

USE OF GENETIC TECHNIQUES TO IDENTIFY THE DIET OF COWNOSE RAYS,
RHINOPTERA BONASUS, IN NORTH CAROLINA AND VIRGINIA: AN ANALYSIS OF
SHELLFISH PREY ITEMS.

by

Lyndell M. Bade

June, 2013

Chair: Joseph J. Luczkovich, Ph.D.

Major Department: Biology

Abstract: Cownose rays, *Rhinoptera bonasus*, are considered voracious predators on mollusks and have been implicated in the consumption of commercially important species of shellfish on the East Coast. Digestive tracts of the 32 rays collected for this study from Pamlico Sound, NC and Chesapeake Bay, VA contained pieces of partially-digested tissue, well-digested tissue and fluid (chyme), and very small shell fragments which made identification to the species level nearly impossible. Visual content analysis of the stomach yielded 80.78% average unknown total tissue by weight and in spiral valves the total average unknown tissue was 94.39% by weight. Shell fragments were found in digestive tracts of specimens collected by bowfishing and haul seine (4.54% and 3.02% averages by weight, respectively). Tissue identifiable as bivalve was found in stomachs from cownose rays (> 90 cm disc width) collected by bowfishing. Fish parts were found in digestive tracts of cownose rays collected by haul seine but not by bowfishing or cast net (10.24% average by weight in stomachs and 2.53% average by weight in spiral valves). To provide a solution to the difficulty of identification of digestive tract contents

by visual methods, molecular identification techniques, using the sequence of the cytochrome oxidase subunit I (COI) gene, were applied to a variety of samples to determine the presence of key bivalve species of commercial and ecological importance in North Carolina and Chesapeake Bay. Species chosen were Atlantic bay scallop (*Argopecten irradians concentricus*), Baltic macoma clam (*Macoma balthica*), cross-barred venus clam (*Chione cancellata*), Eastern oyster (*Crassostrea virginica*), hard clam (*Mercenaria mercenaria*), soft shell clam (*Mya arenaria*), and stout tagelus clam (*Tagelus plebicus*). Species-specific primers were designed from the COI sequences for each bivalve species. Primers were designed to amplify polymerase chain reaction (PCR) products of differing sizes that were distinguishable from the other species of interest. Based on the primer design, multiplexing of several species in one reaction was possible, reducing testing time and cost. Results indicated that cownose rays in Chesapeake Bay ate stout tagelus clams and soft shell clams. Baltic macoma positives were most likely cross-amplification with stout tagelus DNA and further work is required to test for Baltic macoma clams without cross-amplification. There was no evidence of commercially important bivalves (hard clams, oysters, and bay scallops) being consumed by the rays in this study. Ontogenetic shifts in prey were difficult to determine based on uneven sample sizes, but large adult females (disc width over 90 cm) were found with a large proportion of stout tagelus clams in their digestive tracts. All young-of-year (YOY) individuals were male and caught in North Carolina. The sample size was small (n = 3) but the YOY individuals did not eat any of the species tested for in this study. Further sampling over an extended period of time and locations is required to confirm these results. Best-use practices of tissue extraction, manipulation, and handling techniques are discussed to help inform methodologies for forensic testing on marine species.

USE OF GENETIC TECHNIQUES TO IDENTIFY THE DIET OF COWNOSE RAYS,
RHINOPTERA BONASUS, IN NORTH CAROLINA AND VIRGINIA: AN ANALYSIS OF
SHELLFISH PREY ITEMS

A Thesis
Presented To the Faculty of the Department of Biology
East Carolina University

In Partial Fulfillment of the Requirements for the Degree of
Master of Science

By

Lyndell M. Bade
June, 2013

© COPYRIGHT BY LYNDELL M. BADE 2013
ALL RIGHTS RESERVED

Use of Genetic Techniques to Identify the Diet of Cownose Rays, *Rhinoptera bonasus*, in North Carolina and Virginia: An Analysis of Shellfish Prey Items

by

Lyndell M. Bade

APPROVED BY:

DIRECTOR OF THESIS: _____
Joseph J. Luczkovich, Ph.D.

COMMITTEE MEMBER: _____
Christopher N. Balakrishnan, Ph.D.

COMMITTEE MEMBER: _____
Susan B. McRae, Ph.D.

COMMITTEE MEMBER: _____
Kevin O'Brien, Ph.D.

COMMITTEE MEMBER: _____
Roger A. Rulifson, Ph.D.

DIRECTOR, DEPARTMENT OF BIOLOGY:

Jeffrey S. McKinnon, Ph.D.

DEAN OF THE GRADUATE SCHOOL: _____
Paul J. Gemperline, Ph.D.

I dedicate this to the memory of my parents,
Norman Dee and Lyndell Eileen Bade,
my granny Twilia Neill Dorland,
and
In honor of my grandfather Maurice Dorland:

They gifted me with their strength, love, encouragement, stubbornness, joy in the world around me, strolls on the beach at sunrise, horseback rides in the pasture, travel to far flung corners of the globe, and a holistic view of the natural world.

and

To Mateya, Elliott, and Sascha,
My darling nieces,
For whom I am grateful every day.

Acknowledgements

I am thankful for the help and encouragement of so many people during the last few years. I am grateful to my committee for all their support. I am grateful for Dr. Kevin O'Brien's insight, kindness, and enthusiasm, and I am thankful for Dr. Roger Rulifson's fishery expertise, teaching me about gear bias, and the opportunities he provided to work with spiny dogfish and commercial fishermen. I am grateful for Dr. Chris Balakrishnan's generosity with his time and assistance, and for teaching me to design primers. Before Chris was even on my committee, he mentored and guided me in the last year of my research, and I am very grateful for all his help. I am thankful for Dr. Sue McRae and Dr. Joe Luczkovich's support, ideas, assistance, training, and funding support for this project. Dr. Luczkovich read a paper on oysters and multiplexed PCR that spurred the creation of this project, and although outside his area of expertise, he handled the new content and genetics techniques with aplomb and humor. Dr. McRae helped me to iron out the methods and protocol, and I am grateful for her genetics expertise and the use of her lab for this research.

I would like to thank the Institute for Coastal Science and Policy, especially Dr. John Rummel, Kay Evans, and Cindy Harper for their care, kindness, encouragement, help, and advice...on all things professional and personal! I thank the Department of Biology, including Dr. Terry West, Barbara Beltran, and Taria Crenshaw for the support, teaching opportunities, and experience.

For their immeasurable help with genetics methods, techniques, sequencing, and troubleshooting, I am indebted to Dr. Erik Pilgrim, Denise Mayer, Dr. Trip Lamb, Dr. Ashley Egan, and Dr. Chris Balakrishnan. Erik endured panicked emails and random phone conversations about

PCR protocol with patience and kindness; he was very generous and thoughtful in his time and help, and even sequenced shellfish samples for me. None of this would have been possible without all of their help and kindness.

For assistance in the collection of bivalve samples, I am grateful for the kindness and generosity of Robert Fisher, Dr. Joel Fodrie and his lab, UNC Institute of Marine Science, Nathan Geraldi, Christine Addison, Dr. Roger Newell, Dr. David Kimmel, Drew Cathey, Laura McKenna, and Cecilia Krahforst.

For their cownose ray expertise and experience, kindness, encouragement, lengthy discussions, and help with experimental design, I am indebted to Joseph Smith, Robert Fisher, Dr. Dean Grubbs, Dr. James Gelslechter, Matthew Kolmann, Dr. Neil Aschliman, and Dr. Roger Rulifson. For help in the collection of cownose ray specimens, discussion about capture locations and techniques, and dissections, I thank Captain George Beckwith, Anna Beckwith, Robert Fisher, Dr. John Morrissey, the bowfishing tournament team in Virginia, Dr. Shona Paterson, Chuck Bangle, Dr. Andrew Thaler, Will Flannery, the people of the town of Oriental, and Jason Rock of NCDMF. The North Carolina Division of Marine Fisheries shared sampling stations and data from their fishery-independent gill net survey to help me determine best time frame and capture locations for cownose rays. I am thankful to April Goodman Hall of NOAA for her kindness and generosity with maps of cownose ray collection locations and aerial surveys from her publication. Lena Keller was instrumental in the processing of digestive tract contents for a large portion of my samples, and I greatly appreciate her perspective and assistance.

To my friends and housemates over the past few years, I thank them for their support, cheer, and discussions on methodology and results at all hours of the day or night, with a cup of

coffee, brunch at the pub, or chatting around the campfire with a glass of wine. I am grateful for the support, help, and friendship of the Luczkovich, McRae, and Rulifson labs. I would especially like to mention the encouragement and friendship of Guillaume Bagal, Shruti Saxena, Lisa Beth Robinson, Tricia Kimmel, Dr. David Kimmel, and Dr. Jamie DeWitt. I am very thankful for my family, who endured my long absences and infrequent communication with humor and grace, and I am grateful for their love and patience.

Last but certainly not least, Shona K. Paterson was my number one fan, my best friend, and a pillar of strength, support, and encouragement throughout this process. She assisted me in every aspect of this project, from collecting bivalves to dissecting cownose rays, from sorting through stomach contents to discussing PCR protocol, from celebrating my results to organizing references. I will be forever grateful for her friendship, loyalty, kindness, and generosity of spirit.

Table of Contents

Introduction.....	1
Natural History.....	2
Feeding Behaviour & Ecology.....	6
Ecological Impact	10
COI (Barcode of Life) gene and use for species identification	13
Objectives & Goals	17
Methods and Materials.....	19
Collection Methods.....	19
Digestive Tract Analysis Methods.....	21
Genetics Methods.....	24
Step 1: Specimen & Tissue Collection and Step 2: DNA Extraction.....	26
Step 3: Cytochrome Oxidase I (COI) Amplification	26
i. Polymerase Chain Reaction.....	27
ii. Gel electrophoresis.....	27
iii. PCR Product Processing	28
Step 4. COI Sequencing.....	29
i. Sequencing reactions	29
Step 5. Species-Specific Primer Design.....	31
Step 6. Species-Specific Primer Testing.....	32
i. PCR with Species-Specific Primers	32
ii. Gel Electrophoresis.....	33
iii. Primer Optimization.....	34
Step 7: Multiplex Testing & Optimization	34
Step 8: Application of Multiplex Tests to Digestive Tract Samples	35
PCR Results and Analysis	36
Known-Tissue Mixtures.....	37
Statistical Methods.....	38
Results.....	41
Genetics Results.....	41
Primer Testing on Known Species Samples	46
Storage Treatment of Digestive Tract Samples	49
Testing on Digestive Tract Samples	51
Cownose Ray Visual Diet Analysis.....	57

Cownose Ray Genetic Diet Analysis	59
.....	69
Discussion	72
Genetics Discussion	72
Cownose Ray Feeding Ecology	72
Best Practices	75
Sex Ratio, Ontogenetic Shift, and Ecomorphology of Cownose Rays.....	77
Ecological Impact of Cownose Rays	79
References	83
Appendix A.....	91

List of Figures

Figure 1. Capture locations of cownose rays in North Carolina and Virginia.....	20
Figure 2. An overview to the genetics methods.....	25
Figure 3. Alignment of a portion of the COI gene sequences for oyster, hard clam, and soft-shell clam with positions shown of forward primers for each species (orientation is 5' to 3').....	44
Figure 4. Gel image of the stout tagelus clam (Tpl) and bay scallop (Aic) multiplex test.....	47
Figure 5. Gel image of the mixed-tissue combinations of hard clam (Mme), soft-clam (Mar), and oyster (Cvi)	49
Figure 6. Gel image of positives for soft shell clams (Mar) from cownose ray number 20110618014.....	52
Figure 7. Gel image of tests on a subsample of 16 “smeary” samples	55
Figure 8. Gel image of Baltic macoma and cross-barred venus (Mba & Cca) multiplex tests with diluted control samples found on the bottom row and cross-amplification testing of non-target species on the top row.....	56
Figure 9. Gel images of digestive tract samples from cownose ray 20110618083 tested with bay scallop and stout tagelus clam (Aic & Tpl) and Baltic macoma and cross-barred venus (Mba & Cca) multiplexes	57
Figure 10. The visual examination process of stomach and spiral valves involved separating the contents into categories of tissue type and obtaining weights of identifiable parts of fish, bivalves, shell pieces, and detritus.....	58
Figure 11. Percentage of wet weight of stomach contents that could be identified visually from cownose rays of different sizes.....	59
Figure 12. Proportion of all digestive tract samples positive (y-axis) for each bivalve species tested, by disc width in cm (x-axis).....	62
Figure 13. Proportion positive for stout tagelus clams in digestive tract samples for each cownose ray, by disc width and separated by female rays and males rays.....	63
Figure 14. MDS plot of cownose rays positive for any bivalve species by disc width (cm).....	64
Figure 15. MDS plot of cownose rays by stomach content results (multiplex testing results) and the method of capture used.....	65
Figure 16. MDS plot of cownose rays by stomach contents results (multiplex testing results) and showing the sex of the rays.....	66

Figure 17. MDS plot of cownose rays by stomach contents results (multiplex testing results) showing the location of capture of the rays.	67
Figure 18. MDS plot combining visual stomach analysis results with genetic testing results, by capture method (bowfishing vs haul seine).	69
Figure 19. MDS plot of combined stomach content analysis and genetic testing results, by capture method, and indicating percent weight of fish found in stomachs.....	70
Figure 20. MDS plot of combined stomach content analysis and genetic testing results, by sex of cownose ray and fraction positive for stout tagelus clam (by genetic testing).	71

List of Tables

Table 1. Target bivalve mollusk species in this study, each reportedly eaten by cownose rays.....	18
Table 2. Species name, location of sample, and sequence number for all sequences obtained from locally-acquired specimens.	43
Table 3. Genus and species-specific PCR primers, primer name, amplification specificity, primer sequence from the Cytochrome Oxidase I (COI) gene, and base pair size of the PCR product	45
Table 4. Master mix protocols designed, optimized, and used for each multiplex set	46
Table 5. DNA concentrations of samples treated with cold, ethanol, and re-frozen storage treatments.....	50
Table 6. Comparison of average DNA concentrations per treatment group.....	51
Table 7. Samples positive for any of the target bivalve species, divided by the stomach and spiral valve and type of sample taken from each.....	53
Table 8. Number of cownose rays with digestive tracts containing samples positive for the species tested.....	61

Introduction

Cownose rays, *Rhinoptera bonasus*, are cartilaginous fishes related to manta rays, eagle rays, and other cownose rays. 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). Cownose rays are known to over-summer in Chesapeake Bay, using the bay for pupping and reproductive efforts (Smith & Merriner 1986, 1987, Fisher 2010). Due to interference in human fishing efforts, they are considered a nuisance species in North Carolina and Chesapeake Bay, with calls 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, and they are voracious predators on mollusks (Smith & Merriner 1985, Fisher et al. 2011). Because of the crushing action of their jaws, contents found in the digestive tract are predominantly pieces of masticated tissue (boluses), shell fragments, and partially digested tissue (chyme) of a viscous and sludge-like nature. This has made species-specific stomach content analysis very difficult by traditional visual methods; other diet studies were able to identify prey to the order, family, or genus level but still found high unidentified quantities of tissue and goo (Collins et al. 2008, Craig et al. 2010, Fisher 2010, Ajemian & Powers 2011). Species-level identification of the contents of digestive tracts of cownose rays has been achieved, but the majority of findings are at higher classification level or still unknown. A large portion of the diet of cownose rays is missed because we are unable to identify the origin of the tissue and chyme found in their tracts. In order to determine whether or not cownose rays are directly depredating bivalves of commercial importance, digestive tract content analysis must be species-specific. To answer this question, I used direct sequencing

techniques to develop species-specific polymerase chain reaction (PCR)-based diagnostic tests that were used to identify species origin of tissue and chyme from the stomachs and spiral valves of cownose rays. To better understand the impact of cownose rays on shellfish populations and their role in the food web, I review the natural history, feeding ecology, and ecological impacts in the following sections. I will also review the use of the COI gene (Barcode of Life gene) as a forensic tool for fisheries and diet studies.

Natural History

Cownose rays are benthic-pelagic, and are found worldwide in warm temperate and tropical oceans and estuaries. They are euryhaline, utilizing river and estuarine habitats as well as the open ocean, where they are found on continental and inshore shelves. They can tolerate salinities as low as 5-7 ppt, frequently are encountered in salinities ranging from 17 to 37 ppt (Neer et al. 2007), and utilize rivers for a substantial part of the year in southern Florida (Collins et al. 2008). They can tolerate very low levels of dissolved oxygen in the Gulf of Mexico and were positively associated with hypoxic habitats and found near the edges of the hypoxic zones, where low dissolved oxygen (1-2 mg/L) levels were recorded at the bottom (Craig et al. 2010). Cownose rays also spend time at the water's surface, which makes them fairly easy to spot, especially while migrating. They are gregarious, forming very large aggregations for migrations (Blaylock 1989, Blaylock 1993). They range from southern New England to South America (Brazil) in the Atlantic and throughout the Gulf of Mexico and Cuba (Bigelow et al. 2002). Two separate populations of *Rhinoptera bonasus* have been identified in the United States: one along the Western Atlantic and the other in the Gulf of Mexico (Neer & Thompson 2005).

There has been a phylogenetic controversy regarding the classification of cownose rays since competing classifications have existed for all batoids. This has made life history and comparative studies difficult. Until very recently, there was a critical need for a more thorough phylogenetic and morphological analysis of batoids (Rocco et al. 2007). According to recent work by Aschliman and colleagues (2012), *Rhinoptera* species make up their own family, Rhinopteridae. The cownose rays are also most closely related to the Mobula rays (ie. *Mobula hypostoma* of the Family Mobulidae) (Deagle et al. 2009). Estimated divergence times between the two genera ranges from 30-13 million years ago (MYA), putting their emergence solidly in the Tertiary period (Aschliman et al. 2012). The origin of the major clade of the pelagic rays (bat rays, eagle rays, manta rays, and cownose rays) was approximately 75 to 65 MYA, at the boundary of the Cretaceous and Tertiary periods (Aschliman et al. 2012). There are seven species in *Rhinoptera*, but little is known about many of the other species. Most of the classification work is based on number of teeth plate rows or series, and there is a critical need for phylogenetic revision of the family (Last & Compagno 1997).

Longevity estimates based on vertebral sections indicate an age range of 0-18 years for individuals captured; theoretical life expectancy is estimated at 20-40 years (Neer & Thompson 2005, Fisher 2010). Maximum recorded length of disc width is 213.3 cm. Mean size at maturity and age of sexual maturity differs between the two populations. In the Gulf of Mexico population, sexual maturity is at approximately 4-6 years old, indicated by a mean disc width of 64.2 cm for males and 65.3 cm for females (Neer & Thompson 2005). Cownose rays in the Atlantic population reach sexual maturity at mean disc width of 75-85 cm for males and 85-92 cm for females (Smith & Merriner 1986).

Cownose rays are sexually dimorphic, with the females being larger than the males. Females exhibit uterine viviparity, also known as aplacental viviparity, as a reproductive system (Smith & Merriner 1986, Fisher 2010). Fertilization is internal and young develop internally until birthed. Females usually have litters of 1 young at a time, although twins have been observed in approximately 1-3 % of cases (personal observation, Fisher 2010). Females have paired ovaries and uteri, but only the left ovary develops fully upon sexual maturity. Although there are exceptions, in most cases it is the left reproductive tract (uterus and ovary) that is the only one functional (Fisher 2010). The right ovary does not develop or has reduced functioning, and the right uterus is under-developed or reduced in size. In males, paired testes are both functional, although the left is larger than the right (Fisher 2010). Gestation of young is 11-12 months (Smith & Merriner 1986, Neer & Thompson 2005). The reproductive mode in cownose rays is ovoviviparity, with the developing embryos absorbing the yolk sac for nourishment. Once the yolk sac is absorbed entirely, the embryos are fed by secretions called histotroph through the uterine wall (Smith & Merriner 1986, Compagno 1990, Fisher 2010). Chesapeake Bay is utilized for birthing and fertilization, with young born in June and July and gestation of the next set of embryos beginning in August (Smith & Merriner 1986, Fisher 2010). Initial evidence indicates that cownose rays are also using North Carolina estuaries for birthing and fertilization; pregnant females and neonates have been collected in North Carolina (personal observation). With such low numbers of young, late age at maturity, and long gestation periods, cownose rays have some of the lowest fecundity rates of sharks and rays (Fisher et al. 2013).

Cownose rays undertake cyclic migrations in the spring and fall. The migratory groups have been estimated between 10,000 – 5,000,000 individuals and are generally segregated by age (Smith & Merriner 1985, Blaylock 1989). The Gulf of Mexico population migrates clockwise in

the Gulf while the Western 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 & Merriner 1986, Craig et al. 2010). The extent of the southward migration is relatively unknown; tagging evidence and nursery area research indicates that they may head as far south as Brazil (Schwartz 1964, Yokota & Lessa 2006), but overwintering in southern Florida is more likely (Smith & Merriner 1987, Grusha 2005). Although data from the tags were incomplete or corrupted, in one study there was enough information to place 3 of 5 adult females tagged with Pop-up Satellite Archival Tags (PSAT) in Chesapeake Bay as far south as southern Florida, where they likely overwintered (Grusha 2005). It appears as though temperature plays a significant role in migratory timing, with individuals rarely being captured in water temperatures below 15°C or above 30°C (Collins et al. 2008). Lethal minimum temperature for the cownose ray is 12°C (Schwartz 1964, Collins et al. 2008). Neer and colleagues (2007) found that the population of cownose rays in northern Florida migrates once the water becomes too warm. Evidence suggests that there is a predominantly resident population in southwest Florida, and within this population, smaller scale migrations take place within Florida. Salinity levels, flow rate, reproduction, predator avoidance, prey availability, or other factors may be contributing to small-scale and oceanic migrations (Collins et al. 2008).

