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A genetic technique to identify the diet of cownose rays, *Rhinoptera bonasus*: analysis of shellfish prey items from North Carolina and Virginia

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Abstract Cownose rays are implicated in the consumption of commercially important shellfish on the U.S. East Coast. We tested this assumption by developing a molecular technique for species identification from cownose ray gut contents. Digestive tracts sampled from 33 rays in Pamlico Sound, NC and Chesapeake Bay, VA contained pieces of partially-digested tissue, well-digested tissue, fluid, and minute shell fragments which made visual identification to the species level nearly impossible. We sequenced the cytochrome oxidase subunit I (COI) for seven locally acquired bivalve species, chosen for their commercial and ecological importance in NC and VA. Sequences were used to design species-specific primers for each bivalve species to amplify polymerase chain reaction (PCR) products. We designed primers such that PCR products were sufficiently different in size to be distinguishable from one another when resolved on an agarose gel, and multiplexing of several species in one reaction was possible. Digestive tract sample testing revealed that cownose rays in Chesapeake Bay ate stout tagelus and soft shell clams. There was no evidence of the rays in the study consuming commercially important oysters, hard clams, and bay scallops. Further sampling over an extended period of time and additional locations is required to confirm these results. Our diagnostic tests could easily be expanded to elucidate the impact of cownose ray predation on prey populations.

Keywords Elasmobranch · Diet · Feeding ecology · Molecular techniques · COI

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Introduction

Cownose rays, *Rhinoptera bonasus*, are cartilaginous fishes related to manta rays, eagle rays, and other species of cownose rays. They range from southern New England to South America (Brazil) in the Atlantic and throughout the Gulf of Mexico and Cuba (Bigelow and Schroeder 2002). Cownose rays undertake cyclic migrations; the migratory groups have been estimated between 10,000 and 5,000,000 individuals (Smith and Merriner 1985; Blaylock 1989). The Atlantic population migrates from the Southeast to the North in the spring and early summer, and then returns from the Northeast southwards towards Florida in the fall (Smith and Merriner 1986; Craig et al. 2010; Bade 2013). They are found in North Carolina waters during the spring and summer, when they migrate through in large schools and also use the estuaries of North Carolina for feeding on mollusks and crustaceans (Peterson et al. 2001;

Goodman et al. 2010). Chesapeake Bay is a historical summer residence for cownose rays (Blaylock 1993). Cownose rays are known to use Chesapeake Bay for pupping and reproductive efforts (Smith and Merriner 1986, 1987; Fisher 2010); recent evidence indicates that there may also be a semi-permanent population in North Carolina or that the estuaries of North Carolina are used as a nursery area for juveniles (Smith and Merriner 1987; Goodman et al. 2010) (personal observation).

Cownose rays are considered a nuisance species in North Carolina and Chesapeake Bay, because of their potential to consume commercially important shellfish species, with subsequent calls from fishermen and aquaculturists for a directed fishery on cownose rays to cull the population. As durophagous rays, they crush the shell and hard parts of the prey they consume (Smith and Merriner 1985; Fisher et al. 2011). Cownose rays primarily eat shellfish and crustaceans, which they excavate from infaunal and epifaunal benthic habitats. In Chesapeake Bay, primary prey species are thought to be oysters (*Crassostrea virginica*) and hard clam (*Mercenaria mercenaria*) (Smith and Merriner 1985), although a small study found soft shell clams (*Mya arenaria*) to be the dominant prey item in seagrass beds (Orth 1975). Fisher (2010) found primary prey species to be thin-shelled bivalves like the soft shell, macoma (*Macoma balthica*), and stout tagelus clams (*Tagelus plebeius*), along with crustaceans (crabs, shrimp, and worms). In North Carolina, razor clams, oysters, and unidentified mollusks have been described as traditional sources of prey (Smith and Merriner 1985); a key food source for cownose rays during migration has been Atlantic bay scallops (*Argopecten irradians concentricus*) (Peterson et al. 2001).

Because of the crushing action of their jaws, contents found in the digestive tract are predominantly pieces of tissue, shell fragments, and well-digested tissue (chyme). This has made species-specific identification of stomach contents using traditional visual methods very difficult; previous diet studies on cownose rays were able to identify prey to the order, family, or genus level but still found high unidentified quantities of tissue and chyme (Collins et al. 2008; Craig et al. 2010; Fisher 2010; Ajemian and Powers 2011). Studies in Chesapeake Bay and the Gulf of Mexico found that anywhere between 20 and 70 % of prey items were unidentifiable (% Frequency of Occurrence or % Index of Importance), depending upon prey type and due to level of mastication and/or stage of digestion (Collins et al. 2007; Fisher 2010; Ajemian and Powers 2011). Another study in the Gulf of Mexico found that up to 80 % of the stomach and spiral valve contents by weight consisted of unidentifiable matter (Craig et al. 2010). Species-level identification of the contents of digestive tracts of cownose rays have been possible for some prey types, but the majority of findings is at a higher classification level or remains unknown.

To address this problem, we used direct sequencing techniques to develop species-specific polymerase chain reaction (PCR)-based diagnostic tests to identify prey tissue and chyme from the stomachs and spiral valves of cownose rays. The cytochrome oxidase subunit I (COI) gene was used as the basis of these tests. The COI gene is one of the 13 protein-coding genes in animal mitochondrial DNA, which is responsible for respiration processes (Saccone et al. 1999). The mitochondrial genome is useful for species-level identification because of the lack of introns in the sequence, the limited opportunities for recombination and subsequent mutations, simplistic replication processes, and the uniparental mode of inheritance (Saccone et al. 1999). The COI is described as the "barcoding gene" because of its usefulness in species identification (Hebert et al. 2003). COI sequences have been found to be highly conserved within species but sufficiently different between species, even closely related species, to allow for species-level identification based on the COI sequence (Hebert et al. 2003). The Barcode of Life Data System (BOLD) is a barcode reference library for collection of COI sequences (Ratnasingham and Hebert 2007), and this has also made conservation of genetic biodiversity, as well as species identification, more readily accessible.

Genetic analysis, including the use of the COI gene, has been used to identify unknown prey types in diet studies. The problem of unidentified or uncertain prey identification in diet studies of fishes is well known, and one source of error in food web analyses. The COI gene was amplified from unknown tissue from stomachs of broadnose sevengill (*Notorynchus cepedianus*) sharks and compared to a reference library for species-specific identification (Barnett et al. 2010). In a study of multiple species of deep water sharks in New Zealand, both a traditional stomach content analysis based on visual and microscopic identification of tissues and identification by COI gene sequence was applied to identify prey in the diets of these rare species (Dunn et al. 2010). Other genetic methods and techniques have been applied to analyze diet contents. In one example, next-generation,

high-throughput sequencing of the DNA in Australian fur seal (*Arctocephalus pusillus doriferus*) faeces was completed and utilized to identify prey (Deagle et al. 2009). These studies required identification of unknown prey by matching sequences to a library or database; we were interested in developing PCR tests to identify the presence or absence of specific prey items.

