Ecol Evol 4:219–229

Joly S, Davies TJ, Bruneau A, Derry A, Kembel SW, Peres-Neto P, Vamosi J, Wheeler TA (2014) Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. Mol Ecol Resour 14(2):221–232

Kress WJ, García-Robledo C, Uriarte M, Erickson DL (2015) DNA barcodes for ecology, evolution and conservation. Trends Ecol Evol 30(1):25–35

Legler ND, Johnson TB, Heath DD, Ludsin S (2010) Water temperature and prey size effects on the rate of digestion of larval and early juvenile fish. Trans Am Fish Soc 139:868–875

Leray M, Knowlton N (2015) DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proc Natl Acad Sci U S A 112(7):2076–2081

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ (2013) A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool 10:34

Leray M, Meyer CP, Mills SC (2015) Metabarcoding dietary analysis of coral dwelling predatory fish demonstrates the minor contribution of coral mutualists to their highly partitioned, generalist diet. PeerJ 3: e1047

MacAvoy SE, Macko SA, McIninch SP, Garman GC (2000) Marine nutrient contributions to freshwater apex predators. Oecologia 122:568–573

MacAvoy SE, Garman GC, Macko SA (2009) Anadromous fish as marine nutrient vectors. Fish B-NOAA 107:165–174

Méheust E, Alfonsi E, Le Ménec P, Hassani S, Jung J (2015) DNA barcoding for the identification of soft remains of prey in the stomach contents of grey seals (*Halichoerus grypus*) and harbour porpoises (*Phocoena phocoena*. Mar Biol Res 11(4):385–395

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R Soc Open Sci 2: 150088

Moran Z, Orth DJ, Schmitt JD, Hallerman EM, Aguilar R (2015) Effectiveness of DNA barcoding for identifying piscine prey items in stomach contents of piscivorous catfishes. Environ Biol Fish 99(1):161–167

Murdy EO, Birdsong RS, Musick JA (1997) Fishes of Chesapeake Bay. Smithsonian Institution Press, Washington D. C

Nagy ZT, Sonet G, Glaw F, Vences M (2012) First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. PLoS One 7(3): e34506

Paquin MM, Buckley TW, Hibpshman RE, Canino MF (2014) DNA-based identification methods of prey fish from stomach contents of 12 species of eastern North Pacific groundfish. Deep-Sea Res 85:110–117

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Mol Ecol 21:1931–1950

Ratnasingham S, Hebert PDN (2007) BOLD: the barcode of life data system (www.Barcodinglife.Org). Mol Ecol Notes 7: 355–364

Ratnasingham S, Hebert PDN (2013) A DNA-based registry for all animal species: the barcode index number (BIN) system. PLoS One 8(8): e66213

Rocha LA, Rocha CR, Baldwin CC, Weigt LA (2015) Invasive lionfish preying on critically endangered reef fish. Coral Reefs 34:803–806

Ross ST (2001) Inland Fishes of Mississippi. University Press of Mississippi, Jackson, MS. 624 pp.

Schloesser RW, Fabrizio MC, Latour RJ, Garman GC, Greenlee B, Groves M, Gartland J (2011) Ecological role of blue catfish in Chesapeake Bay communities and implications for management. *In:* Michaletz PH, Travnichek VH (ed.) conservation, ecology, and Management of Catfish: the second international symposium. Am Fish Soc Symp 77, Bethesda, Maryland. pp. 369–382

Schooley JD, Karam AP, Kresner BR, Marsh PC, Pacey CA, Thornbrugh DJ (2008) Detection of larval remains after consumption by fishes. Trans Am Fish Soc 137:1044–1049

Tuckey TD, Fabrizio MC (2010) Estimating relative juvenile abundance of ecologically important finfish in the Virginia portion of Chesapeake Bay. Project #F-104-R-14. Annual Report to Virginia Marine Resources Commission. Virginia Institute of Marine Science, Gloucester Point.

Valdez-Moreno M, Quintal-Lizama C, Gómez-Lozano R, García-Rivas MC (2012) Monitoring an alien invasion: DNA barcoding and the identification of lionfish and their prey on coral reefs of the Mexican Caribbean. PLoS One 7(6): e36636

Vestheim H, Jarman SN (2008) Blocking primers to enhance PCR amplification of rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs. Front Zool 5:12

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. Philos Trans R Soc Lond Ser B Biol Sci 360(1462):1847–1857

Ward RD, Hanner R, Hebert PDN (2009) The campaign to DNA barcode all fishes FISH-BOL. J Fish Biol 74:329–356

Weigt LA, Baldwin CC, Driskell A, Smith DG, Ormos A, Reyier EA (2012a) Using DNA barcoding to assess Caribbean reef fish biodiversity: expanding taxonomic and geographic coverage. PLoS One 7(7): e41059

Weigt LA, Driskell AC, Baldwin CC, Ormos A (2012b) DNA barcoding fishes. In: Lopez Erickson DL (ed) DNA barcodes: Methods and Protocols. Humana Press, New York City, NY, pp. 109–126

Wirta HK, Hebert PDN, Kaartinena R, Prosser SW, Várkonyi G, Roslin T (2014) Complementary molecular information changes our perception of food web structure. Proc Natl Acad Sci U S A 111(5):1885–1890

Worth S (1893) Report on the propagation and distribution of food-fishes. Rep U S Commissioner Fish Fish 1893:78–138