In Chesapeake Bay, the migratory pattern includes a migration into the Bay in May and over-summering until October. Chesapeake Bay is known as a historical summer seasonal residence for cownose rays (Blaylock 1993). Migratory groups are age- and sexually- segregated upon entrance into Chesapeake Bay (Blaylock 1989, Smith and Merriner 1985, Fisher 2010); aggregations in Chesapeake Bay during the summer are based on size, sex, and reproductive stage (Smith and Merriner 1987, Fisher, 2010). Cownose rays migrate northwards along the

North Carolina coast in the late spring and early summer, and then southwards in the late summer to fall, although recent evidence indicates that there may also be a semi-permanent population 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, communication with fishermen, residents, and other researchers). Cownose rays are sighted in North Carolina waters from April to October and possibly even through the winter.

Feeding Behaviour & Ecology

Cownose rays exhibit the feeding strategy of durophagy, where the majority of the diet is made up of hard or shelled organisms. Durophagous predators feed on hard-shelled prey, and in the case of cownose rays, the shell is crushed during predation (Fisher et al. 2011). Cownose rays primarily prey upon shellfish and crustaceans, which they excavate from infaunal and epifaunal benthic habitats. Historically in Chesapeake Bay, primary prey species are thought to be oysters (*Crassostrea virginica*) and hard clam (*Mercenaria mercenaria*) (Smith & Merriner 1985), although a small study found soft shell clams (*Mya arenaria*) to be the dominant prey item on seagrass beds (Orth 1975). Fisher (2010) found primary prey species to be thin-shelled bivalves like the soft shell (*Mya arenaria*), macoma (*Macoma balthica*), and stout tagelus clams (*Tagelus plebeius*), along with crustaceans (crabs, shrimp, and worms). Commercially important oysters and hard clams were found only in rays collected over commercial oyster aquaculture areas; oysters made up 1% of bivalves found in rays collected by fishery-dependent methods and 7% of bivalves found in rays collected by fishery-independent methods (Fisher 2010). Hard clams made up 3% of the bivalves found in rays caught in commercial oyster areas and by fishery-independent methods. Dominant prey items found in the cownose ray digestive tracts

were site-specific, in that the dominant prey items reflected the dominant types of benthic organisms found at sites sampled (Fisher 2010). Thin-shelled bivalves and crustaceans dominated diets of cownose rays and benthos at the sites sampled. Quantity and distribution of specimens suggests that cownose rays selectively depredate high abundance species (Fisher 2010). This is consistent in Smith & Merriner (1985) findings of soft shell clam, Baltic macoma clam, and stout tagelus clam as being found in highest frequency; oysters were found in only 2.5% of all stomachs and hard clams in 7.5% of all stomachs in that study. Diet studies in the Gulf of Mexico found that cownose rays exhibit feeding strategies across the range from generalist to specialist, often exhibiting ontogenetic-specific and habitat-specific feeding preferences (Ajemian & Powers 2011). In North Carolina, razor clams, oysters, and unidentified mollusks have been identified as traditional sources of prey (Smith & Merriner 1985); a key food source for cownose rays during migration has been Atlantic bay scallops (*Argopecten irradians concentricus*) (Peterson et al. 2001). However, the extraction and mastication feeding process of cownose rays makes species-specific prey identification difficult through traditional visual methods, with many prey items only being identified to the family or genus level. Studies in Chesapeake Bay and the Gulf of Mexico found that anywhere between 20-70% 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 & 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).

Cownose rays feed on intertidal and subtidal flats during high tide, and this shallow-water feeding is observed by humans as water roiling or boiling on the surface as well as the exposure

of the pectoral fins. Feeding behaviour is characterized by circular depressions in the sand, sediment clouds, and sediment plumes observed downstream from the feeding area. Feeding depressions are bowl-shaped with a cavity in the center of the bowl-shape (Smith & Merriner 1985). Seagrass beds are key nursery areas for juvenile fish and crustaceans, and key habitat for bivalve species like Atlantic bay scallops (Peterson et al. 2001). Damage to seagrass beds can be caused during the foraging and feeding behaviour of cownose rays (Smith & Merriner 1985).

Cownose rays have modified pectoral fins with fleshy cephalic fin lobes that are paired anterior fin extensions and that extend past the head itself; the fin lobes have mechanotactile and electrosensory pores on the surface and are used to locate prey in the sediment (Sasko et al. 2006). Pectoral fins are used to excavate the sand through stirring motions, and this movement appears to create part of the feeding depressions (Smith & Merriner 1985). Although it was previously thought that the pectoral fins were entirely responsible for the feeding depression, Sasko and colleagues (2006) found that the actual excavation activity is caused by hydrodynamic processes by the jaws. However, on seagrass beds, the feeding depressions can be one meter in diameter (Orth 1975), and so pectoral fin activity almost certainly contributes to the overall size of the depression. The use of pectoral fins to help excavate prey is probably specific to substrate type and depth of prey, but this relationship has yet to be tested. The cavity at the center of the bowl-shape in the depression appears to be caused by the suction feeding mechanisms of the rays (Smith & Merriner 1985). Suction feeding occurs when the cownose ray places its mouth over the prey and opens its jaws quickly, creating pressure in the orobranchial chamber.

After locating the prey, the excavation process occurs through hydraulic action by the jaws. Water is repeatedly ejected from the jaws to soften and move the sand. This occurs through a series of opening and closing of the jaws. After being excavated, the prey item is

enclosed by the cephalic fins and the suctioning mechanism of the jaws causes the prey to move from the cephalic fins to the mouth. Water, sand, and prey are sucked into the mouth only, the prey item is crushed and the indigestible parts removed from the digestible parts through a series of mouth movements and internal hydraulic action, and the sand is expelled out the gill slits as the ray swims away (Sasko et al. 2006).

Cownose rays have plate-like teeth used for crushing shells and appropriate for the durophagous diet. Depending upon the type of mollusk and hardness of shell, the shell is either spit out or crushed and swallowed (Smith & Merriner 1985). Shell fragments and other hard parts of prey items are found in the digestive tracts of cownose rays, in both the stomachs and spiral valves (Fisher 2010, personal observation). Soft tissue of prey items are crushed during the mastication process, and so appear in a stomach content analysis as chunks of tissue, hard parts, sludge, and fluid. The freshly masticated tissue is known as bolus and the partially-digested tissue and sludge found in the stomach and spiral valve is chyme. The crushed nature of shell fragments, hard parts, and soft tissue in stomach contents makes species-specific identification very difficult through traditional, visual methods. Over the last 40 years, cownose rays have been blamed for the wholesale depletion and destruction of commercially important shellfish populations, yet current diet studies do not support that claim (Fisher et al. 2011). Further, those claims cannot be properly investigated, nor can cownose ray feeding ecology be better understood, without species-level identification of stomach contents.

Ecological Impact

Throughout history, humans have targeted and hunted apex predators, or top predators, thereby decreasing their population numbers and creating an imbalance in the ecosystem. When the apex predator is removed from a system, or the numbers severely decreased, the effect is that the prey species for that predator is left unchecked (Myers et al. 2007, Prugh et al. 2009). This is referred to as a trophic cascade (Pauly et al. 1998, Terborgh et al. 2001). In most cases, the population of the prey species booms until it reaches carrying capacity and population size is limited again by other ecological factors.

Cownose rays are thought of as a classic example of a prey species that has increased due to a trophic cascade and the phenomenon known as “The Rise of the Mesopredator” (Prugh et al. 2009). Mesopredator is the term used for the species directly preyed upon by apex predators, and therefore the “middle level” predator in a food chain. A boom in the mesopredator population impacts the prey species of the mesopredator: predation pressure and consumption increases and population levels subsequently decrease. In some cases, this is a very dramatic cycle. The imbalance created by removing or limiting apex predators has cascading impacts throughout all the trophic levels in the food web. To further confound the issue, there are also indications that decreased numbers of apex predators can have an impact on the predatory behaviour of the mesopredator and their perception of risk (Heithaus et al. 2008). With decreasing apex predation pressure, it is “safer” for mesopredators to access feeding grounds or areas that they did not previously utilize due to high predation risks. Additionally, under decreased apex predation pressure, more time may be spent foraging and feeding than was spent while the risk of predation was higher (Heithaus et al. 2008). Thus, mesopredators may actually

change their predatory behaviours while under less direct predation pressure themselves. This may contribute to increased predation efficiency as well as decreased prey population sizes.

Some studies in the last decade found that numbers of large sharks (apex predators) have declined dramatically from the 1970s in East Coast waters of the United States (Myers et al. 2007), although these findings are highly contested (Burgess et al. 2005, Myers et al. 2007). Myers et al. (2007) found that mesopredator population levels increased in turn, especially the population numbers of cownose rays. The population size of cownose rays on the East Coast has been reputed to have increased in size to as many as 40 million individuals from 1970 to 2007, possibly due to a reduction of predation pressure (Myers et al. 2007). It is also possible that numbers of cownose rays in North Carolina and Chesapeake Bay have increased due to the addition of turtle exclusionary devices (TEDs) on shrimp trawls, thereby decreasing bycatch mortality from commercial fishing (personal communication with J. Smith and shrimp fishermen). It was hypothesized in the 1970s that population increases of cownose rays in Chesapeake Bay were due to the decline in commercial haul seine and pound net fisheries (Oesterling 2006).

Numbers of cownose rays in North Carolina were found to have increased in 2008-2009, but from 2003 to 2007 the population numbers held steady (Goodman et al. 2010). That study used aerial surveys and the North Carolina Division of Marine Fisheries (NCDMF) fishery independent gill net survey to investigate the cownose ray population variability and seasonality in North Carolina estuaries. The reported increase in population levels of cownose rays could be related to a number of environmental variables, and further study is needed to determine if the population is indeed increasing in North Carolina (Goodman et al. 2010). It has been hypothesized that low fecundity and late age at maturity indicate that population growth is due to

a decrease in predation (Myers et al. 2007). However, changing environmental conditions, commercial fishing regulations, as well as changes in habitat use during the summer months in North Carolina, may also have resulted in increased population numbers in North Carolina that do not reflect an actual increase in the overall East Coast population size (Goodman et al. 2010, personal observation).

One particularly popular and well-reported finding from the Myers et al. (2007) study was that the Atlantic bay scallop population numbers decreased as the number of cownose rays increased. In 2004, the bay scallop fishery closed to human harvesting. The NCDMF Bay Scallop Fishery Management Plan Amendment 2 lists the bay scallop stocks as a concern, because the abundance of scallops is low and an assessment is incomplete or unavailable. Furthermore, the bay scallop has been classified as a species of concern or a depleted species by the NCDMF since 2000 (NCDMF 2013). Bay scallop increases in abundance were observed in 2009, indicating a slow recovery is in place as of 2010 (NCDMF 2010). As of January 2013, limited areas were re-opened for bay scallop harvest (NCDENR-Proclamation SC-01-2013) (NCDENR 2013). It is important to point out that this increase in bay scallop stocks coincides with the reported increase in cownose rays in North Carolina waters.

Atlantic bay scallops inhabit seagrass beds that are patchily distributed and found in coastal lagoons of high salinity and low turbidity. The timing of cownose ray migrations (mid-August through September) and localized bay scallop crashes were found to coincide in North Carolina (Peterson et al. 2001). This assumes that cownose rays are specialist predators on bay scallops, but the scientific evidence for this is lacking (Ajemian & Powers 2011). In fact, more recent cownose ray diet studies indicate that they are generalist or opportunistic predators (Collins et al. 2008, Fisher 2010, Ajemian & Powers 2011). Furthermore, if cownose rays are

over-summering in North Carolina, and they are specialist predators on bay scallops, we would expect to see a dramatic decline in bay scallop abundances from 2008 to the present. The reported large increase in population numbers of cownose rays would cause an extensive coast-wide problem during the migratory season, if in fact they do consume commercially important shellfish as reputed. They would also cause large-scale disturbance and wide-spread destruction to seagrass beds in the region, which has not been demonstrated.

Substantial efforts toward restoration of seagrass beds, oyster reefs, and recovery of shellfish fisheries have been a conservation focus in recent years. If cownose rays are negatively affecting shellfish stock recovery and habitat restoration, additional life history and feeding ecology data about cownose rays could be used in regional bivalve and seagrass conservation efforts. Life history and feeding ecology of cownose rays in the estuaries of North Carolina have yet to be fully investigated, all of which would provide important regional multi-species conservation and management information.

COI (Barcode of Life) gene and use for species identification

The cytochrome oxidase subunit I (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 generally found as a circular molecule, and it exhibits gene arrangement that is compact, but also shows a large amount of variation of gene order among and between taxa (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). Mitochondrial DNA is typically inherited from the

maternal line, so the organism inherits its mother's mitochondrial sequence. A notable exception to this is the doubly uniparental inheritance (DUI) of mitochondrial DNA found in bivalves, where the male form of the genome is passed from father to son while the female form is passed to both sons and daughters (Doucet-Beaupre et al. 2010).

The COI is described as the "barcoding gene" because of the usefulness of this gene in species identification (Hebert et al. 2003). Ribosomal genes 12S and 16S are also very useful for phylogenetic analyses and identification, but the rate of evolution in the COI gene is three times higher than that of the 12S or 16S (Knowlton & Weigt 1998, Hebert et al. 2003). This is due to the base substitutions that tend to occur at the 3rd position nucleotide (Hebert et al. 2003), which changes the nucleotide sequence but not necessarily the amino acid coded by those nucleotides (codon) (Folmer et al. 1994). Changes in the amino acid sequence of the COI gene happen at a slower rate than other mitochondrial genes (Lynch & Jarrell 1993). As a result of all these characteristics, 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).

Another benefit to using the COI gene for species identification is the availability of universal primers LCO1490 and HCO2198 (Folmer et al. 1994), which successfully amplify a region of the COI that is approximately 700 base pairs in size. These primers were shown to work across diverse phyla, from Echinodermata to Annelida and Arthropoda to Coelenterata (Folmer et al. 1994). This portion of the COI gene is used in barcoding efforts, phylogenetic analyses, and for species identification.

All of these characteristics have made the COI an important tool for species-specific identification of unknown specimens, as well as a useful tool for assignment of organisms to

higher taxonomic levels. Differences in the amino acid sequence of the transcribed COI gene are adequate to designate organisms to high taxonomic levels, such as Order and Phylum (Hebert et al. 2003). After assigning an organism to a higher taxonomic group, it is possible to identify and analyze nucleotide substitutions in the “barcode of life” region of the COI, and thereby determine the species of that organism based on the substitutions found in the sequence. It is also possible to use a COI sequence of an individual organism and assign that organism to a higher-order taxonomic group, based on COI profiles compiled from that group, as well as to the appropriate species designation. These species assignments, based on the COI sequence, were successful at a 96% success rate for assignment to higher order level and 100% success rate for assignment to the species level for Lepidoptera (Hebert et al. 2003). The Barcode of Life Data System (BOLD) is a barcode reference library for collection of COI sequences (Ratnasingham & Hebert 2007), and this has also made conservation of genetic biodiversity, as well as species identification, more readily accessible.

The use of the barcode of life region for identification of species is appropriate for my study of cownose rays diets for some specific and important reasons. Firstly, cownose rays eat mollusks, but species-level identification is difficult based on traditional diet analysis methods. Secondly, the barcode of life approach has been widely used to identify mollusks. The barcoding region of the COI gene has been used to identify oysters to the species level in a multiplex PCR reaction (Wang & Guo 2008), determine evolutionary relationships between groups of clams found in extreme habitats (Peek et al. 1997), and test for specific target species of bivalve larvae from a mixed species plankton sample (Hare et al. 2000). Thirdly, the approach has been used to identify various species of fishes, and the COI sequence of cownose rays needed to be incorporated into this study to test for presence of predator DNA. The barcode

region of the COI gene has been used for identification of fish and fish products, particularly meat and eggs sold commercially (Ward et al. 2005). Finally, 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. Previously, genetic approaches to this identification problem included the use of mitochondrial DNA, which was extracted and amplified using restriction PCR analysis to identify stone flounder (*Kareius bicoloratus*) tissue in sand shrimp (*Crangon affinis*) stomachs (Asahida et al. 1997). More recently, the “barcode of life” approach has been applied to the problem. This approach was used to amplify the COI gene of unknown tissue from stomachs of broadnose sevengill (*Notorynchus cepedianus*) sharks and compare it 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). Putative whale tissue in Greenland shark (*Somniosus microcephalus*) stomach samples were screened against microsatellite markers and used to identify samples as minke whale, when compared with a register of individual minke whale markers (Leclerc et al. 2011). These new techniques are dependent upon a pre-existing library of known species sequences and primers. No one approach or technique is best for all systems, species, or questions. I used the barcode of life region of the COI gene (Hebert et al. 2003) because of the widely available BOLD database and

a published set of universal primers to amplify the COI gene (Folmer et al. 1994) in order to obtain presence or absence data for each of the target bivalve species. Once developed, this genetic approach would allow me, rapidly and conclusively, to determine the trophic links to cownose rays, for the bivalve species tested, and could be applied to food web models.

Objectives & Goals

The goal of the project is 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 include 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 unique test for identifying specific species in mixed species samples from North Carolina estuaries and coastal waters as well as samples from Chesapeake Bay. Based upon previous studies of cownose rays, I predict that there will be an ontogenetic or size-related change in feeding preferences toward these bivalves. Small cownose rays are predicted

to consume mollusks, but only the small non-commercial species of my target species of bivalves, such as cross-barred venus clam, Baltic macoma, and soft-shell clams; the large rays are predicted to eat large commercially valuable bivalves, such as hard clams, soft-shell clams, and bay scallops, as well as the others. I do not predict that cownose rays regularly consume Eastern oysters in the wild when on natural oyster habitats (reefs and hard substrate). Cownose rays have been observed eating oysters in laboratory settings, enclosure studies, and aquaculture sites, where oysters are small and placed horizontally on the sediment, but that does not replicate the natural oyster habitat.

Table 1. Target bivalve mollusk species in this study, each reportedly eaten by cownose rays.

Common name	Scientific name (abbreviation used)	Commercial importance	Habitat
Atlantic bay scallop	<i>Argopecten irradians concentricus</i> (<i>Aic</i>)	Yes, US fishery \$1,957,430 in 2011 NC landings \$1,107,072 in 1980, fishery closed in NC and VA 2011.	High-salinity seagrass areas
Baltic macoma	<i>Macoma balthica</i> (<i>Mba</i>)	None, but ecologically important	Low-salinity unvegetated areas
Cross-barred venus	<i>Chione cancellata</i> (<i>Cca</i>)	None, but ecologically important	High-salinity seagrass areas
Eastern oyster	<i>Crassostrea virginica</i> (<i>Cvi</i>)	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	<i>Mercenaria mercenaria</i> (<i>Mme</i>)	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	<i>Mya arenaria</i> (<i>Mar</i>)	Yes, US fishery \$21 million VA fishery \$86,715 in 1966, none landed in NC in 2011	High and low salinity unvegetated and seagrass areas
Stout tagelus clam	<i>Tagelus plebius</i> (<i>Tpl</i>)	Undocumented, human consumption reported	High-salinity unvegetated and seagrass areas

Methods and Materials

Due to the multiple and interconnected objectives of this study, the methods used fell into the general categories of collection methods, digestive tract content analysis, and genetic methods.

Collection Methods

Cownose rays were captured and collected in waters of North Carolina and Chesapeake Bay (*Figure 1*). Capture locations were determined by locating schools of feeding cownose rays and capturing them during feeding. Capture locations were selected based on conversations with commercial and recreational fishermen and discussion with the NCDMF staff and identification of successful capture sites from their fishery-independent gillnet sampling program results (Goodman et al. 2010). Capture occurred by commercial fishing, recreational fishing, and scientific collection techniques. 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 (Appendix A). Individual cownose rays were captured and collected by bowfishing, haul seine, gill net, longline, or rod and reel. Latitude and longitude were taken at capture locations if at all possible. After collection, individuals were sexed, weighed, measured, and tissue samples taken. Measurements included disc width, disc length or standard length, and total length. Individuals with broken or short tails were measured and duly noted as such. Weights were not taken for individuals if more than 6 hours passed between capture by fishermen and collection and dissection of the specimen. In some cases, collection of the specimens occurred many hours or days after capture by fishermen. Varying degrees of

decomposition and bloating were of concern, especially as that could unduly influence weights measured so long after death.