The goal of the project was to better understand the ecological role and trophic impact of cownose rays in North Carolina and Virginia by collecting dietary consumption data in North Carolina waters and Chesapeake Bay. Specific objectives included 1) the analysis of digestive tract contents to identify which shellfish species were consumed using both visual identification and genetic techniques as a function of cownose ray size and capture location; 2) sequencing the COI gene from each of seven commercially and ecologically important target bivalve species (Table 1) and from cownose rays to design species-specific PCR primers; and 3) the development of a multiplex protocol for PCR-based molecular diagnostic tests to be applied to digestive tract samples from cownose rays. The purpose of these genetic tests is to assess whether cownose rays are eating shellfish of commercial value in North Carolina and Chesapeake Bay, as well as provide information about cownose ray feeding ecology. This methodology will provide a useful genetic test for identification of partially digested prey tissue in digestive tract samples, with application to stomach analyses, predator–prey relationships, and food web studies within the mid-Atlantic range of the cownose ray and their potential bivalve prey.

Methods

Collection of animals

Cownose rays were captured and collected in waters of North Carolina and Chesapeake Bay (Fig. 1). Capture occurred by commercial fishing, recreational fishing, and scientific collection techniques (bowfishing, haul seine, gill net, cast net, longline, or rod and reel). After collection, individuals were sexed, weighed, measured, and tissue samples taken. Measurements included disc width, disc length or standard length, and total length. All animal collection and handling procedures were in accordance with Animal Use Protocol #D268, approved by the East Carolina University Animal Use and Care Committee. Collections were predominantly opportunistic, and so a goal was set of a minimum of 30 individual cownose rays to be used for the development of the multiplex PCR procedure and diet analyses. Thirty-three cownose rays were collected, sacrificed, and dissected from North Carolina (n=8) and Chesapeake Bay (n=25). Cownose rays in the two size classes of juvenile (20–74 cm) and mature (75–120 cm) were

Table 1 Target bivalve species in this study, each reportedly eaten by cownose rays. Data reported are from NMFS, NCDMF, and VAMRC

Common name	Scientific name (abbreviation used)	Commercial importance	Habitat
Atlantic bay scallop	*Argopecten irradians concentricus* (*Aic*)	Yes, US fishery $1,957,430 in 2011 NC landings $1,107,072 in 1980, fishery closed in NC and VA 2011. Limited reopening 2013.	High-salinity seagrass areas
Baltic macoma	*Macoma balthica* (*Mba*)	None, but ecologically important	Low-salinity unvegetated areas
Cross-barred venus	*Chione cancellata* (*Cca*)	None, but ecologically important	High-salinity seagrass areas
Eastern oyster	*Crassostrea virginica* (*Cvi*)	Yes, US fishery $90,563,881 NC fishery $4,486,236 in 2011, VA fishery $6,253,606 in 2011	High-salinity reef building species
Hard clam	*Mercenaria mercenaria* (*Mme*)	Yes, US fishery $3,643,094 NC fishery $1,895,345 in 2011; VA fishery $184,706 in 2006; aquaculture species	High-salinity seagrass areas
Soft-shell clam	*Mya arenaria* (*Mar*)	Yes, US fishery $21 million VA fishery $86,715 in1966, none landed in NC in 2011	High and low salinity unvegetated and seagrass areas
Stout tagelus clam	*Tagelus plebius* (*Tpl*)	Undocumented, human consumption reported	High-salinity unvegetated and seagrass areas

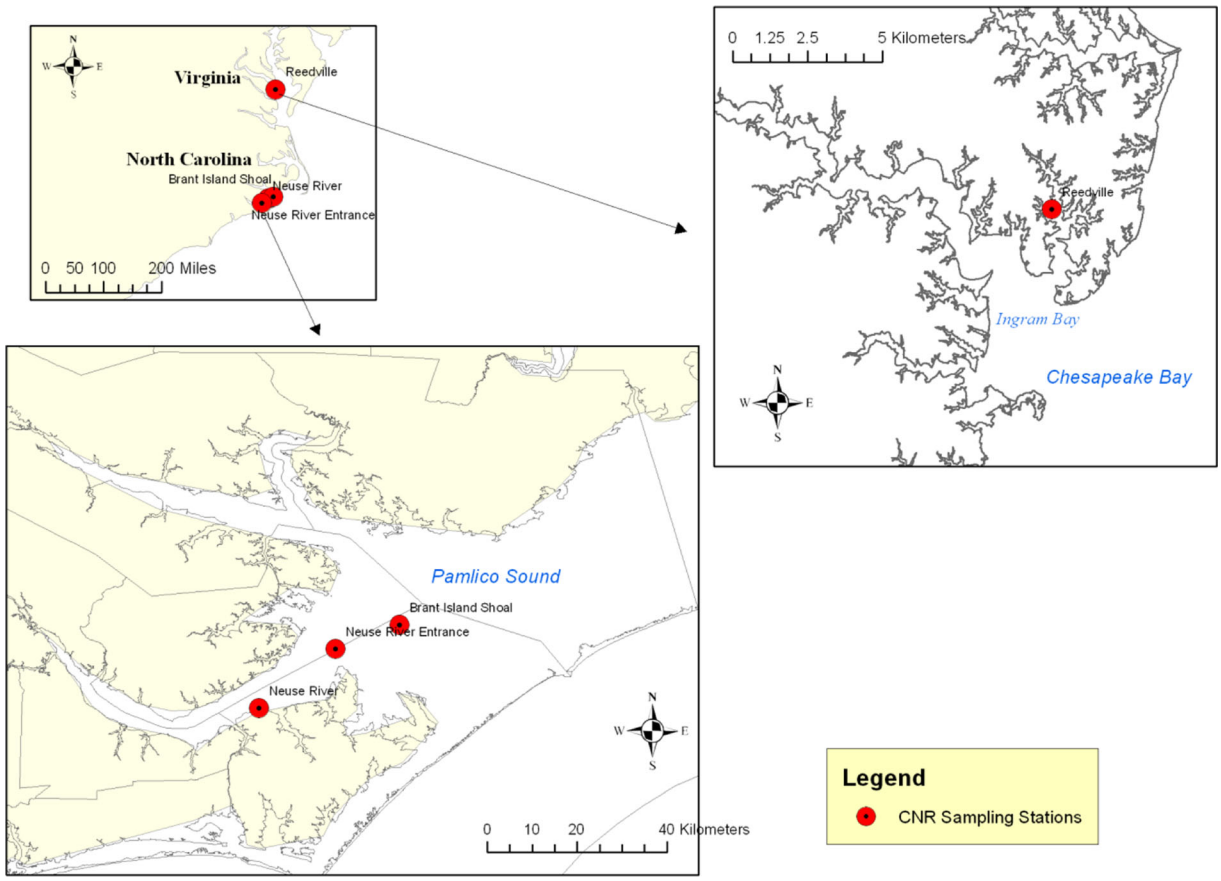

Fig. 1 Capture locations of cownose rays in North Carolina and Virginia. All cownose rays captured in North Carolina were caught in the Neuse River area. The majority of the rays from Chesapeake Bay were caught at a bowfishing tournament, which occurred in the waters around Reedville, Virginia (inset, above right)

represented. The entire digestive tract was removed, bagged, and frozen.

Digestive tract analysis methods

Digestive tracts (stomachs and spiral valves) were stored frozen at −20 °C until the contents were analyzed, at which time they were allowed to thaw overnight in ice or in cold water for 2–3 h. The stomach and spiral valve were analyzed separately, and overall stomach and spiral valve content weights were taken. Any shell fragments, exoskeleton parts, fish bones, and scales were separated from the rest of the contents and stored in formalin for identification and proportion analysis. Solid tissue fragments found in the contents were visually categorized and weights were taken for each type of tissue or content. Contents were divided into general categories of tissue type, detritus/organic matter, hard shell parts, hard fish parts, and unidentified tissue, chyme, and fluid. Subsamples of unidentified prey tissue (approximately 5 mm by 5 mm or smaller) were removed from the larger sample for DNA extraction. Samples of fluid and chyme were also collected for genetic analysis. Depending upon the contents and fullness found in the digestive tracts, between 1 and 8 samples were taken from the stomachs and 1–10 samples taken from the spiral valves. On average, four samples were taken from stomachs and three samples were taken from spiral valves of each cownose ray. Weights of total stomach contents and spiral valve contents were taken for all rays, and weights for each prey category were recorded for stomach and spiral valve contents (23 of 33 individuals).

After the initial survey of the digestive tract contents, samples of the different types of tissues were homogenized by blender or food processor. Stomach and spiral

valve contents were homogenized separately, with multiple samples collected from the homogenized contents (homogenate). The purpose of homogenization was to obtain samples of mixed prey tissues from the digestive tract of the cownose ray. In some cases homogenization was not necessary (or possible) due to the well-mixed, digested, and fluid-like nature of the chyme. In other cases, homogenization was not possible due to the minute size and excessive quantity of shell fragments.

After testing for the most effective sample storage method, samples were kept thawed and stored in the refrigerator at 4 °C until DNA extraction, which occurred the same day (see Bade 2013 for more detail). Genomic DNA was extracted using a DNeasy Tissue kit (Qiagen), following standardized Qiagen extraction protocols. Excess tissue pieces, fluid, chyme, and homogenized contents were stored in ethanol, and hard parts were stored in formalin. Photographs were taken of bivalve shell fragments, shells, fish scales, and any distinctive tissue samples or unusual contents. When possible, prey items were identified macroscopically to the lowest taxonomic level.

Genetics methods

For the purposes of sequencing the COI gene from locally-acquired shellfish, individual bivalve samples were collected in locations throughout North Carolina and Chesapeake Bay. The seven target bivalve species were collected in both locations, when ecologically relevant, with a minimum of two individual specimens per species collected (Table 2). Specimens were stored on ice until frozen and then stored in a −20 °C manual defrost freezer. Tissue samples of approximately 2 mm square were collected from each specimen, from the inside of the adductor muscle, or from the foot or mantle of the bivalve if the adductor muscle was compromised. Care was taken to avoid the inner organs and digestive tract of the bivalve, in order to avoid contamination and sequence confusion from gonadal tissue or bivalve prey. To control for self-identification of cownose ray tissue in stomach content samples, the COI gene was also sequenced from cownose rays (n=5). Cownose ray tissue samples were removed from the frozen inner tissue of the esophagus. At least two tissue samples were taken from each individual, and used for sequencing the COI

Table 2 Bivalve and cownose ray specimens collected and used for cytochrome oxidase subunit I (COI) sequencing

Species	Location	Sequence Name	Accession Number
Chione cancellata	North Carolina	Chi2_COI	KF245610
	North Carolina	Chi3_COI	KF245611
	North Carolina	Chi4_COI	KF245612
Crassostrea virginica	North Carolina	Cvi1_COI	KF245599
	North Carolina	Cvi2_COI	KF245600
	Virginia	Vir3aV_COI	KF245601
Macoma balthica	North Carolina	Bma2_COI	KF245607
	North Carolina	Bma3_COI	KF245608
	North Carolina	Bma5_COI	KF245609
Mercenaria mercenaria	North Carolina	Mer1_COI	KF245605
	North Carolina	Mer2_COI	KF245606
	Virginia	Merc1aV_COI	KF245604
Mya arenaria	Virginia	Mya1aV_COI	KF245602
	Virginia	Mya2aV_COI	KF245603
Rhinoptera bonasus	Virginia	041CNR1_COI	KF245596
	Virginia	083CNR1_COI	KF245597
	Virginia	089CNR1_COI	KF245598
Tagelus plebeius	Virginia	Tpl1aV_COI	KF245613
	Virginia	Tpl2aV_COI	KF245614

Species name, location where sample was obtained, sequence name, and GenBank Accession number for all DNA sequences obtained from locally-acquired specimens

gene. DNA was extracted using a DNeasy Tissue Kit (Qiagen), following standardized protocols.