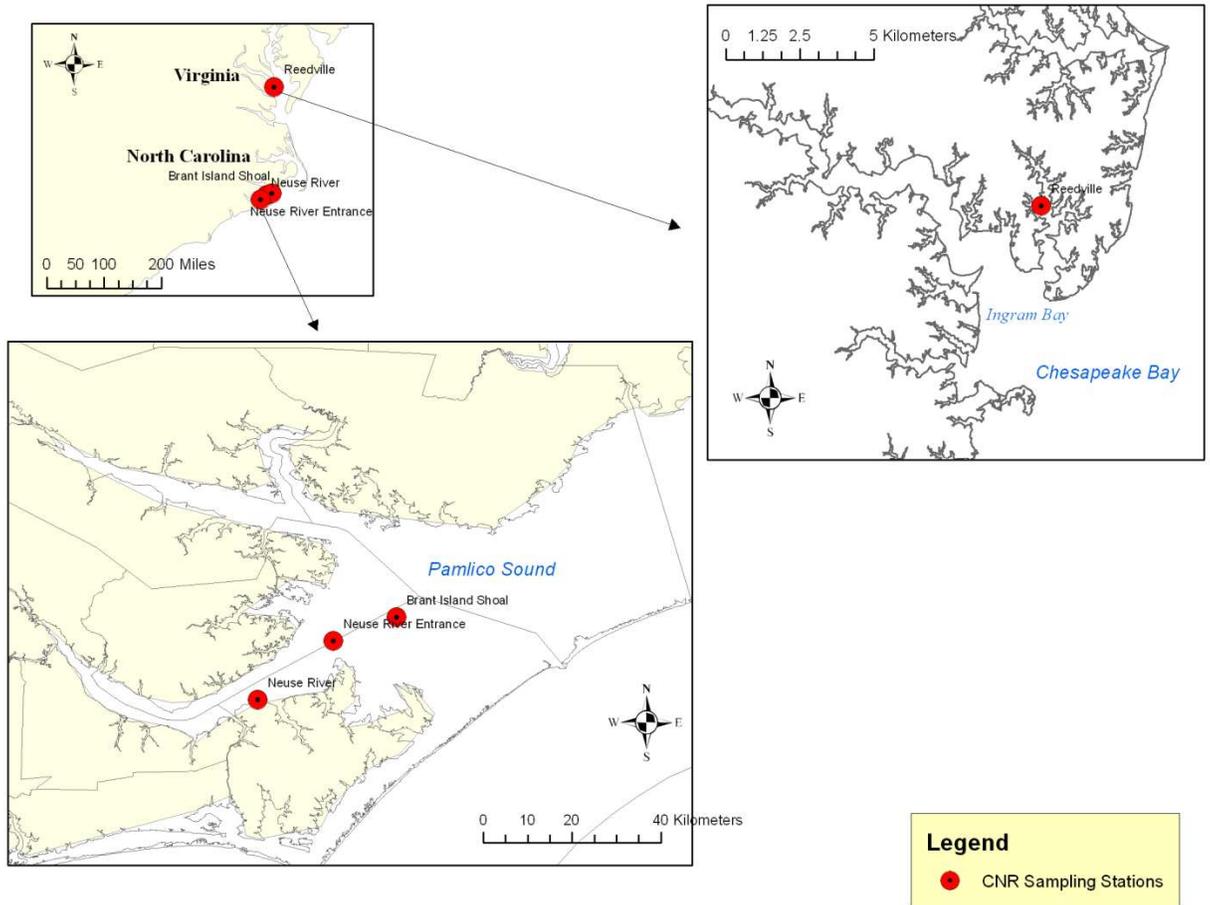


Figure 1. Capture locations of cownose rays in North Carolina and Virginia. All cownose rays captured in North Carolina were caught in the Neuse River area. The majority of the rays from Chesapeake Bay were caught at the bowfishing tournament, which occurred in the waters around Reedville, Virginia (inset,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

A minimum of 30 cownose rays were collected, sacrificed, and dissected, with individuals in two size classes (20-74 cm and 75-120 cm) represented. Sacrificed individuals were dissected in order to remove and collect the entire digestive tract, which was then bagged and frozen. In most cases, dissections occurred a few hours after death. If at all possible, those individuals were kept on ice until the dissection. In some cases, individuals were frozen or kept

on ice during transport to the laboratory facilities, where they were then stored in a freezer or dissected as soon as possible upon arrival. Fin clip and other tissue samples were frozen or stored in 95-100% ethanol and saved for future genetic studies. Sexual maturity was estimated using disc width measurement or clasper length and calcification for males and determination of pregnancy or reproductive tract development for females. This methodology followed techniques used by Fisher (2010) to maintain consistent maturity evaluation; R. Fisher measured and dissected some specimens for use in this study. When needed, the female reproductive tract and any young present were removed during dissection and stored in the freezer for future analysis. This information was cross-referenced with disc width to help determine age and size at maturity.

Digestive Tract Analysis Methods

After dissection, the stomach and spiral valve were bagged, labeled, placed on ice, and then stored frozen to stop digestion. Digestive tracts were stored frozen at -20°C until the contents were analyzed. Digestive tracts were allowed to thaw overnight in ice or in cold water for 2-3 hours. The stomach and spiral valve were analyzed separately, with contents removed from each into a separate container. Overall stomach and spiral valve content weights were taken. Upon removal of digestive tract contents, 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 categorized and organized according to color or type; weights were taken for each representative type of tissue or content. Samples were taken from as many representative types of tissue as practical. A very small (approximately 5 mm by 5 mm or smaller) piece of tissue was removed

from the larger tissue piece for DNA extraction. In cases where it was clear that the tissue was molluscan, and it was still possible to identify the inner organs of the bivalve, care was taken to avoid taking a sample from that region. The digestive tract and sex organs of the identifiable bivalves were avoided to reduce contamination of samples taken with DNA from the bivalve prey and gonadal tissue. Samples of fluid and chyme were also taken for analysis. Some digestive tracts were heavily sampled, with a sample taken from every piece of tissue found in the contents. This was only possible for some digestive tracts and was used to develop best practices.

Weights of total stomach contents and spiral valve contents were taken for all rays. However, out of 33 rays, weights per category were only recorded for stomach and spiral valve contents for 23 individuals. Contents were divided into general categories of tissue color or type, detritus/organic matter, hard shell parts, hard fish parts, and remainder of tissue, chyme, and fluid. For visual analysis, the weights of tissue categorized by color were combined with unknown chyme and fluid weights to create an unknown total tissue category. The categories of tissue color were useful for sampling for genetic analysis but not biologically relevant for a visual analysis.

After sampling was completed, the different types of tissues were recombined and the total contents were then homogenized by blender or food processor. Both stomach and spiral valve contents were homogenized, with multiple samples taken from the homogenized contents (homogenate). In some cases homogenization was not necessary (or even 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; removal of all shell fragments was not possible and so the contents could not be homogenized by blender. For a

subsample of digestive tracts, homogenization was added to the contents analysis methods as a way of testing if positive results could be obtained from a sample from which the target tissue may have been in very small proportion. This method was expected to increase the chance that all types of tissue or species consumed were more likely to be sampled. Homogenization was added into the methods in order to develop best practices, so not all digestive tracts were homogenized after sampling.

Tissue samples removed from the digestive tract contents were collected in a micro-centrifuge tube, labeled, and reserved for genetic analysis. Initially, samples were taken and stored in ethanol, until it was decided that storage treatment of samples should be tested. To develop best practices, samples were taken in triplicate and stored in different treatment methods. Samples were kept thawed and stored in the refrigerator at 4°C, in 95-100% ethanol, or refrozen and stored in the freezer at -20°C until DNA extraction. This triplicate treatment was used while processing six digestive tracts. 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 other unusual contents. When possible, prey items were identified macroscopically to the lowest taxonomic level.

Genomic DNA was extracted using a DNeasy Tissue kit (Qiagen), following standardized Qiagen extraction protocols. The cold tissue samples, stored at 4°C before processing, were extracted within 4-6 hours of sampling. Samples in ethanol or re-frozen were extracted at a later time. DNA elutions were stored in mini-centrifuge tubes at -20°C. Genomic DNA concentrations for a subsample of triplicate samples were ascertained using the BioPhotometer (Eppendorf), and multiple concentration values were acquired and averaged.

After that determination, the samples from the rest of the digestive tracts were stored and treated in the same way.

Genetics Methods

The genetics research of this project involved several related steps that built upon each other: specimen/tissue collection, DNA extraction, COI amplification, COI sequencing, primer design, primer testing and optimization, multiplex testing and optimization, and application to digestive tract contents. Methodology for some steps was a final process, whereas testing of conditions and achieving exclusivity of amplification required an iterative process of different techniques and then re-testing to determine results and any follow-up steps. The flow diagram illustrates the general components and steps to this research, as well as the order of the steps, and the techniques or tests used for each step (Figure 2).

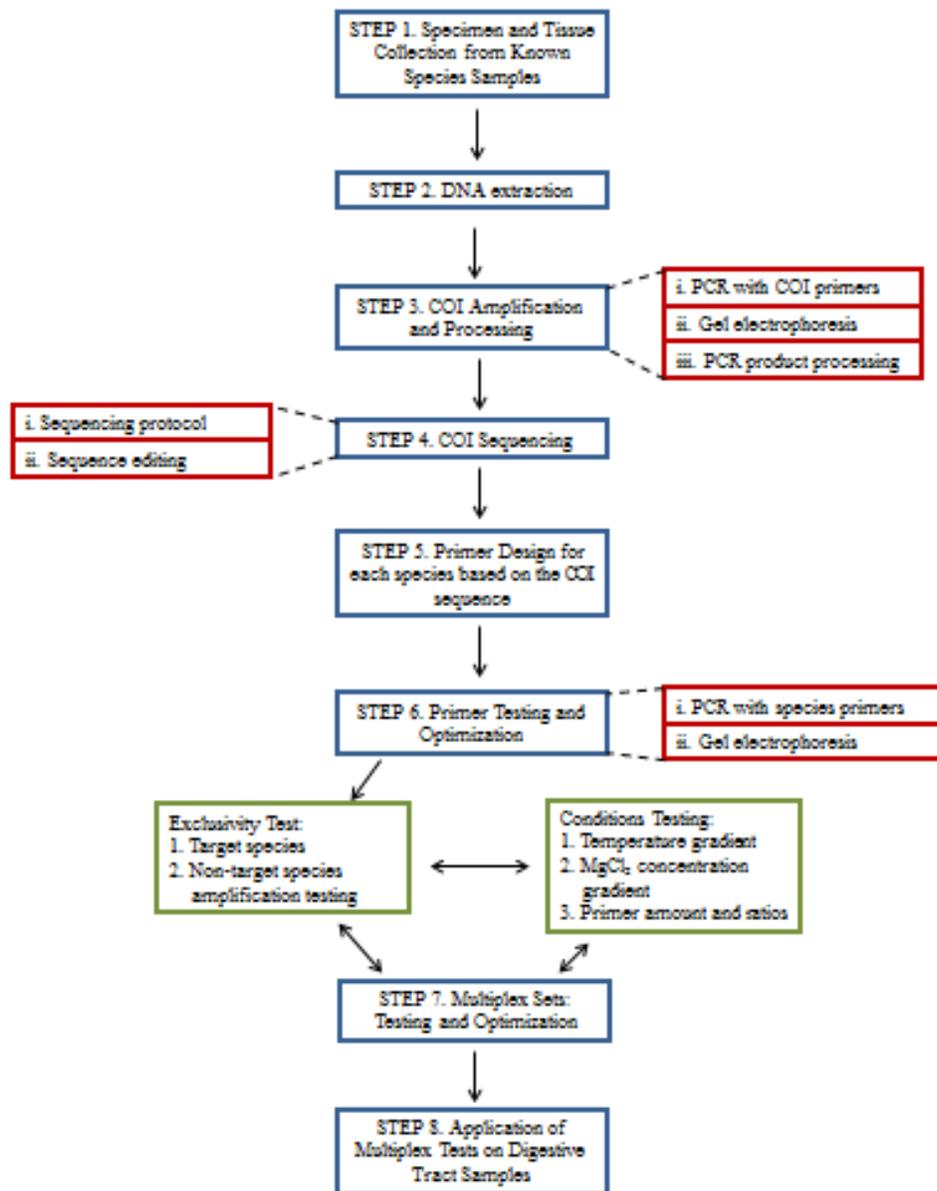


Figure 2. An overview to the genetics methods of this research are illustrated in this flow diagram, from tissue collection through to testing digestive tract samples with the multiplex sets. The arrows indicate the order followed between the steps. The blue boxes are the main steps, in the order required to complete this project. The boxes in red provide detail about the techniques or processes used to achieve that particular step. Boxes outlined in green are testing components and techniques (in this case, exclusivity tests and conditions testing) applied to achieve both primer testing and optimization (Step 6) and testing and optimization of multiplex sets (Step 7). In some cases, testing and development was an iterative process involving the use of multiple and different techniques to ensure the best results, which is indicated by the use of double-headed arrows.

Step 1: Specimen & Tissue Collection and Step 2: DNA Extraction

Individual shellfish samples were collected in locations throughout North Carolina and Chesapeake Bay, primarily in Virginia and Maryland. Whenever possible, the seven target bivalve species were collected in both locations and a minimum of two individual specimens per species were collected. 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 whenever possible. If not possible, or difficult to verify that the adductor muscle was not compromised, tissue was taken from the foot or the mantle of the bivalve. 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. Cownose ray tissue samples were removed from the frozen inner tissue of the esophagus, being careful to not use the outer tissue, which was exposed to air and bacteria, or the esophageal lining. At least two tissue samples were taken from each individual. Tissue samples were placed in mini-centrifuge tubes, labeled, and stored in a -20°C freezer. DNA was extracted using a DNeasy Tissue Kit (Qiagen), following the manufacturer's Animal Tissue Protocol. Genomic DNA samples were stored at -20°C.

Step 3: Cytochrome Oxidase I (COI) Amplification

Published sequences of the cytochrome oxidase I (COI) gene from all bivalve species except cross-barred venus and stout tagelus clams were acquired from GenBank and saved for alignment. To sequence specimens from North Carolina and Chesapeake Bay, I amplified the COI gene, using polymerase chain reaction, from each locally-collected sample. Universal primer pairs LCO1490 and HCO2198 were used on all species as a starting point (Folmer et al.

1994). Custom oligonucleotide primers were ordered from Bioneer, and concentrated primer stocks of 100 μM were made from the dried primers per manufacturer directions. Tris-EDTA (10 mM Tris and 0.1 mM EDTA) buffer was used to resuspend the primers for concentrated stock, and working stocks were diluted in distilled, deionized, DNA-free water (Fisher Scientific) to a working stock of 10 μM . Concentrated primer stocks and diluted working stocks were stored at -20°C .

i. *Polymerase Chain Reaction:*

PCR for the amplification of the COI gene totaled 10 μl volume reactions and consisted of: 5.5 μl of ddH₂O, 1 μl of 10x PCR buffer (20mM Tris-HCL at pH 8.4, 50 mM KCL), 20 mM MgCl₂, 2 mM dNTPs, 10 μM concentrations of primers HCO2198 and LCO1490, and 0.5 unit of *Taq* polymerase (Invitrogen). Reagents were mixed well upon thawing by vortex for a few seconds. All reagents were mixed by pipette upon addition to the master mix stock, followed by vortex for a few seconds to ensure complete mixing of the reagents in the master mix. Following the completion of the master mix stock, 10 μl each was added to individual PCR tubes already containing 1 μl of DNA template from different samples. The following thermocycler protocols were used as a starting point for the PCR process: 94°C for 2 min, 94°C for 30 s, 50°C for 45 s, 72°C for 1 min, and repeated for 29 cycles. A final extension step of 72°C for 5 minutes was used, followed by a 15°C hold.

ii. *Gel electrophoresis:*

PCR products were separated by electrophoresis on a 2% agarose gel stained with Ethidium Bromide. PCR products, either the entire 11 μl of product or 3 μl of the product, were loaded

into the gel with a small amount of loading buffer (0.5-2 μ l 6X buffer). If only a portion of the product was loaded in the gel, the remainder of the product was stored at -4°C for future use. One hundred base pair ladders were used as a reference. Gel electrophoresis was generally set to an initial securing setting of 96 or 103 volts and then lowered to 90 or 70 volts, depending upon the size of the gel. The gel was allowed to run for 1-2 hours, and then removed for imaging. The gel was imaged using the Kodak Gel Logic 100 with Carestream molecular imaging software or the Alpha Innotech Red imaging machine. Images were obtained, saved electronically, and printed for future reference.

Only the PCR product from specimens that amplified and imaged bands clearly and sharply were reserved and stored for future sequencing. COI sequences are approximately 700 base pairs in size, so if amplification occurred successfully during PCR, the band on the gel would be at the 700 base pair location in comparison to the reference ladder. If the sample did not have a distinct band at that size location, the amplification temperature and master mix reagent amounts (i.e., MgCl_2) were changed to optimize the PCR conditions for that species or specimen. If necessary, a temperature gradient in the thermocycler was applied to test for ideal amplification temperatures.

iii. *PCR Product Processing:*

PCR product clean-up and preparation for sequencing occurred through the use of two different methods, gel purification by the UltraClean GelSpin DNA purification kit (MO-BIO) and ExoSAP-IT (Affymetrix). In the first method, the band of interest on the gel was cut out and DNA isolated from it using an UltraClean GelSpin DNA purification kit (MO-BIO) per

specification of the manufacturer's protocol. DNA re-isolated from the gel was measured for concentration using a BioPhotometer (Eppendorf) before the sequencing process. The alternative process, used later in the project due to initial difficulties obtaining clean sequence results, involved the use of small volumes (1-2 μ l) of ExoSAP-IT (Affymetrix) to clean up PCR products for sequencing; procedures were completed using manufacturer's protocols. The ExoSAP-IT cleaned up the excess primers and free nucleotides in the PCR product, which was then used directly in sequencing reactions.

Step 4. COI Sequencing

i. Sequencing reactions

Sequencing reactions and procedures occurred in-house at the Core Genomics Facility at East Carolina University, using BigDye Terminator v3.1 chemistry and the 3130xl Genetic Analyzer (Applied Biosystems). Sequencing of the COI gene on all samples occurred with the use of the Folmer et al. 1994 primers, HCO2198 and LCO1490. The veneroidLCO primer, designed by Dr. Erik Pilgrim for COI amplification in veneroid clams, was also used to amplify and sequence the COI gene. Follow up sequencing of a select number of samples of each species was conducted using protocol of Dr. Erik Pilgrim of the Molecular Ecology EPA lab in Cincinnati, Ohio. Dr. Pilgrim's master mix protocol included 9.9 μ l of ddH₂O, 4 μ M dNTPs, 2.0 μ l of 10x buffer with MgCl₂, 9 mM MgCl₂, 5 μ M each of primer 1 and 2, 4.0 μ l of 1x BSA, and 0.5 unit of *Taq* (Qiagen). Total volume of 18 μ l master mix was added to 2 μ l of DNA template. The thermocycler program used an initial start setting of 94°C for 2:30 minutes, then 35 cycles of 94°C for 30 seconds, 46°C for 60 seconds, and 72°C for 60 seconds. An extension step of 72°C for 10 minutes concluded the program. PCR products were loaded into a 2% agarose gel,

products separated by electrophoresis, and imaged by Dr. Pilgrim. Dr. Pilgrim used primer sets LCO1490 and HCO2198, veneroidLCO and HCO2198, and primer sets for the 16s gene on all samples. If PCR products amplified, then PCR products for those samples were cleaned and sequenced by the Sequencing facility at the Environmental Protection Agency lab in Cincinnati, Ohio. Sequence results were edited and assembled by Dr. Pilgrim using Sequencher software (GeneCodes).

ii. *Sequence Editing:*

All sequence results were trimmed and assembled using Sequencher software. Sequence results for each species, as well as published sequences from GenBank (referred to as reference sequences), were compared to each other using Sequencher. Alignment of reference sequences and sequence results was conducted using the MUSCLE alignment program (Edgar 2004) and the Se-Al program (Rambaut 1995). Alignment was conducted using the MUSCLE alignment program and then imported into Se-Al software for further manipulation. Alignment positioning was confirmed using the amino acid and protein settings on Se-Al. Any necessary adjustments to the alignment were made manually in Se-Al. Alignment to reference sequences was conducted, as well as alignment of all sequences for each species to all other sequences for all species. For each species, a minimum of one reference sequence and three sequences from known individuals were aligned and used to design primers. Any and all genetic diversity found in the sequences, i.e.) nucleotides differing between individuals of the same species, was recorded as locations to avoid in primer design. Also noted were obvious gaps or positions in the sequence different enough from the other species so as to be a good location for primer design.

Step 5. Species-Specific Primer Design

Primers were designed to amplify PCR product sizes sufficiently large enough, and different enough in size to the other species, to be visualized and identified on an agarose gel. Primers were also designed to result in products of different sizes so that some primer sets could be combined in one reaction. Based on our sequencing results, I designed primers that were likely to amplify only a single species among the target species of interest. I visually inspected alignments to identify regions where each species had a distinctive sequence, and designed the 3' primer ends to target these mismatched regions (Wang and Guo 2008). Sequences and respective alignments in Se-AI were used to cross-reference primer candidates. Primers were designed using the Primer3 website; conditions selected included optimum annealing temperature of 60°C, minimum at 57°C and maximum at 63°C. Parameters for primer size included minimum base pair size of 18 and maximum of 27; the parameter for GC% content was set to an optimum of 50%. Product size ranges were listed at 600-100 base pairs to start with, and then subsequently smaller size ranges from there. Size ranges requested changed based upon the species and multiplex set. All other parameters were left at the default settings. The OligoCalc: Oligonucleotide Properties Calculator website was used to check for hairpin turns and self-complementarity, as well as swapping strands in order to view the reverse complement strand.

Primer candidate sequences, for the forward and reverse primers, were imported into Se-AI to compare the candidate primer sequence with sequences of the target species and all other non-target species. The ideal primer did not match the sequence of any other non-target species, most particularly at the 3' end for forward primers and the 5' end for reverse primers. If the candidate primers matched too closely, they were discarded. Occasionally, the Primer3

candidate primers were tailored or manually altered in Se-AL, in order to be adequately different to the non-target species sequences, and then imported back into Primer3 and OligoCalc to check for annealing temperature, GC content, and self-complementarity. In most cases, the forward primer was the first to be designed, and once a good match was found for that species, then the reverse primer was designed and product size determined.