To sequence specimens from North Carolina and Chesapeake Bay, the COI gene was amplified, using PCR, from each locally-collected sample. Universal primer pairs LCO1490 and HCO2198 were used on all species (Folmer et al. 1994). PCR amplification of the COI gene was conducted in a reaction volume of 10 μl containing: 5.5 μl of ddH20, 1× PCR buffer (20 mM Tris–HCl at pH 8.4, 50 mM KCL), 20 mM $MgCl_2$, 2 mM dNTPs, 10 μM concentrations of primers HCO2198 and LCO1490, and 0.5 unit of Taq polymerase (Invitrogen). The following thermocycler protocols were used for PCR: hot start of 94 °C for 2 min, followed by 29 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min. A final extension step of 72 °C for 5 min was used, followed by a 15 °C hold. PCR products were separated by electrophoresis on a 2 % agarose gel stained with ethidium bromide. Clean-up of PCR product for sequencing was done through gel purification (UltraClean GelSpin DNA purification kit, MO-BIO) or ExoSAP-IT (Affymetrix), following manufacturer's directions. Sequencing reactions occurred in-house at the Genomics Core Facility at East Carolina University, using BigDye Terminator v3.1 chemistry and the 3130xl Genetic Analyzer (Applied Biosystems). The veneroidLCO primer (Pilgrim, BOLD Systems, Bade 2013) for COI amplification in veneroid clams, was also used in conjunction with the Folmer et al. 1994 primers to amplify and sequence the COI gene. Follow up sequencing of a subset of samples was conducted by the Environmental Protection Agency (EPA) Molecular Ecology lab (Cincinnati, Ohio).

Sequence results were edited and assembled, and compared to reference sequences found on GenBank, using Sequencher versions 5.0 and 5.1 (GeneCodes, Ann Arbor, MI). Alignment of reference sequences and sequence results was conducted using MUSCLE (Edgar 2004) and then manually, by visualizing sequences in the Se-Al program (Rambaut 1995). All species except for the Atlantic bay scallop were sequenced successfully from locally-acquired specimens (Table 2). With the exception of the Altantic bay scallop, a minimum of three sequences from known individuals and one reference sequence were aligned and used to design species-specific primers. Primers were designed using Primer3 and OligoCalc, to amplify PCR products of sufficiently large size, and different enough in size to the other bivalve species in the study, to be separated and identified on an agarose gel. Primers were also designed to have similar annealing temperatures to facilitate multiplex PCR.

Primer pairs were tested on DNA of the species of interest (for which the primers were designed, or the target species) as well as on the other species included in the study (the non-target species) to test for amplification of product. Master mix protocol and thermocycler programs were designed following standardized protocol (see Bade 2013). Initial thermocycler conditions used for testing and optimization were: hot start of 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 60 s, with a final extension step of 72 °C for 5 min and a 12 °C hold. Primers were optimized to amplify target species DNA and not cross-amplify DNA from the non-target species in the study; primers were also optimized and tested in multiplex reactions of mixed species composition. After primer design and testing, the best multiplex PCR primer sets were identified, optimized, and used for digestive tract sample testing (Table 3). Specific details of the master mix conditions are reported in Table 4 and in more detail elsewhere (Bade 2013). In silico analyses of the multiplex primer pairs were conducted by BLAST search (GenBank, NCBI) to check for species-specific primer specificity and identify potential cross-amplification with any of the other sequences entered into GenBank.

Genetic analysis of cownose ray digestive tracts

Each digestive tract sample (unknown sample) and positive control samples were tested with all sets of primers for the seven bivalve species, under the respective multiplex PCR conditions. Control samples included individual template DNA of the target bivalve species, mixed species DNA template of the multiplex species, and a dilution of the mixed species sample. A negative control was also utilized in each PCR reaction to test for contamination. Products were separated using gel electrophoresis, following standardized procedures (see Bade 2013). Positive and negative results for each unknown digestive tract sample, which was tested with each set of multiplexed primers, were determined based on comparison to positive control samples. For each unknown sample, any bands present in that lane had to be the appropriate size for that species, and similar to a band present in the positive control lanes, to be considered a positive result. Positive PCR results indicated that

Table 3 Species-specific PCR primer pairs, primer name, amplification specificity, primer sequence from the Cytochrome Oxidase I (COI) gene, and base pair size of the PCR product

Multiplex set and Primer Name	Specificity	Primer Sequence(5′-> 3′)	Size (bp)
Aic & Tpl			
TPL-F3	stout tagelus clam	GGTCTGGTCTGGTTGGATTG	473
TPL-R	stout tagelus clam	TACGCTGAGGAGCAATACCC	
AIC-F3	Atlantic bay scallop	GTTGGGTGCCATTGATATGAG	342
AIC-R3	Atlantic bay scallop	AGGGAAACCAACAGTAAGAACCTC	
Mme, Mar, Cvi			
MER-F	hard clam	TGGCTATACCTGGAAAGATGTTG	579
MER-R	hard clam	TGGACAAAAAGAATAGGATCACCT	
MYA-F2	soft-shell clam	TAGTTGGGACTGGGCTTAGTGTC	438
MYA-R	soft-shell clam	CACGCATGTTACCCCAAGTTC	
CVI-F	Eastern oyster	TTGTGTATAACGCTGTGGTAACG	218
CVI-R	Eastern oyster	TGACCCAACTCCTCTCTCAGAC	
Mba & Cca			
BMA-F	Baltic macoma clam	GCACAGAGTTAATACATCCTGGC	410
BMA-R	Baltic macoma clam	AGGACGCATATTAGCACCTGTAG	
CHI-F2	cross-barred venus	ATGTGGGTGGTGTGTCTTCA	232
CHI-R3	cross-barred venus	GGATCTCCTAAACCCACAGGA	

Primers are listed in the three multiplexed groups: bay scallop and stout tagelus (*Aic & Tpl*), hard clam, soft-shell clam, and Eastern oyster (*Mme, Mar, Cvi*), and Baltic macoma and cross-barred venus (*Mba & Cca*)

prey species were present in the unknown digestive tract sample. Fraction prey-positive results for each bivalve species were normalized for each cownose ray, based on the total number of samples tested for each ray:

$$\textit{Fraction positive for } i = \frac{\textit{Number of samples positive for species } i}{\text{Number of samples per cownose ray}}$$

Results

Cownose ray visual diet analysis

The majority of all stomach contents were not able to be identified macroscopically due to the high level of mastication and digestion. Visual examination of the contents of stomach and spiral valve revealed mostly unidentifiable tissues, although some identifiable hard parts of prey were observed and weighed. Average percent by weight for each category differed between stomachs and spiral valves: the average unknown total tissue for all stomachs was 80.78 % by weight, while in spiral valves, the average for unknown total tissue was 94.39 % by weight. In stomachs, the average detritus was 0.078 % by weight, and the average bivalve tissue was 3.83 % by weight. Only four of 23 stomachs had contents that were identifiable as bivalve tissue and those four individuals were large individuals (>90 cm disc width) collected by bowfishing in Virginia. Shell

Table 4 Master mix protocols for each multiplex set

Multiplex Master Mix Composition		
Aic & Tpl	*Mme*, *Mar*, *Cvi*	*Mba & Cca*
ddH_20	ddH_20	ddH_20
1xPCR buffer	1xPCR buffer	1xPCR buffer
2 mM $MgCl_2$	2 mM $MgCl_2$	5 mM $MgCl_2$
200 pM dNTPs	200 pM dNTPs	200 pM dNTPs
0.25 mM AicF3 & AicR3	1 mM MerF & MerR	0.25 mM BmaF & BmaR
0.5 mM TplF3 & TplR	0.5 mM MyaF2 & MyaR	0.5 mM ChiF2 & ChiR3
	0.5 mM CviF & CviR	
Taq: 0.5 unit	*Taq*: 0.5 unit	*Taq*: 0.5unit

The protocols for mixing a multiplex set of PCR primers designed and optimized for each of the seven target bivalve prey species: bay scallop and stout tagelus clam (*Aic & Tpl*) multiplex protocols (left), hard clam, soft-shell clam, and Eastern oyster (*Mme*, *Mar*, *Cvi*) multiplex (middle), and Baltic macoma and cross-barred venus (*Mba & Cca*) (right). Each multiplex master mix was brought to a total volume of 10 μl using ddH_2O

fragments were found in stomachs and spiral valves of rays collected by bowfishing and haul seine, and made up 4.54 % and 3.02 % averages by weight for stomachs and spiral valves, respectively. Shell fragments were only seen in digestive tracts of large individuals> 90 cm disc width. Fish parts were found in stomachs and spiral valves of rays collected by haul seine but not by bowfishing or cast net. For fish parts, the average found in stomachs was 10.24 % by weight; in spiral valves the average was 2.53 % by weight for fish parts. Digestive tracts of large individuals (>100 cm disc width) contained fish remains and were captured by haul seine.

Genetic analyses: multiplex testing on known species samples

The hard clam, soft-shell clam, and oyster (*Mme*, *Mar*, *Cvi*) and bay scallop and stout tagelus clam (*Aic & Tpl*) multiplex sets were optimized at 60 °C and 35 cycles. All sets of primers in those multiplexes resulted in species-specific amplification of target species DNA with no cross-amplification of the other species included in the study. In spite of substantial optimization efforts, the Baltic macoma (*Mba*) primers consistently cross-amplified stout tagelus clam DNA and cross-barred venus (*Cca*) primers cross-amplified oyster DNA. This cross-amplification was problematic in uncovering the presence of these species in actual digestive tract samples, in which all of these species could be mixed together in varying concentrations. Consequently, positive results from Baltic macoma primers require further testing and are not discussed in detail. BLAST searches for short, nearly exact matches of the multiplexed primer pairs indicated that there were no significant sequence matches (E<3e-06; product size=expected product size for those primer pairs) to other organisms for hard clam, soft-shell clam, Eastern oyster, bay scallop, and stout tagelus clam. Baltic macoma and cross-barred venus primers significantly matched other species: Baltic macoma primers matched the stout tagelus and *Macoma petalum* sequences; the cross-barred venus primers matched the *Chione elevata* sequence, which is a very closely related species found in Belize.

Primers were tested for sensitivity on known species samples, mixed known species, and differing concentrations of DNA from known species. Known DNA samples of the target species (positive controls) were used with every test of the primers and when digestive tract contents were tested with the multiplex sets. An example of this is the gel image of the bay scallop and stout tagelus clam (*Aic & Tpl*) multiplex testing on positive control samples, mixed DNA samples, and diluted mixed DNA samples (Fig. 2). Concentrations of DNA of the positive controls ranged from 0 to 157 ng/μl, with the average concentration being 31 ng/μl. Concentrations of DNA were found to be below the limits of detection in highly diluted control samples, but still amplified by PCR. The primer sets of the *Mme*, *Mar*, *Cvi* multiplex test were found to amplify even in highly dilute (1/20) control samples with a calculated concentration of 3.82 ng/μl. Primers of the *Aic & Tpl* multiplex set amplified positive control samples with a calculated concentration of 1.20 ng/μl, and primers of the *Mba & Cca* multiplex set were sensitive to positive control samples of 0.35 ng/μl concentration.

Cownose ray genetic diet analysis

Samples taken from the digestive tract contents of 33 cownose rays (n=215) were tested against all three multiplex sets of potential bivalve prey. Samples were found to be positive for stout tagelus clams, soft-shell clams, and Baltic macoma clams.[1] Digestive tract samples were not found to be positive for hard clams, oysters, bay scallops, and cross-barred venus clams (Table 6). Positive results were found from all types of stomach and spiral valve samples, at varying stages of digestion (Table 5). All positive samples were obtained when the digestive tract samples were stored cold with same day DNA extraction (Table 5).

Of the 33 cownose ray digestive tracts sampled, positive results were found in 45 samples from 10 individual rays (total fraction positive=0.303). Two cownose ray digestive tracts had samples positive for both stout tagelus and soft-shell clams, and eight cownose rays tested positive for only stout tagelus clams. Cownose rays with digestive tract samples positive for the species tested in this study were all collected

[1] Baltic macoma primers were found to have amplified digestive tract samples that also tested positive with stout tagelus clam primers. The cross-amplification rate of the Baltic macoma primers with stout tagelus positives was 86 %, making those positives uncertain, and require further testing. To test for species identification of those uncertain samples, we sequenced a subsample (n=8) of the uncertains and all samples were a match for stout tagelus sequence. BLAST searches of the Baltic macoma primers revealed significant matching to stout tagelus DNA sequences, so this cross-amplification is not unexpected.

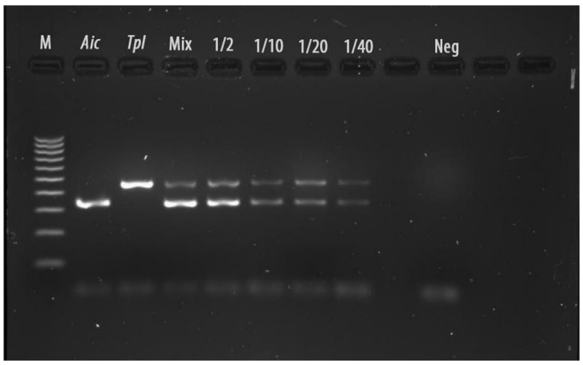

Fig. 2 Gel image of the stout tagelus clam (Tpl) and bay scallop (Aic) multiplex test. The first lane M is the size marker or 100 bp ladder. The next two lanes are single-species DNA tested with the multiplex primers and amplification conditions (*Aic & Tpl*). The following lanes are mixed DNA and diluted mixed DNA samples (1/2, 1/10, 1/40) of the two species, tested with the multiplex PCR conditions to determine primer sensitivity. Neg is a negative control sample