Starting position location in the sequence, product size, annealing temperature, and percent GC content were recorded for each candidate primer during the design process. This allowed for cross-referencing of forward and reverse candidate primers and was especially helpful when determining best-matched primer pairs. Whenever possible, multiple primer pairs were designed for each species in order to optimize success of potential multiplex sets. Once primer pairs were finalized, oligonucleotide primers were custom ordered from Bioneer. Upon arrival, the primers were stored at -20°C until the concentrated stocks were made. Concentrated and working stocks were made using the same protocols as described earlier.

Step 6. Species-Specific Primer Testing

i. PCR with Species-Specific Primers

As an initial trial, all primer sets were tested on the species of interest (target species) as well as on DNA from the non-target species. A negative control was also included in the primer trials. Master mix protocols followed the formula of 5.5 ul of ddH₂O, 1.0 ul 10x PCR buffer, 20 mM of MgCl₂, 2 mM of dNTP's, 10 μM concentrations of each primer, and 0.5 unit of *Taq* (Invitrogen). Volume for one reaction totaled 10 μl and that master mix was added to 1 μl of DNA template. Master mix protocol and procedures were kept consistent with the PCR process

described earlier for amplification of the COI gene. The thermocycler program created was based on other programs used for similar research (Hare et al. 2000, Wang & Guo 2008), and included an initial hot start of 95°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 60 seconds. An extension time of 72°C for 5 minutes ended the program. Primer testing used the annealing temperature of 52°C as a baseline temperature for the initial trials. Trials for all primer sets used the exact same conditions to test the usefulness and amplification success of the primer sets. Primer sets were tried on two individual samples from the target species as well as one individual sample from all the non-target species (all other bivalve species and a cownose ray sample).

ii. Gel Electrophoresis

After the completion of the PCR process, products were separated through gel electrophoresis on a 2% agarose gel stained with Ethidium Bromide. For these trials, PCR products of 3 µl volume were combined with 0.5 µl of 6x loading dye buffer, and then loaded in the gel wells. Gel electrophoresis and imaging procedures followed those described earlier. For especially successful primer trials, the remaining PCR products were saved and stored at -20°C.

After trials on all primer sets were completed, results were recorded and the best candidate primer sets were chosen for each species. The primer sets were also tested on multiple samples from the target species, separated on the same gel, and imaged in order to compare relative brightness of PCR products for each species.

iii. Primer Optimization

Creating more stringent PCR conditions, and determining the ideal or most successful conditions for that set of primers, is known as optimization. To determine the most successful annealing temperature for the primers for that species, a temperature gradient PCR was first utilized. The same individual DNA sample, of a specific species, was tested with one set of species-specific corresponding primers across a gradient of chosen temperatures. The temperature gradient setting on the thermocycler allows for multiple samples to be tested at 8 different annealing temperatures at the same time. In this case, temperatures from 52-62°C were used as the annealing temperature for the PCR reaction. The products were then separated on an agarose gel, visualized, and compared to each other. Once the most successful temperature was chosen for that primer set, then the same primer set was used on all other non-target species, at that particular annealing temperature, to test for cross-amplification product in non-target species.

Step 7: Multiplex Testing & Optimization

The initial step to testing a multiplex reaction involved the addition of all primer sets for the target species to the PCR reaction. Master mix concentrations, PCR conditions, and thermocycler programs followed standardized protocols used earlier in the project and were based on the testing results specific to those species. The multiplex reaction was tested in the PCR, products separated through gel electrophoresis, and visualized. Conditions, success, and any subsequent cross-amplification were recorded for each test. Optimization involved a variety of tools, including testing different volumes of individual primers, testing primers on a temperature gradient, testing a gradient of magnesium chloride concentrations, modifying the

number of PCR cycles, testing at different genomic DNA volumes, and testing of different dilutions of mixed-species template DNA combined with DNA-free water (1/2, 1/10, 1/20, 1/40, 1/60, 1/80). Optimization was deemed complete when the target species (also called positive controls) were successfully amplified in PCR reaction, while the non-target species were not amplified in that same multiplex reaction.

Step 8: Application of Multiplex Tests to Digestive Tract Samples

After DNA extraction of the stomach and spiral valve samples, the template DNA elutions were stored in the -20°C freezer. Each sample was tested with all sets of primers for the seven bivalve species, under the multiplex PCR conditions optimized for each set. In general, the thermocycler conditions followed the settings designed for primer testing, with some minor changes specific to each multiplex set. The annealing temperature for the bay scallop and stout tagelus clam (*Aic and Tpl*) multiplex was 60°C, as well as the hard clam, soft-shell clam, and oyster (*Mme, Mar, and Cvi*) multiplex. The annealing temperature for the Baltic macoma and cross-barred venus clam (*Mba and Cca*) multiplex was 63°C; the thermocycler conditions for that set were modified to total 25 cycles instead of 35 cycles. The higher temperature and fewer cycles were used to reduce the amount of cross-amplification with non-target species DNA.

Samples and positive control samples were tested with each set of multiplex primers. Control samples included individual template DNA of the target species, mixed species DNA samples of the multiplex species, and a dilution of the mixed species sample (a 1/2 dilution or a 1/10 dilution). DNA concentrations for the positive control samples were acquired using the Tecan spectrophotometer system and utilized as a reference. A negative control, or a PCR sample without DNA present, was also utilized in each PCR reaction to test for contamination in

the master mix. The PCR products were stored in the refrigerator or freezer until they could be separated using gel electrophoresis. Product handling, loading, and electrophoresis followed the same protocols as used in previous portions of this research, in order to maintain consistency throughout the project. Gels were imaged by UV light using only the Kodak Gel Logic 100 and Carestream molecular imaging software. Photographs of all gels were printed and saved for analysis. Remaining PCR products were saved and stored at -20°C.

PCR Results and Analysis

Gel photographs were printed and used for analysis; positive results were identified from the printed gel photograph and then compared to the digital photograph using Adobe Photoshop version 11.0.2. Samples positive for one of the target species were identified by the presence of a band of the appropriate base pair size in the lane. Bands were identified as strong or faint, in comparison to the positive controls, and recorded as such. Positives and negatives for each sample, organized by cownose ray specimen number, were recorded for each target species tested.

Some samples, after PCR and gel electrophoresis, appeared on the gel as a smear that started at the well and ran down the lane. In all but two instances, there was no band present for those samples. The “smeary” samples were recorded and cross-referenced between all multiplex sets. Samples consistently smeary were identified and chosen for follow-up analysis. Concentrations of those samples were acquired using the Tecan spectrophotometer system. In cases with high DNA concentrations (> 60 ng/μl), dilutions were made from those samples to replicate an average concentration level (approximately 30 ng/μl) of the positive controls. The

diluted samples were then tested with the multiplex primer sets under the appropriate PCR conditions, products were separated by gel electrophoresis, and then imaged.

Known-Tissue Mixtures

Mixtures of tissue of known species and quantities were created and tested for primer sensitivity. Bivalve specimens of hard clams (*Mme*), soft shell calms (*Mar*), and Eastern oyster (*Cvi*) were dissected and tissues sampled. Samples were taken from the adductor muscle and the rest of the specimen was reserved for use. Adductor muscle samples were weighed and masses recorded. In 1:1:1 ratios, equal parts of adductor muscle tissue were combined together from all three species. The tissue was finely chopped and then ground in a mortar and pestle. The total amount of tissue was too small to use the blender; all effort was made to homogenize the tissue as best as possible. Small quantities of the mixture were sampled and stored for DNA extraction; DNA extractions were started the same day.

The remainder of the bivalve organism was removed from the shell and weighed. The remainder of the specimens were combined and homogenized in the blender; the remaining tissue resulted in 1:2:4 ratios. Samples were taken from the homogenate and stored for DNA extraction; the remainder of the homogenate was stored in the -20°C freezer. DNA extractions were started the same day. No effort was made to avoid the digestive tract or internal organs of the bivalve. When sampling individual tissue samples from the digestive contents, it is sometimes possible to avoid the digestive tract of a bivalve. However, this is not always the case, especially when digestive tract contents are primarily chyme and well-digested tissue. Homogenizing the remainder of the stomach or spiral valve contents would also homogenize any

bivalve digestive tracts and organs. In order to test the primer sensitivity on homogenized known samples, the whole bivalve specimen was used to replicate more natural conditions.

After extractions were completed, DNA elutions were stored in mini-centrifuge tubes at -20°C. The samples were tested with the *Mme*, *Mar*, *Cvi* multiplex primers and PCR protocol; products were separated through gel electrophoresis and imaged.

Statistical Methods

Results from the digestive tract contents tested were analyzed using multidimensional scaling (MDS) procedures. Multidimensional scaling is a visualization and ordination method for multivariate data (Dillon & Goldstein 1984). It is used to create a diagram illustrating the relationships between objects, by using a table of distances between those objects (Manly 2005). Data used in MDS can be categorical or continuous and it is not necessary for the data variables to be normally distributed. MDS is a data reduction technique and a tool used to uncover the story behind the data (Dillon & Goldstein 1984). It is a scaling method that represents the proximity between objects, and in this case, it can suggest which cownose rays are more similar. The typical problem is that of any complex, multi-dimensional problem (p -dimensions where $p > 2$), and reducing it to a lower dimensional configuration of the points that still preserves the similarities or distances between points where the dimension is 2 or 3.

A data matrix is created of the underlying variables, with cases as rows and the variables are found in the columns. Cases have multiple variables. From the data matrix a distance matrix is formed which has distances between each pair of cases. The distance matrix is symmetric with 0 values down the main diagonal. Based on an assumed dimensional configuration, coordinates are assumed for each object in the assumed dimensions, and distances between

objects are calculated (Manly 2005). The configuration distance between objects is calculated as d_{ij} , where i and j are two different objects. A regression of d_{ij} on the data distance (δ_{ij}) is calculated, and the regression used can be linear, polynomial, or monotonic (Manly 2005). The fitted distances between objects, calculated from the regression, are called disparities (\hat{d}_{ij}). Disparities are the data distances scaled to the configuration distance in as close of a match as possible (Manly 2005). “Goodness of fit” (Stress) is measured between the configuration distances and the disparities. Stress is calculated based on Kruskal’s stress index, where low stress values can be thought of as the best “goodness of fit” (Kruskal 1964).

Stress (S) is defined as:

$$S = \sqrt{\frac{\sum_i^n (d_{ij} - \hat{d}_{ij})^2}{\sum_i^n d_{ij}^2}}$$

Where n is the number of samples, d_{ij} is the distance between i and j at each iteration along the two coordinates in MDS space and \hat{d}_{ij} are the disparities, or the monotonic transformation of the data that are attempting to minimize S at each iteration (Kruskal 1964). (The stress equation reported above is the form used for this study.) If the stress measure is too high, the coordinates of each object are modified slightly so that the stress is reduced. This is an iterative process until the stress is reduced between the disparities and the configuration distances (Manly 2005). The resulting matrix of underlying variables produces a scaling with minimal stress (Kruskal 1964). The stress index is minimized and coordinates can be plotted. Those coordinates are used to graphically represent the relationship between the objects. In this study, MDS analyses were done using R (R Core Team 2012) with a multivariate package (Venables & Ripley 2002) and graphed (Wickham 2009).

MDS can be metric or non-metric, and the difference between the two is related to the type of linear regression used. Metric MDS use a linear or polynomial regression equation, whereas non-metric MDS use a monotonic regression (Manly 2005). In a monotonic regression, it is the ordering of the data distances that is important (Manly 2005). A non-metric MDS was used with these data because it is a more flexible MDS and it was more applicable for this study.

In this study, cownose rays were treated as cases and the variables entered for each case in this study were:

% of stomachs with positive results for bivalve species *i*

% of spiral valves with positive results for bivalve species *i*

% of all samples with positive results for bivalve species *i*

Categorical variables were state of origin for each cownose ray (NC or VA), capture method (bowfishing, hook & line, nets), and sex of cownose ray (M or F). Size of cownose ray (disc width in cm) was a continuous variable. The fraction positive results for each bivalve species were normalized for each cownose ray, based on total number of samples tested for each ray:

Fraction positive for i = $\frac{\text{Number of samples positive for species } i}{\text{Number of samples per cownose ray}}$. Bivalve species (shown as *i*) were

represented in the index as each bivalve species tested in this study. Separate MDS analyses were done for % stomachs positive (for each bivalve species separately), % spiral valves positive (for each bivalve species separately), and % all samples positive (for each bivalve species separately).

Results

Genetics Results

Sequences of the cytochrome oxidase I (COI) gene were obtained from locally-acquired specimens, aligned, and compared to reference sequences. Sequences were obtained for all but the Atlantic bay scallop, which I was not able to sequence successfully (Table 2). Sequences obtained were used for species-specific primer design. An example of sequence alignment with primers also aligned is shown in Figure 3. Note that the primers aligned uniquely with each species shown, minimizing any overlap in sequences among species. That allowed me to design primers that were species-specific and to prevent cross-amplification.

Primers were designed for each species and after being tested against known samples of each species, the best primer sets were identified (amplification of target species and low cross amplification of non-target species, see methods for details), optimized, and used for digestive tract sample testing (*Table 3*). The primer sets for the species that were included in a multiplex PCR reaction are listed together in three separate groups (multiplex sets). Master mix protocols for each multiplex set are found in *Table 4*. 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 non-target species. In an effort to remove all amplification of non-target species in the Baltic macoma and cross-barred venus multiplex set (*Mba* & *Cca*), the magnesium chloride levels were increased to a 50 mM concentration, the annealing temperature was increased to 63°C and the PCR protocol was reduced to 25 cycles. None of these efforts were completely successful; the *Mba* primers

consistently cross-amplified stout tagelus clam DNA and *Cca* primers cross-amplified oyster DNA. This was problematic in uncovering the presence of these species in actual digestive tract samples, in which these species could be mixed together in varying concentrations.

Table 2. Species name, location of sample, and sequence number for all sequences obtained from locally-acquired specimens.

Species	Location	Sequence Name	Accession Number
<i>Chione cancellata</i>	North Carolina	Chi2_COI	KF245610
	North Carolina	Chi3_COI	KF245611
	North Carolina	Chi4_COI	KF245612
<i>Crassostrea virginica</i>	North Carolina	Cvi1_COI	KF245599
	North Carolina	Cvi2_COI	KF245600
	Virginia	Vir3av_COI	KF245601
<i>Macoma balthica</i>	North Carolina	Bma2_COI	KF245607
	North Carolina	Bma3_COI	KF245608
	North Carolina	Bma5_COI	KF245609
<i>Mercenaria mercenaria</i>	North Carolina	Mer1_COI	KF245605
	North Carolina	Mer2_COI	KF245606
	Virginia	Merc1av_COI	KF245604
<i>Mya arenaria</i>	Virginia	Mya1av_COI	KF245602
	Virginia	Mya2av_COI	KF245603
<i>Rhinoptera bonasus</i>	Virginia	041CNR1_COI	KF245596
	Virginia	083CNR1_COI	KF245597
	Virginia	089CNR1_COI	KF245598
<i>Tagelus plebicus</i>	Virginia	Tpl1av_COI	KF245613
	Virginia	Tpl2av_COI	KF245614

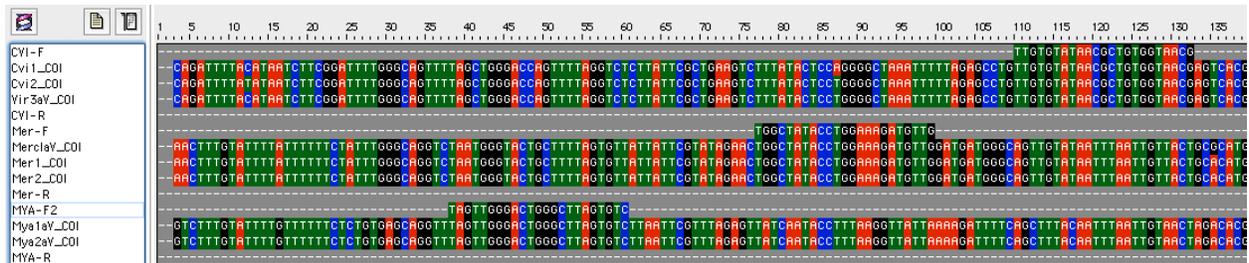


Figure 3. Alignment of a portion of the COI gene sequences for oyster, hard clam, and soft-shell clam with positions shown of forward primers for each species (orientation is 5' to 3'). Multiple sequences for each species were used for alignment and primer design. Abbreviations used in this image are Cvi or Vir for oyster (*Crassostrea virginica*), Mer and Merc for hard clams (*Mercenaria mercenaria*), and Mya for soft-shell clam (*Mya arenaria*). Forward and reverse primers are indicated by the F or R following the species abbreviation, and all primers and abbreviations are listed in Table 3. Sequence abbreviations for each species are listed in Table 2.

Table 3. Genus and species-specific PCR primers, primer name, amplification specificity, primer sequence from the Cytochrome Oxidase I (COI) gene, and base pair size of the PCR product. Primers are listed in the three multiplexed groups.

Multiplex set and Primer Name	Specificity	Primer Sequence (5'-> 3')	Size (bp)
<u>Aic & Tpl</u>			
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	
<u>Mme, Mar, Cvi</u>			
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	TGACCCAACCTCCTCTCTCAGAC	
<u>Mba & Cca</u>			
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	

Table 4. Master mix protocols designed, optimized, and used for each multiplex set. Bay scallop and stout tagelus clam (*Aic* & *Tpl*) multiplex protocols are found on the left, hard clam, soft-shell clam, and Eastern oyster (*Mme*, *Mar*, *Cvi*) multiplex is the middle set, and Baltic macoma and cross-barred venus (*Mba* & *Cca*) is the set on the right.

Multiplex Master Mix Protocols		
<u><i>Aic & Tpl</i></u>	<u><i>Mme, Mar, Cvi</i></u>	<u><i>Mba & Cca</i></u>
ddH ₂ O: 6.0 µl	ddH ₂ O: 3.5 µl	ddH ₂ O: 5.4 µl
10xPCR buffer: 1.0 µl	10xPCR buffer: 1.0 µl	10xPCR buffer: 1.0 µl
20 mM MgCl ₂	20 mM MgCl ₂	50 mM MgCl ₂
2 mM dNTPs	2 mM dNTPs	2 mM dNTPs
2.5 µM <i>AicF3</i> & <i>AicR3</i>	10 µM <i>MerF</i> & <i>MerR</i>	2.5 µM <i>BmaF</i> & <i>BmaR</i>
5 µM <i>TplF3</i> & <i>TplR</i>	5 µM <i>MyaF2</i> & <i>MyaR</i>	5 µM <i>ChiF2</i> & <i>ChiR3</i>
	5 µM <i>CviF</i> & <i>CviR</i>	
<i>Taq</i> : 0.5 unit	<i>Taq</i> : 0.5 unit	<i>Taq</i> : 0.5unit
Total volume: 10 µl	Total volume: 10 µl	Total volume: 10 µl

Primer Testing on Known Species Samples

Primers were tested for sensitivity on known species samples, quantities of 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 (Figure 4). Concentrations of DNA of the positive controls ranged from 0 - 157 ng/ μ l, with the average concentration being 31 ng/ μ l. Concentrations of DNA were found to be below the limits of detection in highly diluted control samples, but still amplified in the PCR reaction. 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/ μ l. Primers of the *Aic* & *Tpl* multiplex set amplified in positive control samples with a calculated concentration of 1.20 ng/ μ l, and primers of the *Mba* & *Cca* multiplex set were sensitive to positive control samples of 0.35 ng/ μ l concentration.

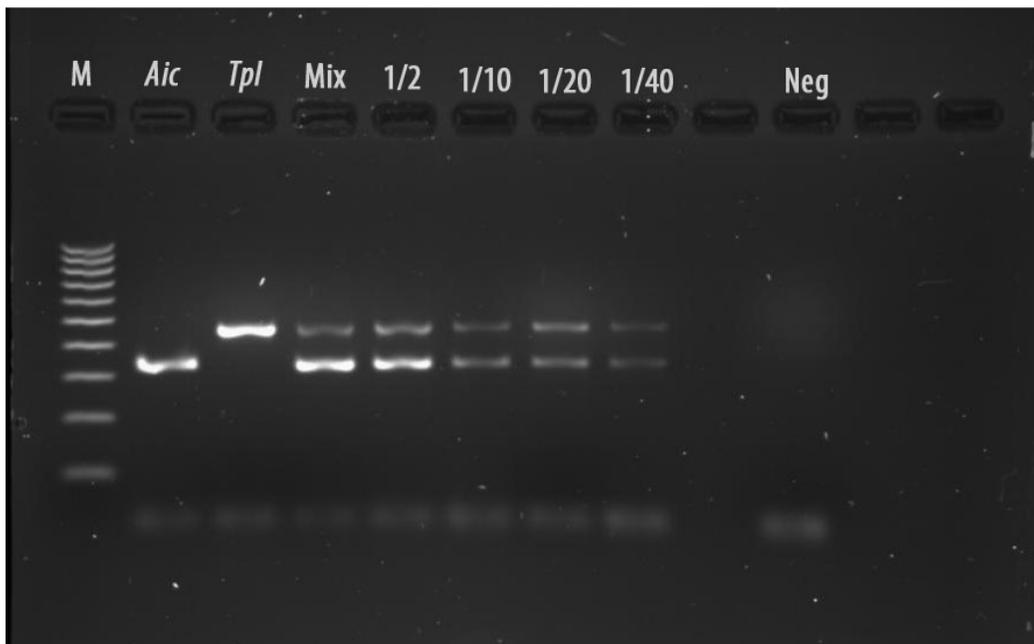


Figure 4. Gel image of the stout tagelus clam (*Tpl*) and bay scallop (*Aic*) multiplex test. The first lane M is the size marker or 100 bp ladder. The next two lanes are single-species DNA tested with the multiplex primers and conditions (*Aic* & *Tpl*). The following lanes are mixed DNA and diluted mixed DNA samples (1/2, 1/10, 1/40) of the target species, tested with the multiplex conditions to determine primer sensitivity. Neg is a negative control sample.