in Virginia during the bowfishing tournament (Table 5, Table 6). Size and sex of individual cownose rays influenced the bivalve species found in their digestive tracts. Cownose rays larger than 90 cm disc width tested positive for soft-shell clams or stout tagelus clams. The eight female cownose rays positive for stout tagelus and soft-shell clams were larger than 94.5 cm in disc width, and two male cownose rays had digestive tract samples positive for stout tagelus clams (disc width> 93 cm). The fraction positive for stout tagelus clams was 0.56, indicating that 50 % of the cownose ray digestive tracts examined from Chesapeake Bay had consumed stout tagelus clams; these rays were captured using the bowfishing method, where actively feeding rays are targeted. Although the sample size was small (N=3), young-of-year cownose rays, all caught in North Carolina, were negative for all of the bivalve species tested for in this study. No cownose ray digestive tract samples were found to be positive for bay scallop, cross-barred venus clams, hard clams, or eastern oysters.

Discussion

This study has established the use of a multiplex PCR testing method based on the DNA barcode region of the COI gene to identify the presence or absence of seven species of potential bivalve prey in the stomach contents of the cownose ray, a predatory and durophagous elasmobranch that consumes mollusks and crustaceans in North Carolina and Virginia. The primers and multiplex sets designed and optimized in this study were successful in amplifying the target species of interest, even at very low DNA concentrations, which should prove useful when attempting to test large numbers of degraded and digested tissue and chyme samples from cownose ray digestive tracts. We found no evidence that cownose rays, albeit in a small sample size, consumed the bivalve species (Atlantic bay scallops, Eastern oysters, and hard clams) of primary commercial interest, and for which they have been previously implicated in the demise of those fisheries. These findings are consistent with other diet studies in Chesapeake Bay (Smith and Merriner 1985; Fisher 2010). However, as our sample size for the development of this genetic testing procedure was both small and temporally and geographically limited, further studies using this method are required to exonerate cownose rays in putative commercial bivalve depredations. Two species of bivalves (stout tagelus and soft-shell clams) were positively detected from unknown digestive tract samples, and this multiplex PCR method of diet analysis could easily be applied to a larger sample of cownose rays caught in different areas, habitats, and times throughout North Carolina and Chesapeake Bay. With an expansion of prey items included in the genetic testing, this approach could be used to help identify diets of cownose rays throughout the Eastern U.S. seaboard and Gulf of Mexico.

Genetic testing

The primer sets designed and optimized for five of the seven bivalve species were 100 % successful in amplifying the target species and in not amplifying DNA of the non-target species included in the study. Two of the three multiplex sets were entirely successful in amplifying target species, mixed DNA samples of the target species, and diluted mixed DNA samples. In some cases, the multiplex sets were able to successfully amplify DNA of the target species in very low concentrations (0.5 ng/μl). Such low concentrations would approximate concentrations expected in digestive tract samples. All three multiplex sets successfully amplified DNA from the target species and from unknown stomach samples. Primers for Baltic macoma and the cross-barred venus clam, however, consistently cross-amplified DNA from two of the non-target species in this study. The primer sets for those species need to be

Table 5 Samples positive by multiplex PCR tests for any of the seven target bivalve species, divided by the stomach and spiral valve and type of sample taken from each

Cownose Ray ID	Location	Gear Type	Positive for Any Target Bivalve Species						Sample Storage
			Stomach Samples			Spiral Valve Samples			
			Tissue	Chyme/Fluid	Homogenate	Tissue	Chyme/Fluid	Homogenate	
2012083101	NC	hook/line		…	…		…	…	EtOH
2012083102	NC	hook/line		…	…		…	…	EtOH
2012083103	NC	hook/line		…	…	…		…	EtOH
2012083104	NC	hook/line			…			…	EtOH
2012083105	NC	hook/line		…	…			…	EtOH
2012090201	NC	gill net	…		…			…	EtOH
2012090202	NC	gill net	…		…	…		…	EtOH
2012100701	NC	cast net			…			…	Cold
20110618179	VA	bowfishing	…	…	…		…		Cold
20110618083	VA	bowfishing	x	…	x			…	Cold
20110618039	VA	bowfishing	x	…	…	…	…	…	Cold
20110618180	VA	bowfishing		…		…	…		Cold
20110618071	VA	bowfishing	…	…		…		…	Cold
20110618040	VA	bowfishing	x	x	…	…		…	Cold
20110618025	VA	bowfishing	…		…	…		…	Cold
20110618021	VA	bowfishing	…		…	…	…	…	Cold
20110618014	VA	bowfishing	x	x	x	x			Cold
20110618061	VA	bowfishing	…		…	…		…	Cold
20110618060	VA	bowfishing	…		…	…		…	Cold
20110618043	VA	bowfishing	x		…	…		…	Cold
20110618042	VA	bowfishing	…		…	…		…	Cold
20110618016	VA	bowfishing	x		…	…		…	Cold
20110618112	VA	bowfishing	…		…	…		…	Cold
20110618041	VA	bowfishing	x	x	…	…		…	Cold
20110618038	VA	bowfishing	x		…	…		…	Cold
20110618089	VA	bowfishing	x		…	…	x	…	Cold
20110618057	VA	bowfishing	…		…	…	x	…	Cold
20120924016	VA	haul seine	…		…	…		…	Cold
20120924022	VA	haul seine	…		…	…		…	Cold
20120924025	VA	haul seine	…		…	…		…	Cold
20120924017	VA	haul seine	…		…	…		…	Cold
20120924026	VA	haul seine	…	…		…		…	Cold
20120924008	VA	haul seine	…	…		…		…	Cold

Samples are listed by cownose ray specimen identification code, location of capture, gear type, and method of sample storage (EtOH=95 % ethanol preservation, Cold=4 °C storage with same-day DNA extraction). Blank cells indicate no evidence of the target species in samples of that type, an x indicates that at least one of the target bivalve species was detected in that sample type, and a dotted line indicates that sample type was not available or taken for that cownose ray digestive tract

redesigned, or an alternate gene used for primer design, in order to remove cross-species amplification in that multiplex set.