Known species combinations of mixed-tissues were used to test for success of the *Mme*, *Mar*, *Cvi* multiplex. Tissue samples from known bivalve specimens were removed from the shell, combined, crushed or homogenized, and tested against the *Mme*, *Mar*, *Cvi* primers. Three crushed samples of hard clam, soft shell clam, and oyster in a 1:1:1 ratio were taken and DNA extracted; those DNA elutions of known, mixed-species were tested with the *Mme*, *Mar*, *Cvi* primers. Using only 1 μ l of template DNA in the PCR reaction still resulted in successful amplification of those products (Figure 5). The remainder of the bivalve specimens were combined and homogenized in a 1:2:4 ratio of soft-shell clam, oyster, and hard clam, and six samples were removed from the homogenate and DNA extracted. The resulting DNA elutions, once tested with the *Mme*, *Mar*, *Cvi* primers, also resulted in successful amplification of those products (Figure 5). These results indicated that the hard clam (*Mme*), oysters (*Cvi*), and soft-shell clam (*Mar*) DNA could be detected using the multiplex PCR approach in a sample with unknown mixtures of these species, even if they were consumed by cownose rays in varying amounts.

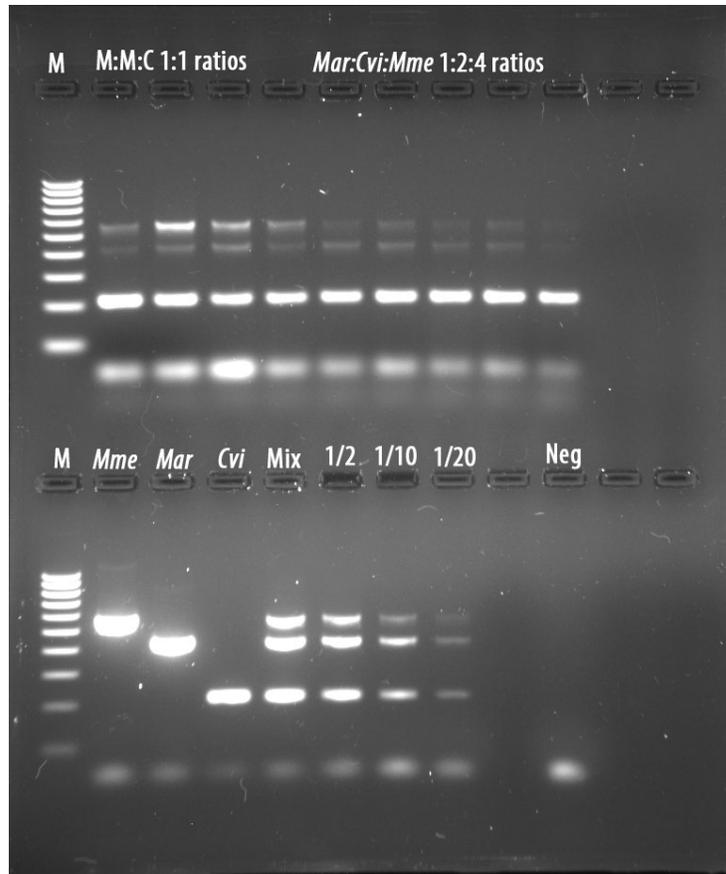


Figure 5. Gel image of the mixed-tissue combinations of hard clam (*Mme*), soft-clam (*Mar*), and oyster (*Cvi*). Mixed-tissue samples of known ratios are found on the top row. M indicates the marker or 100 bp ladder in the first lane. The next three lanes are ratios of 1:1:1 and the rest of the samples are mixed-tissues of soft clam, oyster, and hard clam in ratios of 1:2:4. The bottom row are positive controls for the *Mme*, *Mar*, *Cvi* multiplex tests, along with mixed DNA samples and diluted mixed DNA samples showing sensitivity of primers.

Storage Treatment of Digestive Tract Samples

While sampling the digestive tracts of the cownose rays, three storage methods or treatments were tested. For a subsample of six cownose rays, all samples collected from the digestive tracts were taken in triplicate. For every piece of tissue or fluid sampled, three pieces were collected from each sample. The samples were treated by cold storage, ethanol, and frozen storage. The samples kept thawed were refrigerated and then extracted the same day. After DNA extractions, 24 samples from three digestive tracts were tested for DNA concentrations. In

all but one case, the highest concentrations were found in the samples kept thawed and cold-stored until extraction. In general, the samples re-frozen had the next highest concentrations, and the samples stored in ethanol had the lowest. Average concentrations for each storage treatment were also calculated (*Table 5*). Concentrations of samples in each treatment group were compared with a one-way ANOVA and Tukey’s test and there was no significant difference between groups ($p = 0.114$), which may have been due to the small sample size per treatment group and the wide range of concentration values per group (*Table 6*). The overall trend indicated that samples stored cold with same-day extraction resulted in the higher DNA concentrations. Following testing of storage treatments, all samples were kept thawed, stored cold, and extractions started the same day.

Table 5. DNA concentrations of samples treated with cold, ethanol, and re-frozen storage treatments. Triplicates were taken of each sample and then treated with different storage methods. Multiple DNA concentrations were measured and averages are reported; total average concentrations per treatment are also reported below.

Cownose ray	Sample type	TriPLICATE Samples	DNA concentrations (ng/μl)		
			Average concentration per sample		
			Cold	Ethanol	Re-Frozen
20110618179	SV homogenate	SV3.1, 3.2, 3.3	48.7 (+/- 4.7)	0	35.3 (+/- 4.2)
20110618083	brown SV liquid	SV1.1, 1.2, 1.3	248.3 (+/- 0.6)	150.3 (+/- 1.2)	52.7 (+/- 1.2)
20110618083	red tissue	SS1.1, 1.2, 1.3	128.0 (+/- 1.0)	38.3 (+/- 1.5)	71.3 (+/- 2.1)
20110618083	bivalve tissue	SS3.1, 3.2, 3.3	65.3 (+/- 1.2)	1.7 (+/- 1.2)	20.0 (+/-1.7)
20110618083	SS homogenate	SS6.1, 6.2, 6.3	170.7 (+/- 1.2)	23.0 (+/- 1.0)	57.0 (+/- 6.1)
20110618039	gray/white tissue	SS4.1, 4.2, 4.3	36.3 (+/- 3.4)	28.8 (+/- 3.3)	20.3 (+/- 1.9)
20110618039	white tissue	SS6.1, 6.2, 6.3	51.7 (+/- 0.6)	56.0 (+/- 1.0)	38.3 (+/- 0.6)
20110618039	red tissue	SS7.1, 7.2, 7.3	25.7 (+/- 0.6)	19.5 (+/- 1.3)	23.3 (+/- 0.6)
		Avg concentrations:	96.8 (+/- 78.8)	39.7 (+/- 48.3)	39.8 (+/- 19.0)

Table 6. Comparison of average DNA concentrations per treatment group. There was no significant difference between groups ($p > 0.05$) but the trend indicated that cold storage with same-day extraction resulted in overall higher DNA concentrations.

	<u>Storage Method</u>			<u>ANOVA</u>	
	<u>Cold</u>	<u>Ethanol</u>	<u>Re-Frozen</u>	<u>F</u>	<u>Sig.</u>
Sample Concentrations (ng/ μ l)	<u>96.84</u>	<u>39.70</u>	<u>39.78</u>	2.931	0.114

*Means underscored by the same line are not significantly different ($p > 0.05$) using Tukey's test

Testing on Digestive Tract Samples

All samples taken from the digestive tract contents of 33 cownose rays (215 samples total) were tested against all three multiplex sets. Positive controls, specific to the multiplex species, were also tested at the same time and in the same reaction with the unknown samples. Samples were found to be positive for stout tagelus clams, soft-shell clams (Figure 6), and Baltic macoma clams. Digestive tract samples were not found to be positive for hard clams, oysters, bay scallops, and cross-barred venus clams. Positive results were found from all types of stomach and spiral valve samples (*Table 7*).

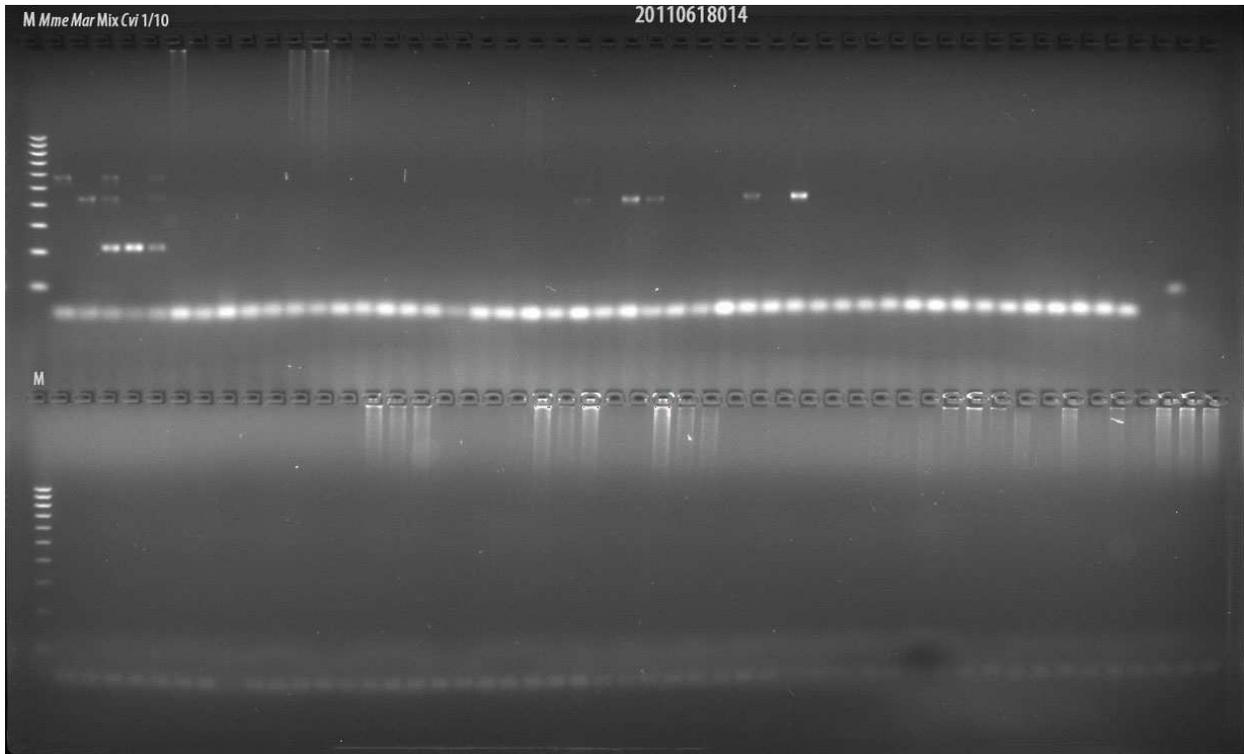


Figure 6. Gel image of positives for soft shell clams (*Mar*) from cownose ray number 20110618014. Positive control samples for each species individually, as well as mixes of all three species and a 1/10 dilution of the mixed sample, were tested in the PCR reaction. Bands for those samples are found in the upper left-hand corner of the image.

Table 7. Samples positive for any of the target bivalve species, divided by the stomach and spiral valve and type of sample taken from each. Samples are listed by cownose ray specimen, location of capture, gear type, and method of sample storage. Blank cells indicate no evidence of the target species in samples of that type, an x indicates the presence of any of the target bivalve species in that sample type, and a dotted line indicates that sample type was not available or taken for that cownose ray digestive tract.

Cownose Ray	Location	Gear Type	Positive for Any 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	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

It was observed that PCR products of some digestive tract samples left smears in the lane, starting from the well, during gel electrophoresis. Those smears, visible in Figure 6, were found in a total of 79 samples and in varying degrees or levels of brightness. The “smeary” samples often resulted after PCR with all three multiplex sets. In all but two cases, there were no positive results from those samples. A subsample of 16 smeary samples was chosen for follow-up testing. DNA concentrations from those samples were found to range from 25 – 365 ng/μl; four samples were in the 20 - 50 ng/μl concentration range, and were similar to the DNA concentrations of the positive control samples. If the smears were based on high DNA concentrations, we would not expect those four samples to be consistently “smeary” after PCR. DNA dilutions were made so that all 16 samples had a concentration of approximately 30 ng/μl; those diluted DNA samples were then tested against the *Aic & Tpl* and *Mme, Mar, Cvi* multiplex sets (Figure 7). Half of the samples were also tested with the Folmer et al. (1994) primers for the COI gene (at 60°C annealing temperature and also under the original conditions used with those primers). No positives for bay scallop, stout tagelus clam, hard clam, soft-shell clam, or oyster were detected from those “smeary” samples. Some residual smears were present, even after the DNA concentrations were diluted. Furthermore, the COI primers worked very well on those samples, indicating that DNA was present but the samples were not one of the species tested for in this study (Figure 7). (The COI primers did not work at the annealing temperature of 60°C but did work with the original conditions and annealing temperature of 52°C.)

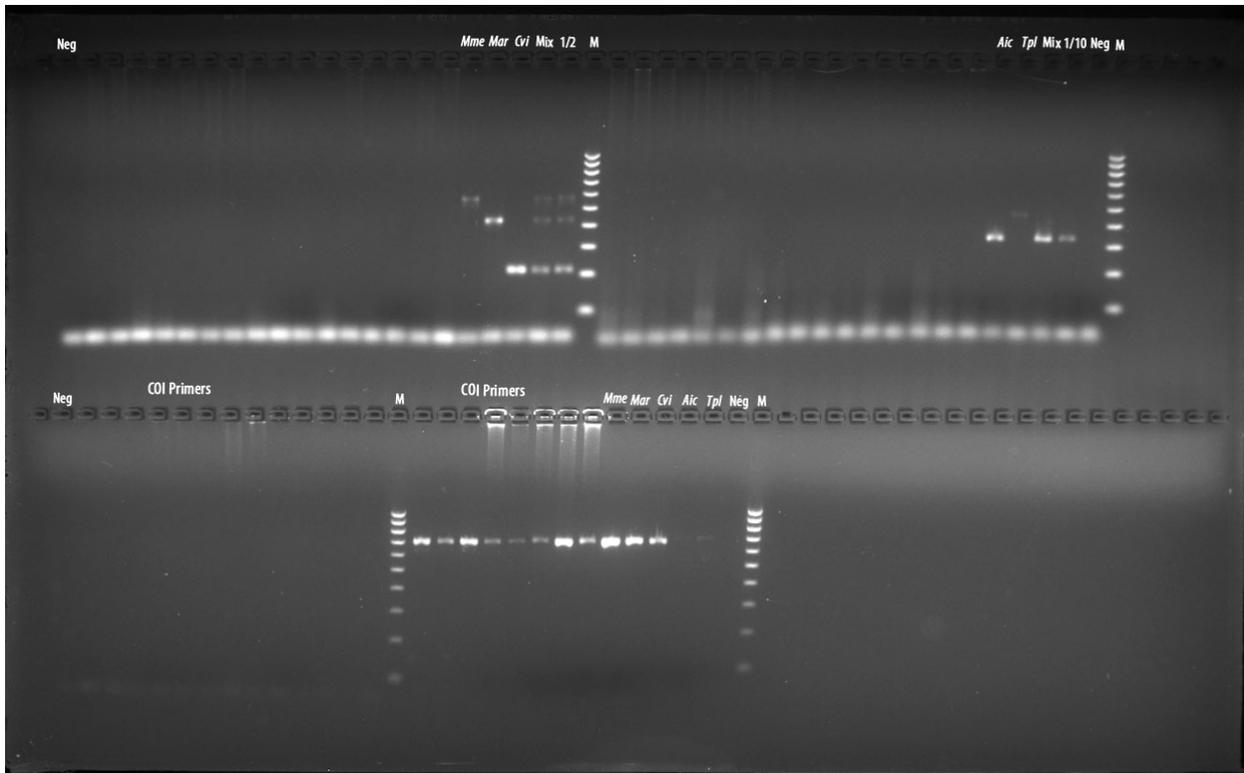


Figure 7. Gel image of tests on a subsample of 16 “smeary” samples. No positives for hard clam (*Mme*), soft-shell clam (*Mar*), oyster (*Cvi*), bay scallop (*Aic*), or stout tagelus clam (*Tpl*) were found (top row). Residual smearing in the lanes is visible, even though the samples were diluted before PCR. Samples tested were positive for the COI gene (bottom row, middle).

Baltic macoma positives must be addressed with caution. Samples positive for Baltic macoma clams were also positive, with only one exception, for stout tagelus clams. The cross-amplification rate of Baltic macoma primers with digestive tract samples also testing positive with stout tagelus clam primers was 86%. A clear illustration of this was visible in the testing of digestive tract samples from cownose ray 20110618083 with both the bay scallop and stout tagelus clam (*Aic* & *Tpl*) and the Baltic macoma and cross-barred venus (*Mba* & *Cca*) multiplexes (Figure 9). All samples positives for stout tagelus clam were also positive for Baltic macoma. Those three stomach samples were each taken from separate tissues identifiable as bivalves in the stomach, making a sample of mixed-species consistency unlikely. Due to the

consistent cross-amplification of stout tagelus clam DNA with Baltic macoma primers, which occurred 100% of the time in trials when tested on known stout tagelus clam DNA (Figure 8), the positive results for Baltic macoma from the digestive tract samples are very likely false positives. However, this cannot be verified at this time and requires further testing. The stout tagelus clam primers had a 0% cross-amplification rate with Baltic macoma DNA. The Baltic macoma positive results are reported in this thesis, but with a strong caution that the majority of the Baltic macoma positives are likely false positives.

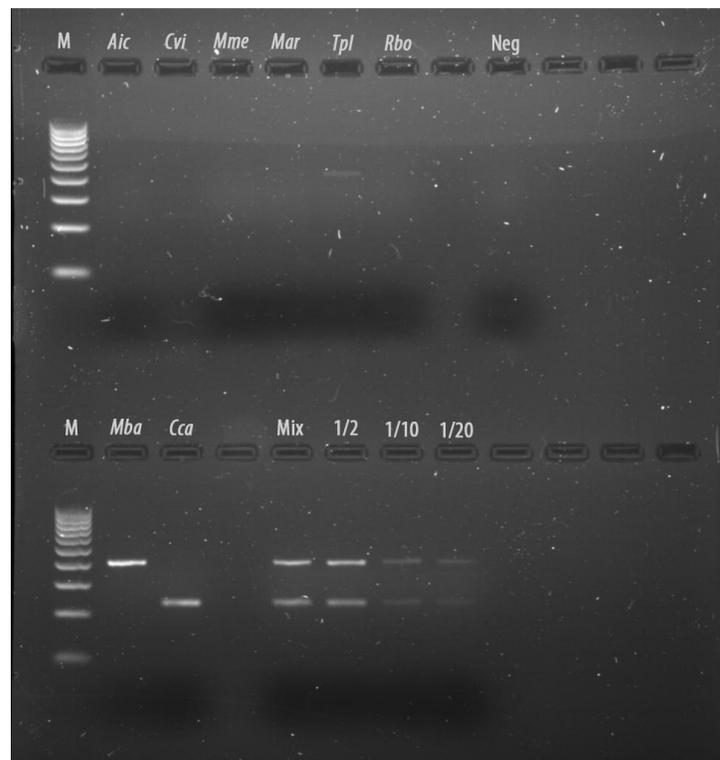


Figure 8. Gel image of Baltic macoma and cross-barred venus (*Mba* & *Cca*) multiplex tests with diluted control samples found on the bottom row and cross-amplification testing of non-target species on the top row. A faint band is visible in the stout tagelus clam lane (*Tpl*) on the top row; the band is the same size (at the same location in the lane) as the Baltic macoma (*Mba*) band.

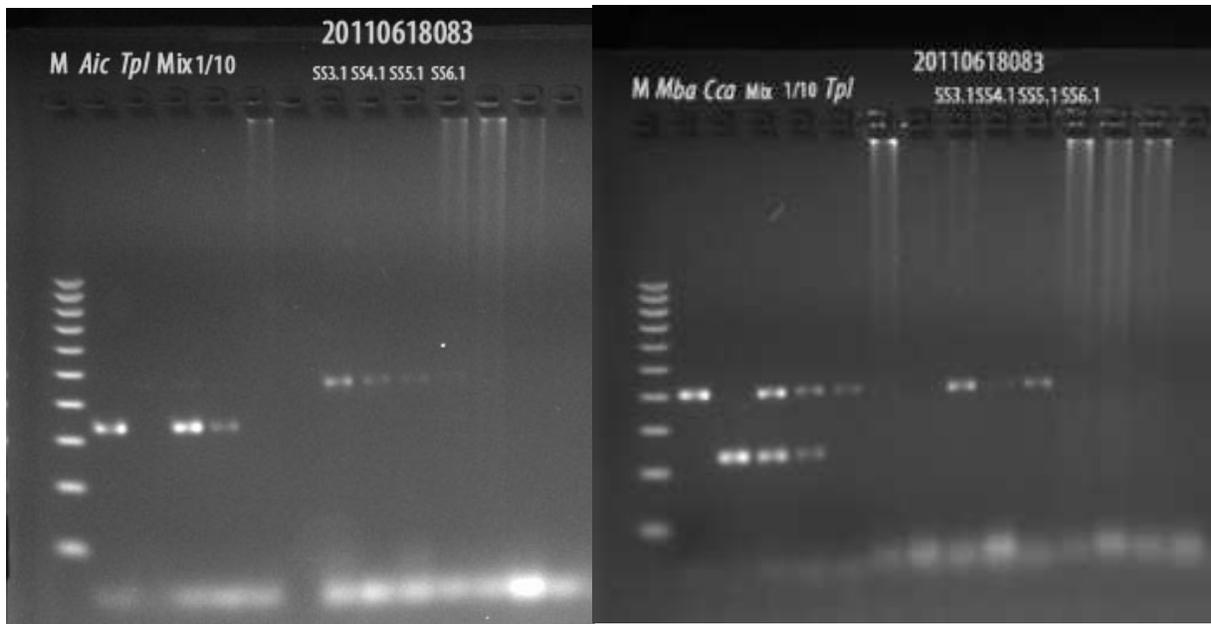


Figure 9. Gel images of digestive tract samples from cownose ray 20110618083 tested with bay scallop and stout tagelus clam (*Aic* & *Tpl*) and Baltic macoma and cross-barred venus (*Mba* & *Cca*) multiplexes. All three stomach samples (SS3.1, SS4.1, and SS5.1) tested positive for both stout tagelus clams and Baltic macoma clams.

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 (Figure 10). The percentage of the weight of the stomach contents that was unidentifiable (unknown) was 100% for small cownose rays (< 90 cm disc width), but larger rays had some identifiable food categories (Figure 11). 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 (Figure 11). Shell fragments (hard parts) were found in stomachs and spiral valves of rays collected by bowfishing and haul seine. Shell fragments made up 4.54% and 3.02% averages by weight for stomachs and spiral valves, respectively. Once again, these shell fragments were only seen in large individuals > 90 cm disc width (Figure 11). 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. Large individuals (> 100 cm disc width) contained fish remains (Figure 11) and were captured by haul seine.



Figure 10. The visual examination process of stomach and spiral valves involved separating the contents into categories of tissue type and obtaining weights of identifiable parts of fish, bivalves, shell pieces, and detritus.

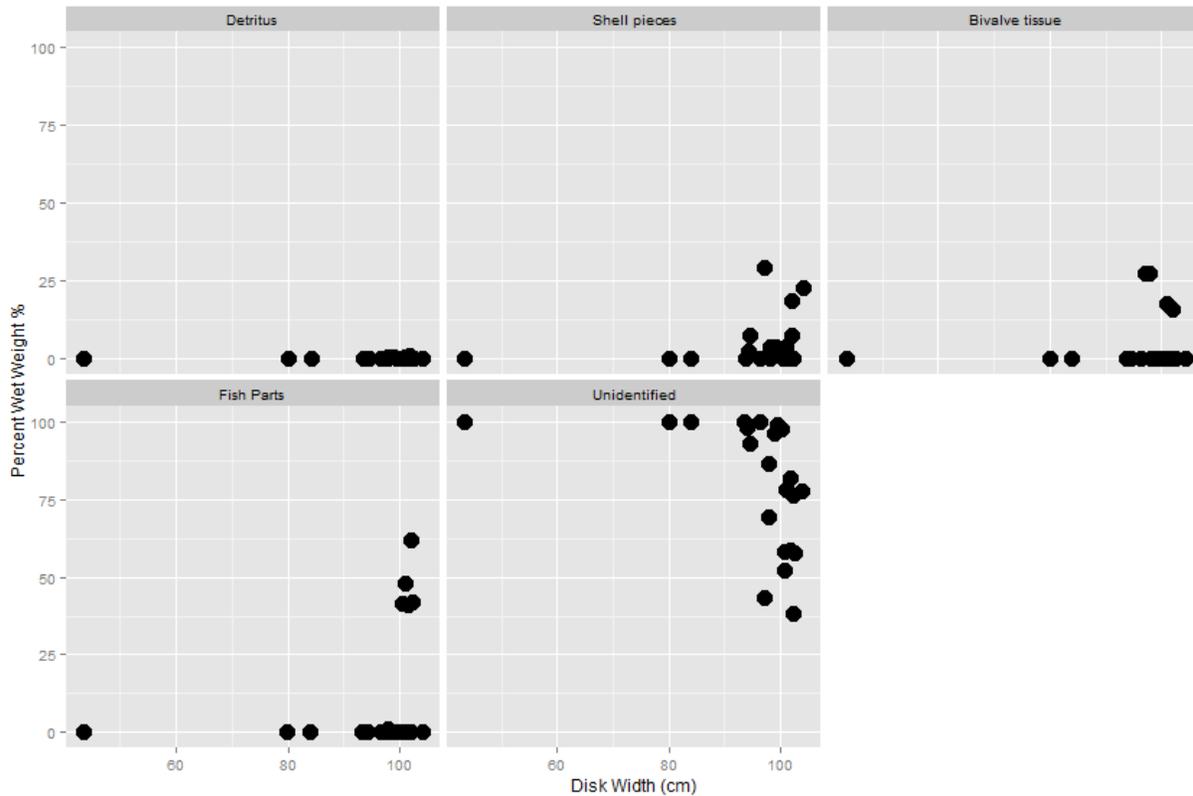


Figure 11. Percentage of wet weight of stomach contents that could be identified visually from cownose rays of different sizes.

Cownose Ray Genetic Diet Analysis

Of the 33 cownose ray digestive tracts sampled, positive results were found in 45 samples from 10 individuals. Samples were positive for stout tagelus clams, soft-shell clams, and Baltic macoma clams (but see Figure 8 and Figure 9 for a caution on Baltic macoma positives).

Cownose rays with digestive tract samples positive for the species tested were all collected in Virginia during the bowfishing tournament (*Table 8*, Table 8). Two cownose ray digestive tracts had samples positive for all three species, while nine individual cownose rays tested positive for both stout tagelus clams and Baltic macoma clams. One individual was positive for only stout

tagelus clams. No cownose ray digestive tracts were found to be positive for bay scallop, cross-barred venus clams, hard clams, or eastern oysters.

Size and sex of individual cownose rays influenced the bivalve species found in their digestive tracts. Generally, some, but not all, cownose rays larger than 90 cm disc width tested positive for Baltic macoma, soft shell clams, or stout tagelus clams (Figure 12). Once above 90 cm disc width, nearly all of the digestive tract samples taken from the individual tested positive for these three species (> 0.5 fraction positive, Figure 12). A small fraction (< 0.5) of two individual cownose ray's stomach samples were positive for soft-shell clams, but negative for the other species. All females positive for any of the bivalve species tested for in this study were larger than 94.5 cm in disc width. Large females (over 90 cm disc width) were found to have consumed more stout tagelus clams than smaller females or males (Figure 13). Only two males had digestive tract samples positive for stout tagelus and Baltic macoma clams, and both of those cownose rays had disc widths larger than 93 cm (Figure 13). Although the sample size was small ($N = 3$), young of year (YOY) cownose rays, all caught in North Carolina, were not positive for any of the bivalve species tested in this study.

A multivariate analysis of these genetic samples, within individual cownose rays, that summarizes the fraction-positive for the seven species of bivalves showed that cownose rays that tested positive for any bivalve species were similar in size, sex, location of capture, and capture method ($Stress = 7.43$). A visual plot of the first two multidimensional scaling axes of fraction positive of stomach samples within each individual showed a group of cownose rays in the upper left quadrant (Figure 14). These individuals were positive for one of the three species of bivalves (stout tagelus, Baltic macoma, or soft-shell clam), were collected in the bowfishing tournament (Figure 15), were large (Figure 14, with disc widths above 90 cm), and were largely

females (Figure 16). Other rays plotting in the MDS in the lower right quadrant of the MDS were negative for the bivalve species tested, were smaller in disc width, and captured by cast net or gill nets. Cownose rays in the upper left quadrant were captured by other methods (haul seine and hook and line) in Virginia and North Carolina (Figure 17). In conclusion, the rays that tested positive for the three species of bivalves were large, mostly females, and taken from the Virginia bowfishing tournament.

Table 8. Number of cownose rays with digestive tracts containing samples positive for the species tested. Numbers of cownose rays are categorized by location of collection and capture method. Percentages were calculated from the total number of rays collected by that gear type and location. Bivalve species tested were Atlantic bay scallop (*Aic*), stout tagelus clams (*Tpl*), hard clams (*Mme*), soft-shell clams (*Mar*), Eastern oyster (*Cvi*), Baltic macoma clam (*Mba*), and cross-barred venus clam (*Cca*).

Location	Gear Type	Number	Bivalve Species						
			<i>Aic</i>	<i>Tpl</i>	<i>Mme</i>	<i>Mar</i>	<i>Cvi</i>	<i>Mba</i>	<i>Cca</i>
NC	Hook & Line	5	0	0	0	0	0	0	0
	Nets	3	0	0	0	0	0	0	0
VA	Bowfishing	19	0	10 (52.6%)	0	2 (10.5%)	0	9 (47.4%)	0
	Haul Seine	6	0	0	0	0	0	0	0
	Totals	33		10 (30.3%)		2 (6%)		9 (27.3%)	

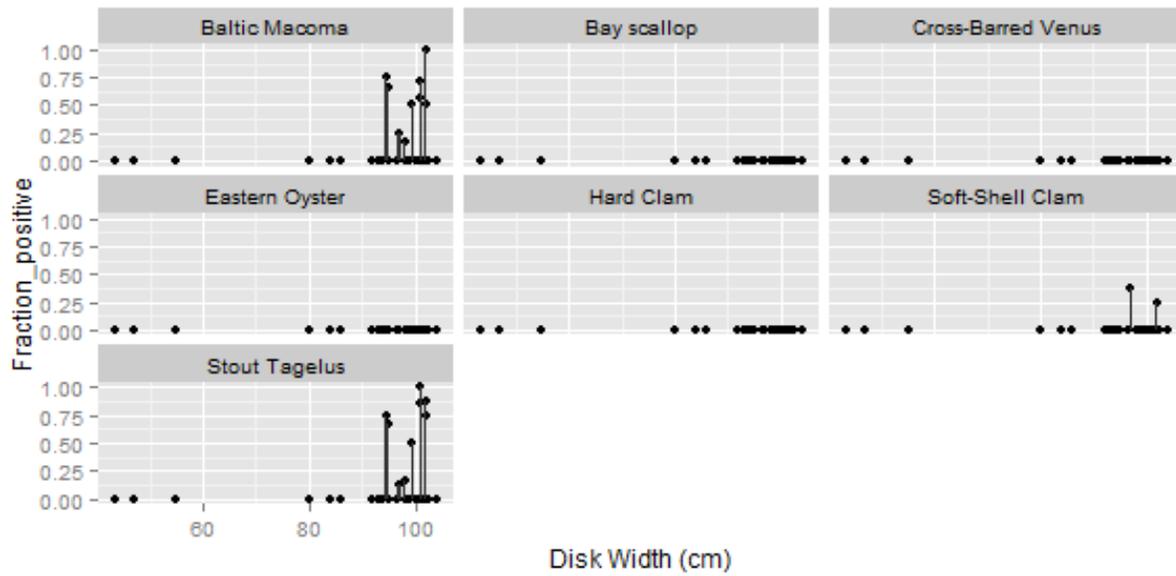


Figure 12. Proportion of all digestive tract samples positive (y-axis) for each bivalve species tested, by disc width in cm (x-axis). Digestive tract samples were found to be positive for stout tagelus, soft-shell clam, and Baltic macoma clams. All rays positive for any of those bivalve species were > 90 cm.

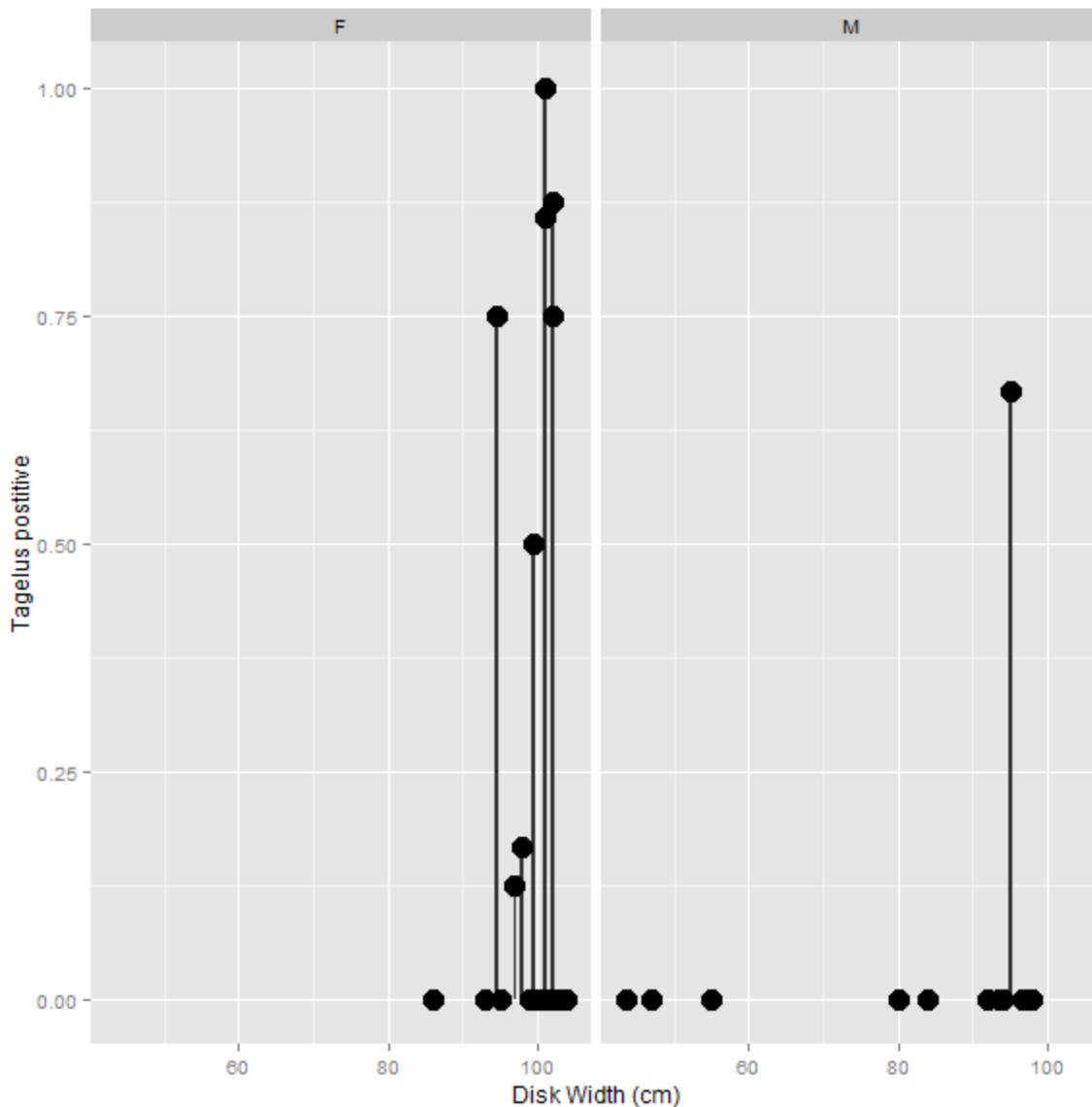


Figure 13. Proportion positive for stout tagelus clams in digestive tract samples for each cownose ray, by disc width and separated by female rays and males rays. More female rays were positive for stout tagelus clams than male rays. All female rays collected were > 80 cm in disc width. Females larger than 90 cm disc width had digestive tracts positive for stout tagelus clams. Male rays positive for stout tagelus clams were larger than 90 cm disc width. Young of year rays collected were all male (found on the left-hand side of the male graph) and had disc widths less than 55 cm.

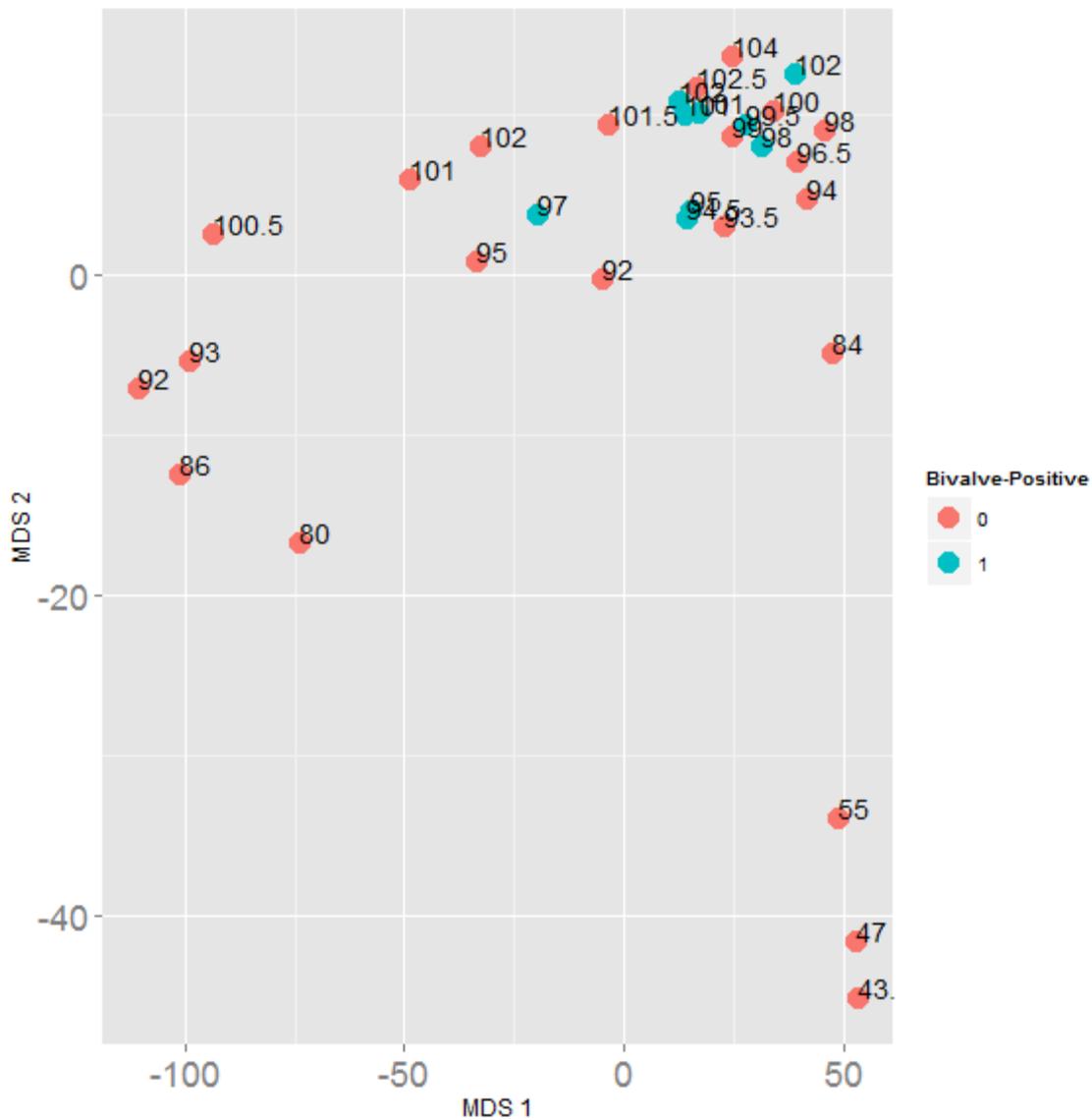


Figure 14. MDS plot of cownose rays positive for any bivalve species by disc width (cm). Disc widths are listed next to each colored circle. Red circles represent cownose rays not positive for the bivalve species tested, and blue circles represent cownose rays with digestive tract samples positive for the species tested. Illustrated in this figure is that all individuals positive are large in size (found in upper right-hand corner).