The majority of the Baltic macoma clam positives found in the unknown digestive tract samples was likely false due to cross-amplification of stout tagelus clam

Table 6 Number of cownose rays with digestive tracts containing samples positive for the species tested

Location	Gear Type	Number	Bivalve Species					
			Aic	*Tpl*	*Mme*	*Mar*	*Cvi*	*Cca*
NC	Hook & Line	5	0	0	0	0	0	0
	Nets	3	0	0	0	0	0	0
VA	Bowfishing	19	0	10 (52.6 %)	0	2 (10.5 %)	0	0
	Haul Seine	6	0	0	0	0	0	0
	Totals	33		10		2		

Numbers of cownose rays are categorized by location of collection and capture method. Percent positives for each prey (parentheses) were calculated from the total number of rays collected by that gear type and at that location. Bivalve species tested were Atlantic bay scallop (*Aic*), stout tagelus clams (*Tpl*), hard clams (*Mme*), soft-shell clams (*Mar*), Eastern oyster (*Cvi*), cross-barred venus clam (*Cca*), and Baltic macoma (*Mba*). Baltic macoma results are not reported here because of uncertainty in the results of the Baltic macoma and cross-barred venus multiplex primer set

DNA by the Baltic macoma primers, and so the Baltic macoma results were not reported here. Sequencing is necessary in order to determine the species identification of those "uncertain" samples, which tested positive for both stout tagelus and Baltic macoma clams. Sequencing of 8 of the uncertain samples revealed that those samples were all stout tagelus clams. However, one unknown digestive tract sample tested positive for Baltic macoma and was not positive for stout tagelus clam (result not reported here), suggesting that one cownose ray consumed Baltic macoma clams. At this time, we cannot estimate the true positive rate of Baltic macoma clams using the current multiplex PCR test. Verification of species identity for the remaining uncertain samples requires sequencing, but these initial results confirm our conclusion that the Baltic macoma primers must be redesigned to eliminate cross-amplification. Baltic macoma clams have been found in other cownose ray diet studies from Chesapeake Bay and North Carolina (Smith and Merriner 1985; Fisher 2010).

A number of samples did not test positive for any of the species included in this study (n=170); furthermore, there were no positive digestive tract samples from 23 of the 33 cownose rays included in this study. Of those 33 cownose rays, five were collected by hook and line in North Carolina, six were collected from haul seine commercial fishers, and the three young-of-year cownose rays were caught by gill net and cast net. The rays collected by hook and line and haul seine had digestive tracts full of fish parts, and those samples would not be expected to amplify with the bivalve primers used in this study. Negative results, or the absence of the potential bivalve prey tested for in this study, means only that those cownose rays ate other prey items than the ones included in our multiplex PCR tests. Samples that did not test positive for the potential bivalve prey in this study could have been the result of the absence of any of those species, degradation of samples from the digestive tract, or problems associated with storage method of the digestive tract samples after removal from the tracts. In this study, we had the most success with samples stored cold after sampling and same-day DNA extraction.

Feeding ecology

Positive results were found in a total of 45 samples from 10 individual cownose rays. The three species of bivalves found in digestive tracts of cownose rays in this study are infaunal, burrowing, thin-shelled bivalves associated with sandy-bottoms and vegetated areas (Ruppert and Fox 1988). Stout tagelus clams are large, with shells up to 7.6 cm in length. They construct burrows in the mud and sandy sediments and can be found as deep as 51 cm into the sediment. Baltic macoma clams are small, infaunal burrowers in muddy, intertidal sediments in waters of low salinity; the adults can be found as deep as 20 cm in burrows in the sediment (Ruppert and Fox 1988). Soft-shelled clams are thin-shelled, infaunal burrowers in silty sediment, and can be found in burrows as deep as 18 cm (Barnes 1974).

In contrast, no digestive tract samples were found to be positive for the Atlantic bay scallop, Eastern oyster, or hard clam, all of which are thick-shelled bivalves associated with seagrass beds or oyster reefs, and all are commercially and ecologically important. No

samples were positive for the cross-barred venus clam, a small, thicker-shelled bivalve that is associated with seagrass beds and makes shallow burrows in fine sediments (Ruppert and Fox 1988). Out of the four species not detected in the cownose ray digestive tracts, two are also infaunal (cross-barred venus and hard clam), but only the hard clam exhibits limited burrowing. The bay scallop is epibenthic, motile, and found in seagrass beds. Oysters are found growing in oyster reefs or attached to hard substrate (Ruppert and Fox 1988). Our study found only infaunal bivalves in the cownose ray digestive tracts. There is no reason to suspect that epifaunal bivalves are being avoided, and they should be easily discovered by foraging rays.

The primary prey species found in this study, the stout tagelus clam, are deeply burrowing and thin-shelled clams. Perhaps a limiting factor for predation by cownose ray is the body size (and therefore orobranchial volume and hydraulic winnowing capacity), disc width, and muscle mass necessary to excavate such deeply-burrowing prey sources. Thus, there may be an interaction between the cownose ray body size and prey burrowing depth. To the best of our knowledge, the relationship between burrowing depth of the clam and cownose ray disc width or body size has not been tested; most feeding morphology studies are conducted in holding tanks or aquariums where food sources are presented on a few inches of sand on the bottom of the tank (Sasko et al. 2006; Fisher 2010). Burrowing depth of prey, and general availability of alternative prey sources, should be accounted for in future diet studies on cownose rays, especially when investigating ontogenetic shifts in diet and prey selection behaviors.

Capture method likely has important impacts on downstream diet analyses. In our study, all cownose rays with digestive tract samples that tested positive were caught near Reedville, Virginia during a bowfishing tournament. Bowfishing is an effective way to capture cownose rays while feeding, or in habitats associated with feeding. Individual cownose rays collected this way are often killed instantly, landed on the boat soon after capture, and do not have a chance to evacuate their stomachs. In contrast, cownose rays captured by hook and line, in gill nets, haul seines, or pound nets will often evacuate their stomachs and will eat other fish and organisms trapped with them in the net (R. Fisher, personal communication). Traditional scientific and recreational capture and collection methods often result in the digestive tract contents containing no food or opportunistic prey sources, which thereby biases natural diet study results. Cownose rays used in this study, captured by haul seine and hook & line, had stomachs full of fish hard parts and tissue, but no visually detectable bivalves. The stomach contents of cownose rays taken from nets was in marked contrast to digestive tract contents from cownose rays caught by bowfishing (while foraging for infaunal prey) and may be due to opportunistic "net" feeding. Previous diet studies in Chesapeake Bay found collection through bowfishing to yield the most unbiased or natural digestive tract contents (Fisher 2010), and our research supports those findings.