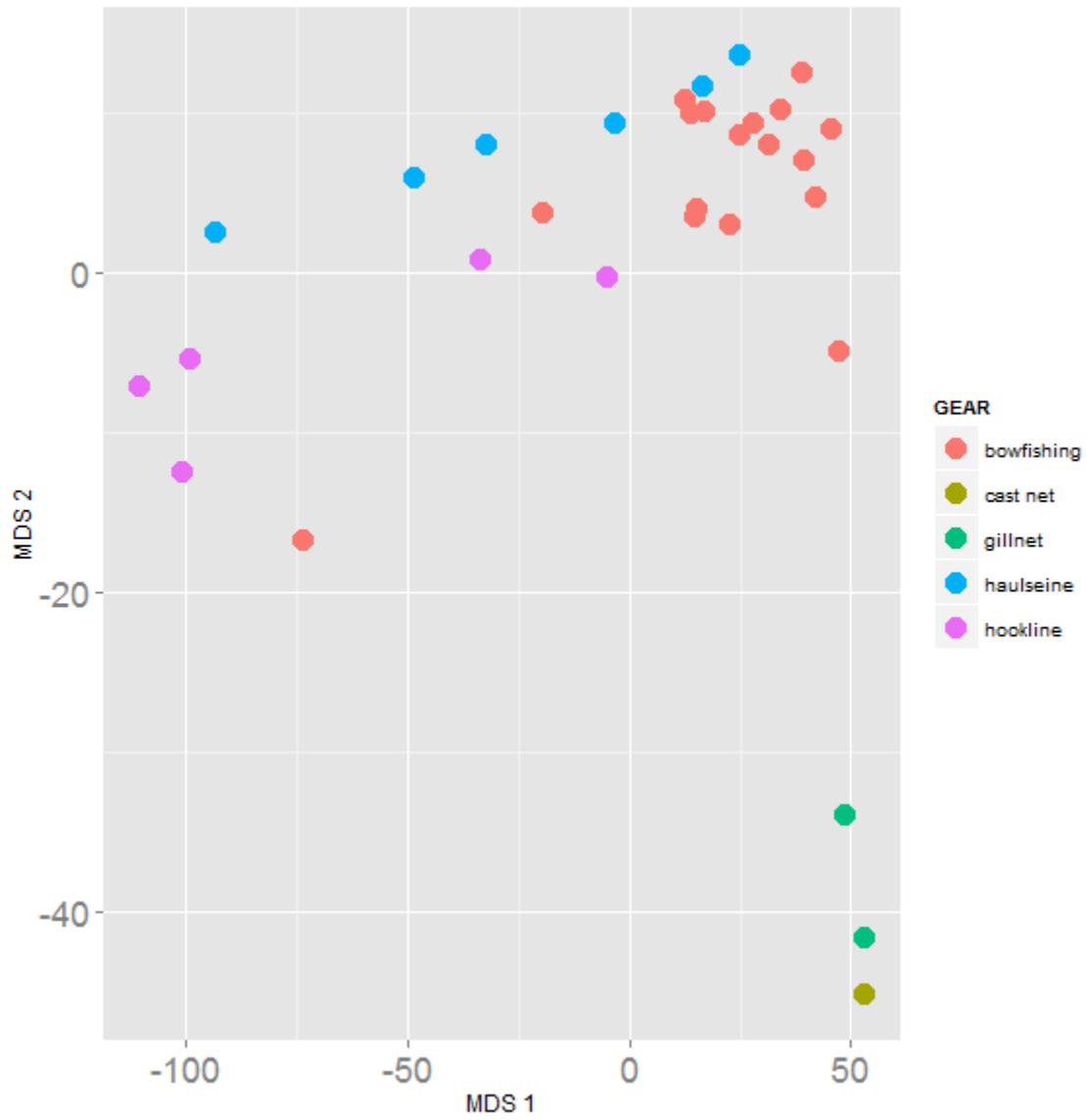


Figure 15. MDS plot of cownose rays by stomach content results (multiplex testing results) and the method of capture used.

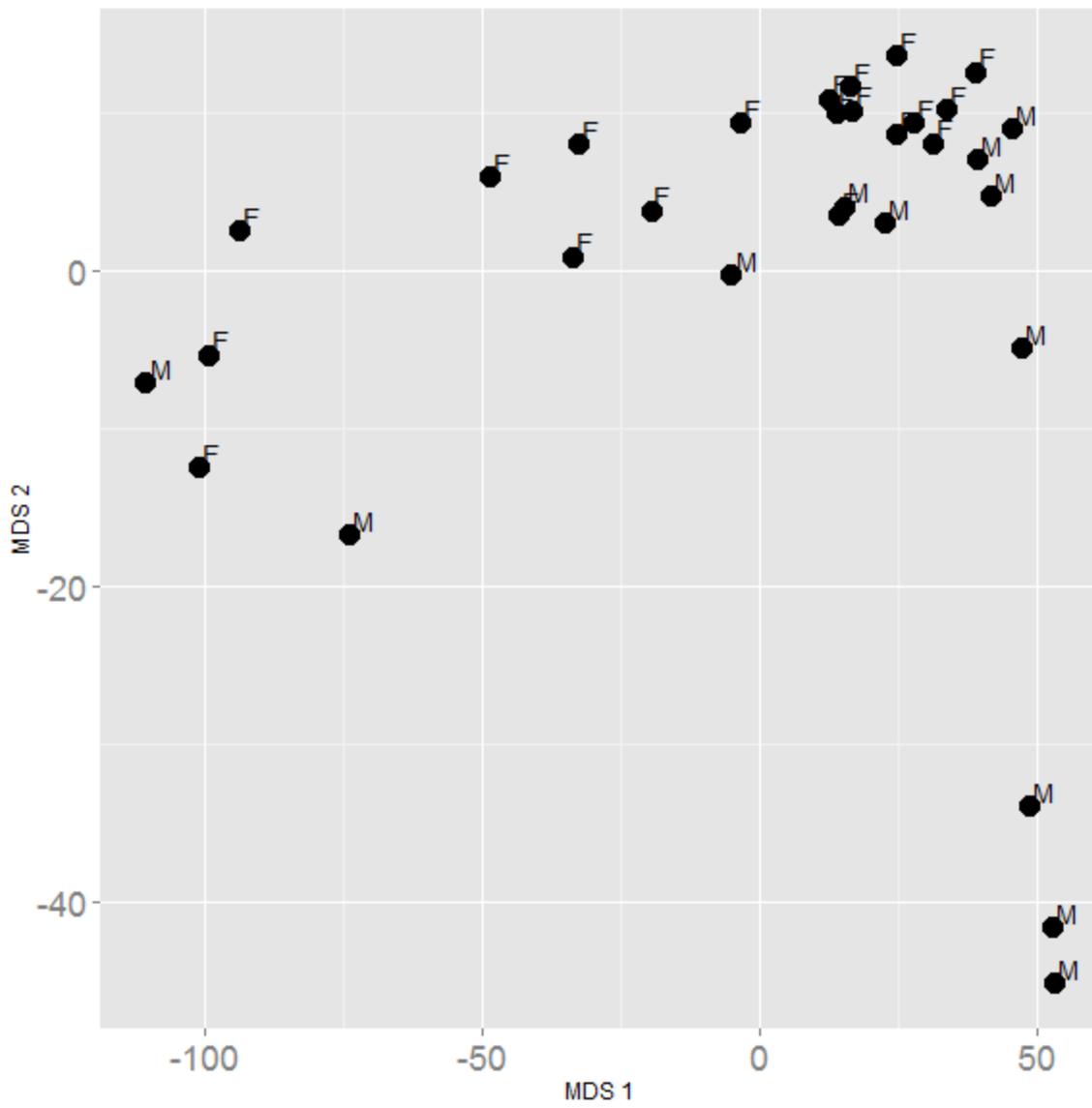


Figure 16. MDS plot of cownose rays by stomach contents results (multiplex testing results) and showing the sex of the rays.

Multidimensional scaling was used with the combined results from genetic testing and visual digestive tract content analysis (*Stress* = 4.60), and visualized in graphs (Figure 18, Figure 19, and Figure 20). Individual circles represent different cownose rays. Capture method (bowfishing and haul seine) was represented by black and green circles, with percent weight of identifiable bivalves indicated next to the appropriate circle (Figure 18). Cownose rays with bivalve tissue visually identifiable in the stomach contents were all caught by bowfishing. Cownose rays with fish tissue found in stomach contents by visual analysis were all caught by haul seine (Figure 19). Percent weight of fish tissue is indicated next to the circle of those cownose rays with fish tissue in the stomach contents. Cownose rays testing positive for stout tagelus clams, by genetic testing, are shown in Figure 20, with the percent fraction of stout tagelus positives indicated next to the circles representing cownose rays. Black circles indicate female cownose rays, with red circles indicating male. Cownose rays testing positive for stout tagelus clams were females (Figure 20).

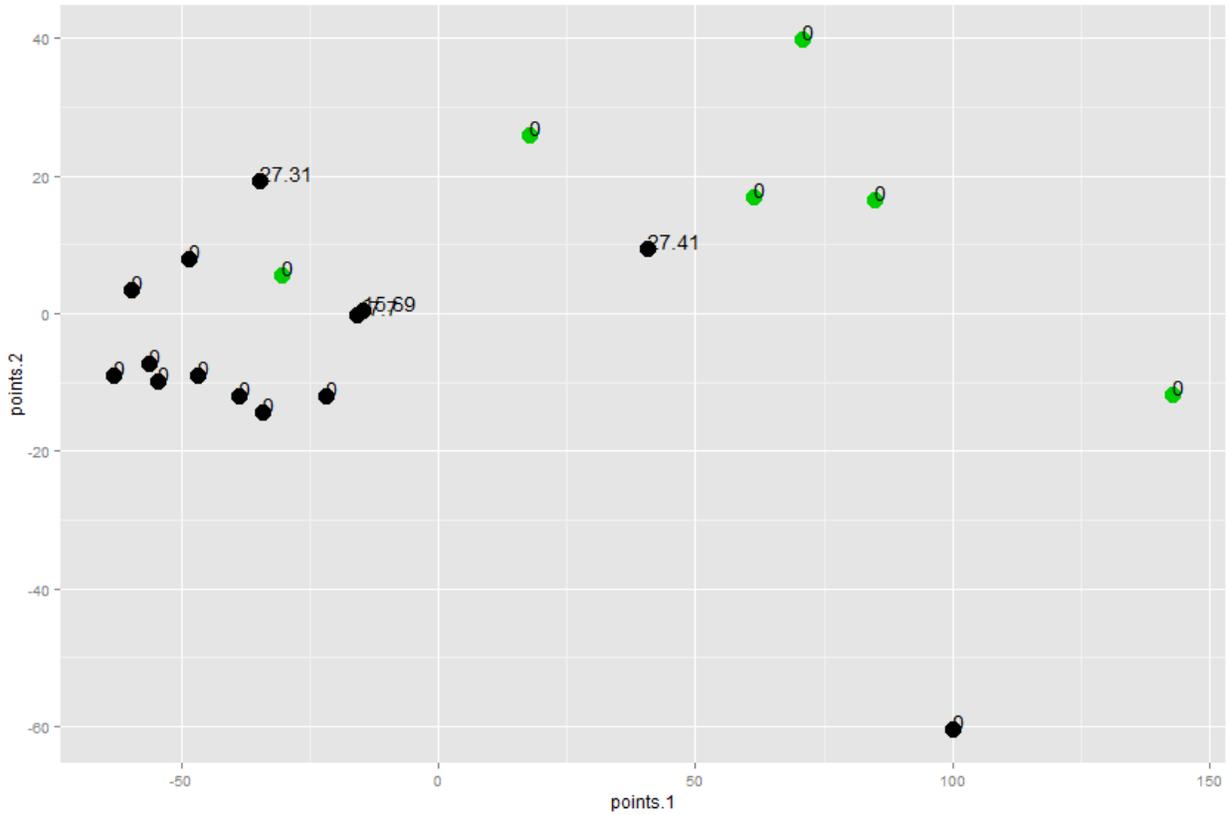


Figure 18. MDS plot combining visual stomach analysis results with genetic testing results, by capture method (bowfishing vs haul seine). Circles on the graph represent individual cownose rays; black circles indicate that the cownose ray was caught by bowfishing and green circles indicate haul seine capture. Numbers next to the circles indicate the percent weight of identifiable bivalve tissue found in stomachs by visual analysis.

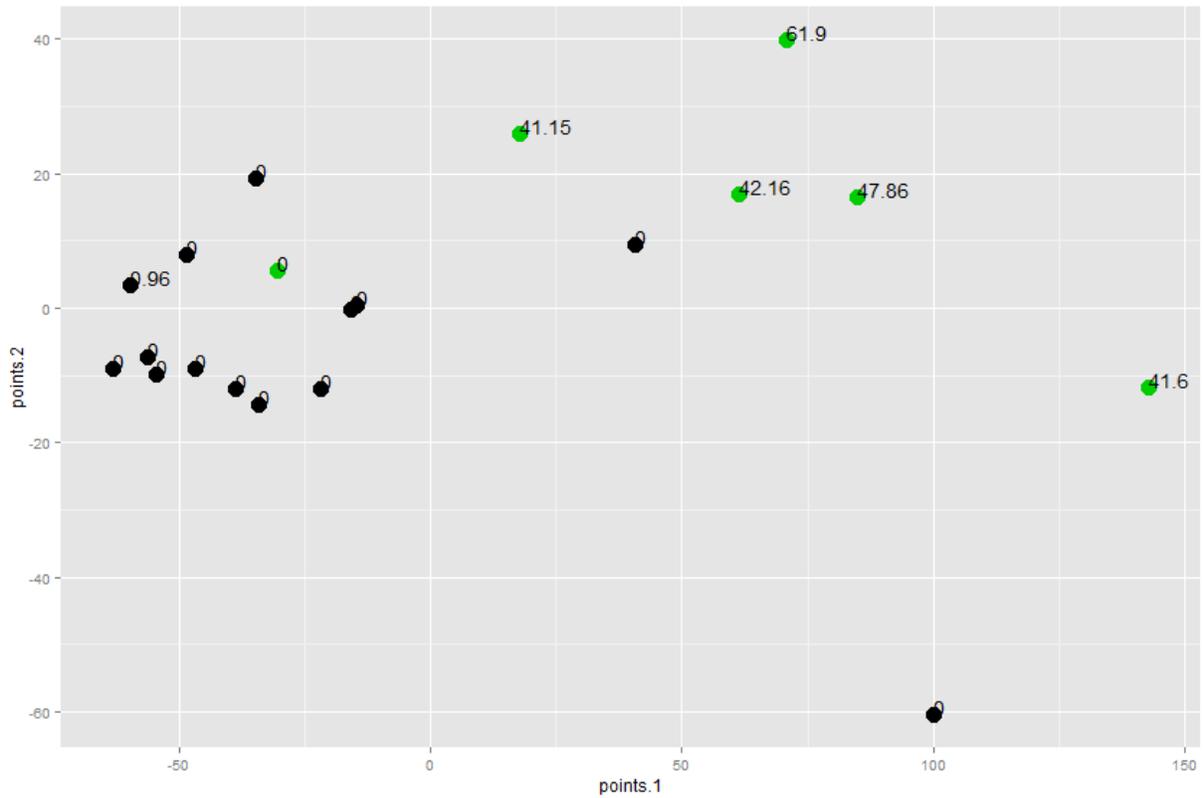


Figure 19. MDS plot of combined stomach content analysis and genetic testing results, by capture method, and indicating percent weight of fish found in stomachs. Black circles indicate the cownose ray was caught by bowfishing and green circles indicate haul seine capture. Numbers associated with circles (representing individual cownose rays) are the percent weight of fish found in stomachs by visual methods.

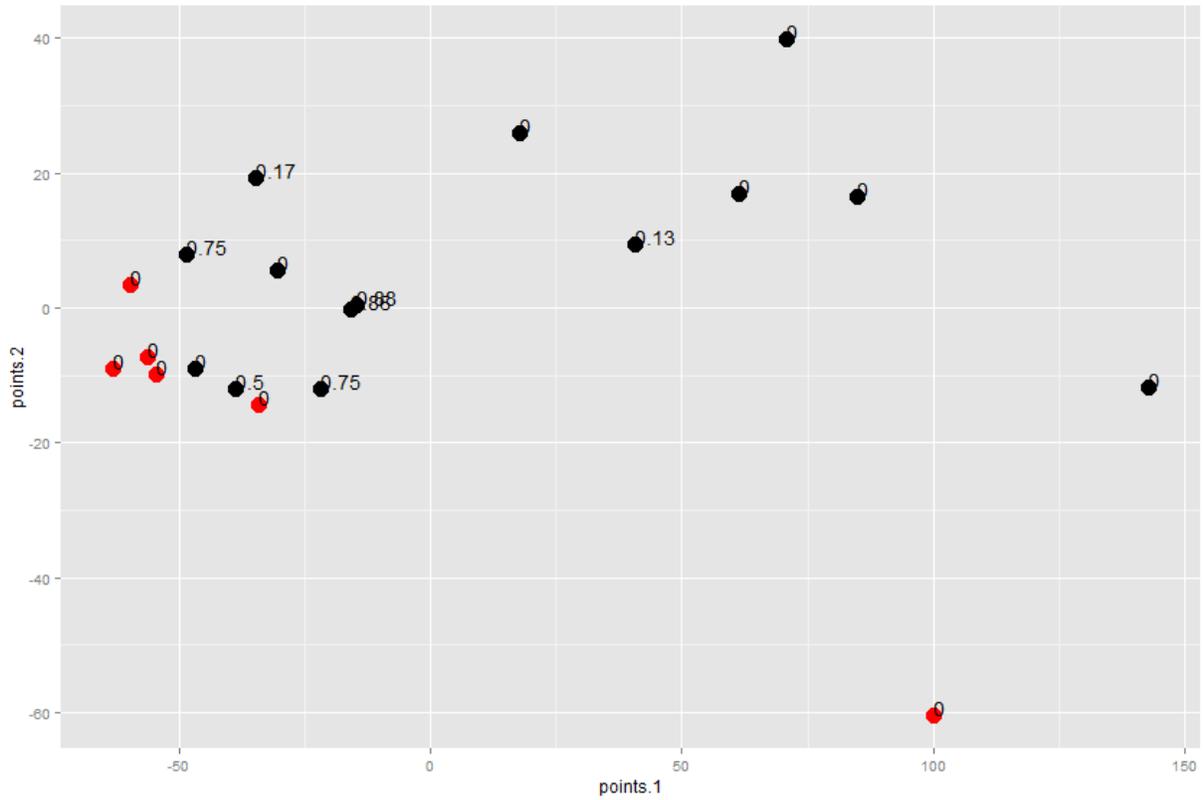


Figure 20. MDS plot of combined stomach content analysis and genetic testing results, by sex of cownose ray and fraction positive for stout tagelus clam (by genetic testing). Black circles indicate female cownose ray and red circles indicate male rays. Numbers next to the circles are the fraction positive for stout tagelus DNA, by individual cownose ray.

Discussion

Genetics Discussion

The primer sets designed and optimized for five of the seven species were 100% successful in amplifying the target species and in not amplifying DNA of the non-target species. 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 target species DNA in very low concentrations (0.5 ng/ μ l). This would translate into very minute amounts of DNA from unknown stomach samples being amplified by the primers designed. All three multiplex sets were successful at amplifying target species and DNA from unknown stomach samples.

The primer pairs for Baltic macoma (*Mba*) and the cross-barred venus clam (*Cca*) consistently cross-amplified DNA from non-target species. The primer sets for those species should be redesigned in such a way so that there are more differences between the primer sequences and the sequences of the non-target species. I acknowledge that this is difficult, as I spent weeks designing primers for these species so that cross-amplification would be minimal. I designed and ordered six potential sets of cross-barred venus primers and two sets of Baltic macoma primers after designing and aligning scores of other potential primers. However, this is the necessary next step to creating a multiplex for those species that will not cross-amplify the non-target species. I am confident that all techniques for optimization of the primers were fully explored and utilized.

Cownose Ray Feeding Ecology

Multiple tissue, chyme, and homogenate samples were found to be positive for stout tagelus clams (*Tpl*), soft-shell clams (*Mar*), and Baltic macoma clams (*Mba*). Baltic macoma positives should be treated with caution. As discussed previously, the majority of the Baltic macoma clams were likely false positives due to cross-amplification of stout tagelus clam DNA by the Baltic macoma primers. However, one unknown tissue sample tested positive for Baltic macoma and was not positive for stout tagelus clam. Some positives may be actual Baltic macoma positives, but that is not able to be teased apart at this time and requires further testing. It is possible that mixed-species samples could have been taken from chyme, fluid, and homogenate samples taken from stomachs and spiral valves, and those mixed-species samples could have tested positive for multiple bivalve species. More testing is necessary in order to determine the species found in those samples positive for both stout tagelus and Baltic macoma clams. Baltic macoma clams have been found in other cownose ray diet studies from Chesapeake Bay and North Carolina, so I did expect to find them in digestive tracts sampled.

Positive results were found in a total of 45 samples from 10 individual cownose rays. A total of 215 samples were tested from 33 cownose rays. No digestive tract samples were found to be positive for the Atlantic bay scallop (*Aic*), Eastern oyster (*Cvi*), or hard clam (*Mme*), 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 & Fox 1988). Out of those four species, only the hard clam exhibits some limited burrowing; the bay scallop is found in seagrass beds and is motile. Oysters are found growing in oyster reefs or attached to hard substrate (Ruppert and Fox 1988).

The three species found in digestive tracts of cownose rays in this study are burrowing, thin-shelled bivalves associated with sandy-bottoms and vegetated areas (Ruppert and Fox 1988). The stout tagelus clam is a rather large animal, 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. The Baltic macoma clam is a small, infaunal burrower 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). The soft-shelled clam is a thin-shelled, infaunal burrower in silty sediment. Soft-shelled clams can be found in burrows as deep as 18 cm (Barnes 1974).

All cownose rays with digestive tracts that tested positive in this study were caught in Virginia during the bowfishing tournament. Bowfishing has been found to be an effective way to capture cownose rays while feeding, or in habitats associated with feeding. Individuals collected this way are often killed instantly, or are landed on the boat soon after capture and do not have a chance to evacuate their stomachs. Cownose rays will often evacuate their stomachs when captured in a net and will eat other fish and organisms trapped with them in a haul seine or pound net (R. Fisher, personal communication). Traditional scientific and recreational capture and collection methods often result in the digestive tract contents containing opportunistic prey sources, which thereby biases 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. When trying to gather “typical” diet data on cownose rays, and address the question of the ecological impact that cownose rays have on shellfish populations, the capture method must not be allowed to bias stomach contents. Previous diet studies in Chesapeake Bay found collection through bowfishing to yield the most unbiased or natural digestive tract contents (Fisher 2010), and my research reflects those findings.