Knowledge of cownose ray diets and feeding ecology has been limited by using only traditional diet study methods. We recommend using visual diet analysis with genetic testing methods as complementary tools in diet studies. Particularly for durophagous rays that crush their prey, the complementary visual analysis and genetic testing methods are highly recommended to identify the hard parts and remaining tissue and chyme found in their stomachs. Handling of digestive tracts, storage of samples, and multiplex optimization was developed during this research (see Bade 2013), and further testing on a larger number of cownose rays is recommended. Using these same multiplex PCR techniques, more species of bivalves, crustaceans, and other invertebrates of interest or potentially available prey sources could be added to the primer sets.

Other techniques, such as sequencing the COI gene from the unknown digestive tract samples and comparing it to the BOLD and GenBank reference libraries, can also be used to determine identity of other unknown prey sources found in stomachs (Dunn et al. 2010). Metagenomic approaches on digestive tract contents will allow for characterization of the entire suite of prey of cownose rays and their entire gut microbiome, as well as other species of marine animals (King et al. 2008; Pompanon et al. 2011; Yoccoz 2012). Next generation sequencing has great potential for the understanding of predator–prey relationships in food webs, but is expensive and labor intensive. The multiplex PCR method we developed is cost-effective and less labor intensive relative to the next generation sequencing metagenomic approach; these multiplex PCR tests can be used as a screening tool for the bivalve prey species of interest, and the potential prey list could easily be expanded to increase its usefulness. This species-specific sample identification method, based on the DNA barcode

region of the COI gene, is potentially of use in elucidation of trophic links in food web studies from many other species of prey and predator.

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References

Ajemian M, Powers S (2011) Habitat-specific feeding by cownose rays (*Rhinoptera bonasus*) of the northern Gulf of Mexico. Environ Biol Fish 95(1):79–97

Bade LM (2013) Use of genetic techniques to identify the diet of cownose rays, Rhinoptera bonasus, in North Carolina and Virginia: an analysis of shellfish prey items. East Carolina University, Greenville

Barnes RD (1974) Invertebrate Zoology 3. Ed. WB Saunders Co, Philadelphia, Pennsylvania

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(1): 188–192

Bigelow HB, Schroeder WC (2002) Bigelow and Schroeder's fishes of the gulf of Maine, 3rd edn. Smithsonian Institution Press, Washington

Blaylock RA (1989) A massive school of cownose rays, rhinoptera bonasus (rhinopteridae), in lower chesapeake Bay, Virginia. Copeia 1989(3):744–748

Blaylock R (1993) Distribution and abundance of the cownose ray, *Rhinoptera bonasus*, in lower Chesapeake Bay. Estuar Coasts 16(2):255–263. doi:10.2307/1352498

Collins AB, Heupel MR, Hueter RE, Motta PJ (2007) Hard prey specialists or opportunistic generalists? An examination of the diet of the cownose ray, *Rhinoptera bonasus*. Mar Freshw Res 58(1):135–144. doi:10.1071/MF05227

Collins A, Heupel M, Simpfendorfer C (2008) Spatial distribution and long-term movement patterns of cownose rays *Rhinoptera bonasus* within an estuarine river. Estuar Coasts 31(6):1174–1183. doi:10.1007/s12237-008-9100-5

Craig JK, Gillikin PC, Magelnicki MA, May LN (2010) Habitat use of cownose rays (*Rhinoptera bonasus*) in a highly productive, hypoxic continental shelf ecosystem. Fish Oceanogr 19(4):301–317. doi:10.1111/j.1365-2419.2010.00545.x

Deagle BE, Kirkwood R, Jarman SN (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Mol Ecol 18(9):2022–2038

Dunn MR, Szabo A, McVeagh MS, Smith PJ (2010) The diet of deepwater sharks and the benefits of using DNA identification of prey. Deep-Sea Res I 157:923–930

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5): 1792–1797

Fisher R (2010) Life History, Trophic Ecology, and Prey Handling by Cownose Ray, *Rhinoptera bonasus*, from Chesapeake Bay. National Oceanic and Atmospheric Administration

Fisher R, Garrett C, Grubbs RD (2011) Cownose ray (*Rhioptera bonasus*) predation relative to bivalve ontogeny. J Shellfish Res 30(1):187–196

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3(5):294–299

Goodman M, Conn P, Fitzpatrick E (2010) Seasonal occurrence of cownose rays (*rhinoptera bonasus*) in north Carolina's estuarine and coastal waters. Estuar Coasts 34(3):640–651. doi: 10.1007/s12237-010-9355-5

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond Ser B Biol Sci 270(1512):313–321. doi:10.1098/rspb.2002.2218

King RA, Read DS, Traugott M, Symondson WOC (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. Mol Ecol 17:947–963

Orth RJ (1975) Destruction of eelgrass, *Zostera marina*, by the cownose ray, *Rhinoptera bonasus*, in the Chesapeake Bay. Chesap Sci 16(3):205–208. doi:10.2307/1350896

Peterson C, Fodrie J, Summerson H, Powers S (2001) Site-specific and density-dependent extinction of prey by schooling rays: generation of a population sink in top-quality habitat for bay scallops. Oecologia 129(3):349–356. doi:10.1007/s004420100742

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P (2011) Who is eating what: diet assessment using next generation sequencing. Mol Ecol 21(8):1931–1950

Rambaut A (1995) Se-Al: Sequence alignment program. Oxford University Press, Oxford

Ratnasingham S, Hebert PDN (2007) bold: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7 (3):355–364. doi:10.1111/j.1471-8286.2007.01678.x

Ruppert EE, Fox RS (1988) Seashore animals of the Southeast: A guide to common shallow-water invertebrates of the southeastern Atlantic coast. Univ of South Carolina Press, Columbia

Saccone C, De Giorgi C, Gissi C, Pesole G, Reyes A (1999) Evolutionary genomics in Metazoa: the mitochondrial DNA as a model system. Gene 238(1):195–209

Sasko DE, Dean MN, Motta PJ, Hueter RE (2006) Prey capture behavior and kinematics of the Atlantic cownose ray, *Rhinoptera bonasus*. Zoology 109(3): 171–181

Smith J, Merriner J (1985) Food habits and feeding behavior of the cownose ray, *Rhinoptera bonasus*, in lower Chesapeake Bay. Estuar Coasts 8(3):305–310. doi:10.2307/1351491

Smith J, Merriner J (1986) Observations on the reproductive biology of the cownose ray, *Rhinoptera bonasus*, in Chesapeake Bay. Fish Bull 84(4):871–877

Smith J, Merriner J (1987) Age and growth, movements and distribution of the cownose ray, *Rhinoptera bonasus*, in Chesapeake Bay. Estuar Coasts 10(2):153–164. doi:10.2307/1352180

Yoccoz NG (2012) The future of environmental DNA in ecology. Mol Ecol 21:2031–2038