Best Practices

A number of different practical aspects of this project were tested to develop the best handling, storage, and treatment methods of cownose ray digestive tract samples. In terms of collection of cownose rays, bowfishing was found to be the best way to gather cownose rays so that the digestive tract contents were less biased by the capture method. It is my recommendation that cownose rays be measured, weighed, and dissected as soon as possible after collection. If that is not possible, storing the cownose ray on ice during transport to laboratory facilities, and then conducting the measurements and dissection that same day, is recommended. That allows for fewer freeze/thaw cycles to degrade the DNA in the digestive tract. The faster the digestive tract can be removed and frozen to stop the digestive process, the better. Digestive tracts were stored frozen at -20°C and allowed to thaw overnight while covered in ice or in a cold water bath for 2-4 hours before contents were removed and analyzed. These storage and handling methods worked well, although care should be taken to analyze the digestive tract contents as soon as thawing has occurred. Waiting too long after thawing before analysis resulted in the contents starting to degrade, which reduced the possibility of visual identification and clean sampling of tissue fragments.

After testing three different treatment and storage methods of digestive tract contents, it was determined that tissue kept thawed and stored cold (4°C), with DNA extractions starting within 4-6 hours of sampling, yielded DNA samples with the highest concentrations. There was no significant difference between the groups, but higher average DNA concentrations were seen from the samples stored cold with same-day extraction. After that, samples stored re-frozen until extraction yielded the second highest DNA concentrations. Overall low yields resulted from

samples stored in 95-100% ethanol. In some cases, the low yields were very low, especially compared to the same samples stored fresh before extraction. Based on these findings, I recommend cold storage with same-day extractions. I do not recommend storing digestive tract contents in ethanol. Caution should be exercised when using ethanol to store degraded or partially digested samples. At this time it is not possible to know whether the ethanol interferes with the extraction process or if it affects the partially-digested tissues and chyme found in digestive tracts. This trend in the DNA concentration data from different storage treatment methods requires further testing and a larger sample size.

Further investigation of the smears visible on the gels after electrophoresis is required, but at this time, it does not appear to be related to high DNA concentrations. Some of the samples, which were found to be consistently “smeary” after testing with all three multiplex sets, were very high in DNA concentration (300 ng/μl) but other smeary samples yielded more acceptable concentration levels (25-60 ng/μl). After diluting the subsample of smeary samples to concentrations of 30 ng/μl, those samples were then tested with two multiplex sets and the COI primers. No positive results were found after testing with the multiplex sets, but the COI primers were successful in amplifying DNA from the unknown samples. After dilution and PCR, residual smearing was still visible in the lanes.

It is possible that the smearing is related to the DNA being extracted from partially digested or degraded samples, or somehow related to the samples being removed from digestive tracts and exposed to enzymes and acids. It is important to note that the positive control samples used in this study showed varying DNA concentrations (0-157 ng/μl) but never resulted in smears in the lane after PCR. The positive control samples were taken from tissue from frozen

bivalve specimens. Further research is required into this issue to determine the cause and techniques to clean up the samples or PCR product during reaction.

Sex Ratio, Ontogenetic Shift, and Ecomorphology of Cownose Rays

A total of 33 cownose rays were used for this study; eight were collected in North Carolina and 25 in Virginia. Out of all cownose rays collected, 20 were females, 12 were males, and one individual was of an unknown size or sex. More females were collected in Virginia than in North Carolina. All young-of-year or neonate cownose rays collected were males and they were captured in North Carolina. All females collected were mature adults, with 19 individuals over 90 cm disc width and one individual over 80 cm. Out of all males, three were young-of-year and nine were mature. All mature males were greater than 80 cm disc width, and seven of the nine had disc widths over 90 cm.

Young-of-year individuals did not have digestive tract samples positive for any of the bivalve species tested for in this study. I predicted that juveniles would consume small, non-commercial species such as the cross-barred venus, *Baltic macoma*, and soft-shell clam. Although I only collected three juvenile rays, there was no evidence of any of the target bivalve species in their digestive tracts. All positive results from digestive tracts were found in a total of 10 individuals, all from Virginia, and all mature adults. Eight females, all over 94.5 cm disc width, had digestive tract samples positive for stout tagelus clams, soft-shell clams, and *Baltic macoma* clams. Two males, both over 90 cm disc width, had digestive tract samples positive for stout tagelus clams and *Baltic macoma* clams. As the bar plots and MDS analyses illustrated (Figure 12, Figure 14), large cownose rays (over 90 cm) ate more stout tagelus clams than the other species. I predicted that the adult cownose rays would consume all the target bivalve

species, but especially prey upon Atlantic bay scallops, hard clams, and soft-shell clams. With the exception of the soft-shell clam, there was no evidence of the cownose rays consuming the commercially-important clams, especially the Atlantic bay scallop and hard clam. Adult cownose rays were found to consume bivalve species, and three of the species tested for in this study. The thin-shelled bivalves, out of all the species tested, were found in digestive tracts samples of cownose rays, whereas the thick-shelled bivalves were not found in digestive tract samples in this study. I predicted that cownose rays would not feed upon oysters in their natural habitat (reefs and hard substrate), and I found no evidence of oysters in the digestive tracts of rays sampled in this study.

Recent morphological research and *in vitro* studies of cownose ray feeding behaviors indicate that the bite pressure of cownose rays changes with other ontogenetic shifts. Very small single (cultchless) and very large oysters, measured by shell height and shell depth, were less likely to be eaten by mature rays in holding experiments; predation was more likely in oysters of shell depths between 8-22 mm and in hard clams of shell depths between 21-26 mm (Fisher 2010). Cownose rays are limited in which bivalve species they can consume through gape limitation and bite pressure, as well as the placement or the habitat in which the bivalve is found (Fisher 2010). Young-of-year rays were able to consume single seed oysters in the range of 10-30 mm shell height and < 10 mm shell depth (Fisher 2010). Based on shell height, depth, and placement, some species of bivalves like hard clams and oysters are very difficult for cownose rays to manipulate and crush, and this is especially dependent upon size of ray (Fisher 2010, M. Kolmann, unpublished data). Hard clams, soft-shell clams, bay scallops, and other clams like the cross-barred venus and stout tagelus are all found in seagrass beds and sandy habitats. Cownose rays were found to preferentially prey upon soft-shell clams over thick-shelled bivalve species

(Fisher 2010). If commercial oyster grounds or clam leases use the same types of habitats, cownose rays may be feeding in those areas but preferentially feeding on thinner-shelled bivalves and crustaceans.

Even when cownose rays have the bite force and jaw gape size to allow predation upon hard clams and oysters, the placement of prey on oyster reefs, hard substrates, and clusters make it difficult for a cownose ray to manipulate (Fisher 2010). Perhaps burrowing depth of bivalve is also a limiting factor in prey selection. Stout tagelus clams are deeply-burrowing clams, and perhaps the 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. To the best of my 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 should be accounted for in future diet studies on cownose rays, especially when investigating ontogenetic shifts in diet and prey selection behaviors.

Ecological Impact of Cownose Rays

The feeding ecology and migratory patterns of an increased cownose ray population effectively interferes with human fishery activities and causes habitat alteration (Peterson et al. 2001). Cownose rays are considered a nuisance species by many fishers and aquaculturists, due to the interference with fishery activities, and the complaints that cownose rays eat commercially important shellfish like oysters, scallops, and hard clams can be traced back to the 1970s (Smith

& Merriner 1985, Blaylock 1993). In Chesapeake Bay, research into the diets of cownose rays started because of concern over oyster stock depletion due to cownose ray predation (Smith & Merriner 1985). Multiple attempts to create a cownose ray fishery in Chesapeake Bay started in the 1970s, predominantly driven by interest from commercial shellfish aquaculturists. Attempts were met with favorable reviews but limited success in finding an active and profitable market (Oesterling 2006). Fishery management plans are created after an active commercial fishery is in place, so care must be taken to appropriately manage harvest of cownose rays while a fishery is being developed. Caution should be used before promoting a cownose ray fishery without a management plan; at this time there are no restrictions or management of cownose ray harvest in North Carolina.

Elasmobranch species with low fecundity and late age at maturity are subject to overfishing, bycatch, and environmental pressures. They can easily move from a “boom” in population level to a crash in population or “bust” cycle. Developing cownose ray fisheries from the Chesapeake Bay and North Carolina populations could easily result in rapid overexploitation of the species. Elasmobranchs are extremely susceptible to intense fishing efforts, with stocks crashing quickly and taking a long time to recover due to the late age at maturity and low fecundity exhibited by many species (Blaylock 1993, Frisk et al. 2004). Cownose rays, having one of the lowest fecundity rates among elasmobranchs species, are thereby very susceptible to overfishing pressures (Blaylock 1993, Fisher et al. 2013).

Improved life history and diet data is crucially important in a fishery management plan for this species. Increasing knowledge of life history, reproductive ecology, age and growth, consumption, and behavioral ecology can only serve to better marine conservation biology, fisheries management, ecosystem-based management, and to protect elasmobranch populations.

Life history and other ecological data are still unknown or severely limited for many elasmobranch species.

The reports that cownose rays decimate commercially important shellfish stocks are one of the primary motivations behind the call for a development of a large-scale fishery. However, knowledge of cownose ray diets and feeding ecology are limited by traditional diet study methods. This study has created new tools to be used to determine the actual impact that cownose rays have on shellfish populations. Although the number of cownose rays sampled in this study is small, results indicate that cownose rays in Virginia are eating soft-shelled bivalve species like stout tagelus clams and soft-shell clams. The association between large cownose rays depredating thin-shelled, deeply burrowing clams is very intriguing and deserves future investigation. No evidence of commercially important species like Atlantic bay scallop, Eastern oyster, or the hard clam were found in digestive tracts in this study. These findings are consistent with other diet studies in Chesapeake Bay (Smith & Merriner 1985, Fisher 2010).

These multiplex tests should be applied to a larger number of cownose rays collected throughout the waters of North Carolina and Chesapeake Bay, from locations of different salinity and habitat type and throughout the migratory season. Now that handling of digestive tracts, storage of samples, and multiplex optimization has been developed, further testing on a larger number of individuals is recommended. Using these same techniques, more species of bivalves and crustaceans of interest could be added to the panels of tests. Other techniques, such as sequencing the COI gene from the unknown digestive tract samples and comparing it to the BOLD and GenBank reference libraries, could also be used to determine identity of other prey sources found in stomachs. Fecal samples could be also used as a non-lethal sampling method.

The fecal sample could be taken from individuals captured and then used to test for prey species of interest.

In conclusion, this study has conclusively established the use of a genetic approach based on the Barcode of Life region of the COI gene to identify unknown prey types 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. This indicates high sensitivity of the primers, which is useful when attempting to test degraded and digested tissue and chyme samples. This method was successful in detecting DNA of bivalve species, even in otherwise unidentifiable stomach samples and homogenized samples and heavily digested samples from the spiral valve. I 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. Three species of bivalves were positively detected from unknown digestive tract samples, and this genetic method of diet analysis could easily be applied to a large 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. There are many other applications for this type of approach, including detection of presence or absence of species from mixed-species samples of bivalve gametes or larval samples in plankton samples, or use in population and species detection of bivalves from water samples. This species-specific sample identification method, based on the Barcode of Life 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.

References

- Ajemian M, Powers S (2011) Habitat-specific feeding by cownose rays (*Rhinoptera bonasus*) of the northern Gulf of Mexico. *Environmental Biology of Fishes* 95:79-97
- Asahida T, Yamashita Y, Kobayashi T (1997) Identification of consumed stone flounder, *Kareius bicoloratus* (Basilewsky), from the stomach contents of sand shrimp, *Crangon affinis* (De Haan) using mitochondrial DNA analysis. *Journal of Experimental Marine Biology and Ecology* 217:153-163
- Aschliman NC, Nishida M, Miya M, Inoue JG, Rosana KM, Naylor GJP (2012) Body plan convergence in the evolution of skates and rays (Chondrichthyes: Batoidea). *Molecular Phylogenetics and Evolution* 63:28-42
- 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. *Journal of Experimental Marine Biology and Ecology* 393:188-192
- Bigelow AF, Schroeder WC, Collette BB, Klein-MacPhee G (2002) *Bigelow and Schroeder's Fishes of the Gulf of Maine*. Smithsonian Institution Press
- Blaylock R (1993) Distribution and abundance of the cownose ray, *Rhinoptera bonasus*, in lower Chesapeake Bay. *Estuaries and Coasts* 16:255-263

Blaylock RA (1989) A Massive School of Cownose Rays, *Rhinoptera bonasus* (Rhinopteridae), in Lower Chesapeake Bay, Virginia. *Copeia* 1989:744-748

Burgess GH, Beerkircher LR, Cailliet GM, Carlson JK, Cortés E, Goldman KJ, Grubbs RD, Musick JA, Musyl MK, Simpfendorfer CA (2005) Is the collapse of shark populations in the Northwest Atlantic Ocean and Gulf of Mexico real? *Fisheries* 30:19-26

Collins A, Heupel M, Simpfendorfer C (2008) Spatial Distribution and Long-term Movement Patterns of Cownose Rays *Rhinoptera bonasus* Within an Estuarine River. *Estuaries and Coasts* 31:1174-1183

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*. *Marine and Freshwater Research* 58:135-144

Compagno LV (1990) Alternative life-history styles of cartilaginous fishes in time and space. *Environmental Biology of Fishes* 28:33-75

Craig JK, Gillikin PC, Magelnicki MA, May LN (2010) Habitat use of cownose rays (*Rhinoptera bonasus*) in a highly productive, hypoxic continental shelf ecosystem. *Fisheries Oceanography* 19:301-317

Deagle BE, Kirkwood R, Jarman SN (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology* 18:2022-2038

- Dillon W, Goldstein M (1984) Multivariate analysis. John Wiley & Sons, New York
- Doucet-Beaupre H, Breton S, Chapman EG, Blier PU, Bogan AE, Stewart DT, Hoeh WR (2010) Mitochondrial phylogenomics of the Bivalvia (Mollusca): searching for the origin and mitogenomic correlates of doubly uniparental inheritance of mtDNA. BMC Evolutionary Biology 10
- 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 Research I 157:923-930
- 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, Call GC, Grubbs RD (2013) Age, Growth, and Reproductive Biology of Cownose Rays (*Rhinoptera bonasus*) in Chesapeake Bay. Marine and Coastal Fisheries (In review)
- Fisher R, Garrett C, Grubbs RD (2011) Cownose ray (*Rhinoptera bonasus*) predation relative to bivalve ontogeny. Journal of Shellfish Research 30:187-196
- 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. Molecular Marine Biology and Biotechnology 3:294-299

- Frisk M, Miller T, Dulvy N (2004) Life histories and vulnerability to exploitation of Elasmobranchs: Inferences from elasticity, perturbation and phylogenetic analyses. *Journal of Northwest Atlantic Fishery Science* 34:1-19
- Goodman M, Conn P, Fitzpatrick E (2010) Seasonal Occurrence of Cownose Rays (*Rhinoptera bonasus*) in North Carolina's Estuarine and Coastal Waters. *Estuaries and Coasts* 34:640-651
- Grusha D (2005) Investigation into the life history of the cownose ray, *Rhinoptera bonasus*, (Mitchill 1815). MS thesis, Virginia Institute of Marine Science, College of William and Mary,
- Hare MP, Palumbi SR, Butman CA (2000) Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. *Marine Biology* 137:953-961
- Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B: Biological Sciences* 270:313-321
- Heithaus MR, Frid A, Wirsing AJ, Worm B (2008) Predicting ecological consequences of marine top predator declines. *Trends in Ecology & Evolution* 23:202-210

- Knowlton N, Weigt LA (1998) New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society of London Series B: Biological Sciences* 265:2257-2263
- Kruskal JB (1964) Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* 29:1-27
- Last P, Compagno L Review of the biodiversity of rays in the South China Sea and adjacent areas. In: Fowler S, Reed T, Dipper F (eds). *Proc Elasmobranch biodiversity, conservation and management: Proceedings of the International Seminar and Workshop in Sabah*. IUCN SSC Shark Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK
- Leclerc L-M, Lydersen C, Haug T, Glover KA, Fisk AT, Kovacs KM (2011) Greenland sharks (*Somniosus microcephalus*) scavenge offal from minke (*Balaenoptera acutorostrata*) whaling operations in Svalbard (Norway). 2011
- Lynch M, Jarrell PE (1993) A method for calibrating molecular clocks and its application to animal mitochondrial DNA. *Genetics* 135:1197-1208
- Myers R, Baum J, Shepherd T, Powers S, Peterson C (2007) Cascading Effects of the Loss of Apex Predatory Sharks from a Coastal Ocean. *Science* 315:1846-1850
- NCDENR (2013) NCDENR Proclamation SC-01-2013. In: NCDMF (ed), Morehead City, NC

NCDMF (2010) Bay Scallop Fishery Management Plan Amendment 1. In: Fisheries NCDOM
(ed), Morehead City, NC

NCDMF (2013) Bay Scallop Fishery Management Plan Amendment 2. In: Fisheries NCDOM
(ed), Morehead City, NC

Neer JA, Kenneth AR, Enric C (2007) Simulating the effects of temperature on individual and
population growth of *Rhinoptera bonasus*: a coupled bioenergetics and matrix modeling
approach. Marine Ecology Progress Series 329:211-223

Neer JA, Thompson BA (2005) Life history of the cownose ray, *Rhinoptera bonasus*, in the
northern Gulf of Mexico, with comments on geographic variability in life history traits.
Environmental Biology of Fishes 73:321-331

Oesterling M (2006) A History of Cownose Ray Interactions in Virginia. In: Fisher R (ed)
Regional Workshop on Cownose Ray Issues Identifying Research and Extension Needs,
Yorktown VA

Orth RJ (1975) Destruction of eelgrass, *Zostera marina*, by the cownose ray, *Rhinoptera
bonasus*, in the Chesapeake Bay. Chesapeake Science 16:205-208

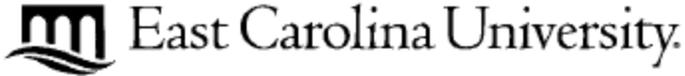
Pauly D, Christensen V, Dalsgaard J, Froese R, Jr FT (1998) Fishing Down Marine Food Webs.
Science 279:860-863

- Peek AS, Gustafson RG, Lutz RA, Vrijenhoek RC (1997) Evolutionary relationships of deep-sea hydrothermal vent and cold-water seep clams (Bivalvia: Vesicomidae): results from the mitochondrial cytochrome oxidase subunit I. *Marine Biology* 130:151-161
- 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* 129:349-356
- Prugh LR, Stoner CJ, Epps CW, Bean WT, Ripple WJ, Laliberte AS, Brashares JS (2009) The Rise of the Mesopredator. *BioScience* 59:779
- Ratnasingham S, Hebert PDN (2007) bold: The Barcode of Life Data System (<http://www.barcodinglife.org>). *Molecular Ecology Notes* 7:355-364
- Rocco L, Liguori I, Costagliola D, Morescalchi MA, Tinti F, Stingo V (2007) Molecular and karyological aspects of Batoidea (Chondrichthyes, Elasmobranchi) phylogeny. *Gene* 389:80-86
- 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, SC
- Saccone C, De Giorgi C, Gissi C, Pesole G, Reyes A (1999) Evolutionary genomics in Metazoa: the mitochondrial DNA as a model system. *Gene* 238:195-209

- Sasko DE, Dean MN, Motta PJ, Hueter RE (2006) Prey capture behavior and kinematics of the Atlantic cownose ray, *Rhinoptera bonasus*. *Zoology* 109:171-181
- Schwartz F (1964) Effects of winter water conditions on fifteen species of captive marine fishes. *American Midland Naturalist* 71:434-444
- Smith J, Merriner J (1985) Food habits and feeding behavior of the cownose ray, *Rhinoptera bonasus*, in lower Chesapeake Bay. *Estuaries and Coasts* 8:305-310
- Smith J, Merriner J (1986) Observations on the reproductive biology of the cownose ray, *Rhinoptera bonasus*, in Chesapeake Bay. *Fisheries Bulletin* 84:871-877
- Smith J, Merriner J (1987) Age and growth, movements and distribution of the cownose ray, *Rhinoptera bonasus*, in Chesapeake Bay. *Estuaries and Coasts* 10:153-164
- Terborgh J, Lopez L, V PNe, Rao M, Shahabuddin G, Orihuela G, Riveros M, Ascanio R, Adler GH, Lambert TD, Balbas L (2001) Ecological Meltdown in Predator-Free Forest Fragments. *Science* 294:1923-1926
- Wang H, Guo X (2008) Identification of *Crassostrea ariakensis* and Related Oysters by Multiplex Species-Specific PCR. *Journal of Shellfish Research* 27:481-487
- Ward RD, Zemplak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B* 360:1847-1857

Yokota L, Lessa R (2006) A Nursery Area for Sharks and Rays in Northeastern Brazil.
Environmental Biology of Fishes 75:349-360

Appendix A



**Animal Care and
Use Committee**

212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

November 14, 2011

Joseph Luczkovich, Ph.D.
Department of ICSP/Biology
Howell Science Complex
East Carolina University

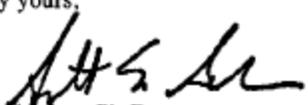
Dear Dr. Luczkovich:

Your Animal Use Protocol entitled, "Consumption Data of Cownose Rays in North Carolina and Virginia: Verification of Prey Items, Shellfish Species, and Consumption Proportion through Direct and Genetic Techniques" (AUP #D268) was reviewed by this institution's Animal Care and Use Committee on 11/14/11. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,



Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

