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

LYNDELL M. BADE, M.Sc.

PRESENTING RESEARCH CONDUCTED AT
EAST CAROLINA UNIVERSITY

Christopher N. Balakrishnan, Erik M. Pilgrim*,
Susan B. McRae, and Joseph J. Luczkovich
East Carolina University, Greenville, North Carolina
Molecular Ecology Research Branch, EPA,
Cincinnati, Ohio*

Outline

Methods:

- Cownose ray collection

Digestive Tract Diets:

- Methods
- Results

Genetics:

- Methods & Procedures
- Results

Conclusions

The Issue?

- Cownose rays implicated in diminished shellfish stocks
- Visual diet analyses can result in high % of unidentifiable and unknown prey types

- Led us to use genetic techniques to investigate cownose ray diets

Potential Prey of Interest:

Eastern oyster

Hard clam

Bay scallop

Soft-shell clam

Stout tagelus

Baltic macoma

Cross-barred venus clam

Collection Methods

Capture of Individuals:

- Longline, hook & line, bowfishing, gill net, cast net, haul seine
- Worked with commercial & recreational fishers

Sampling & measurements:

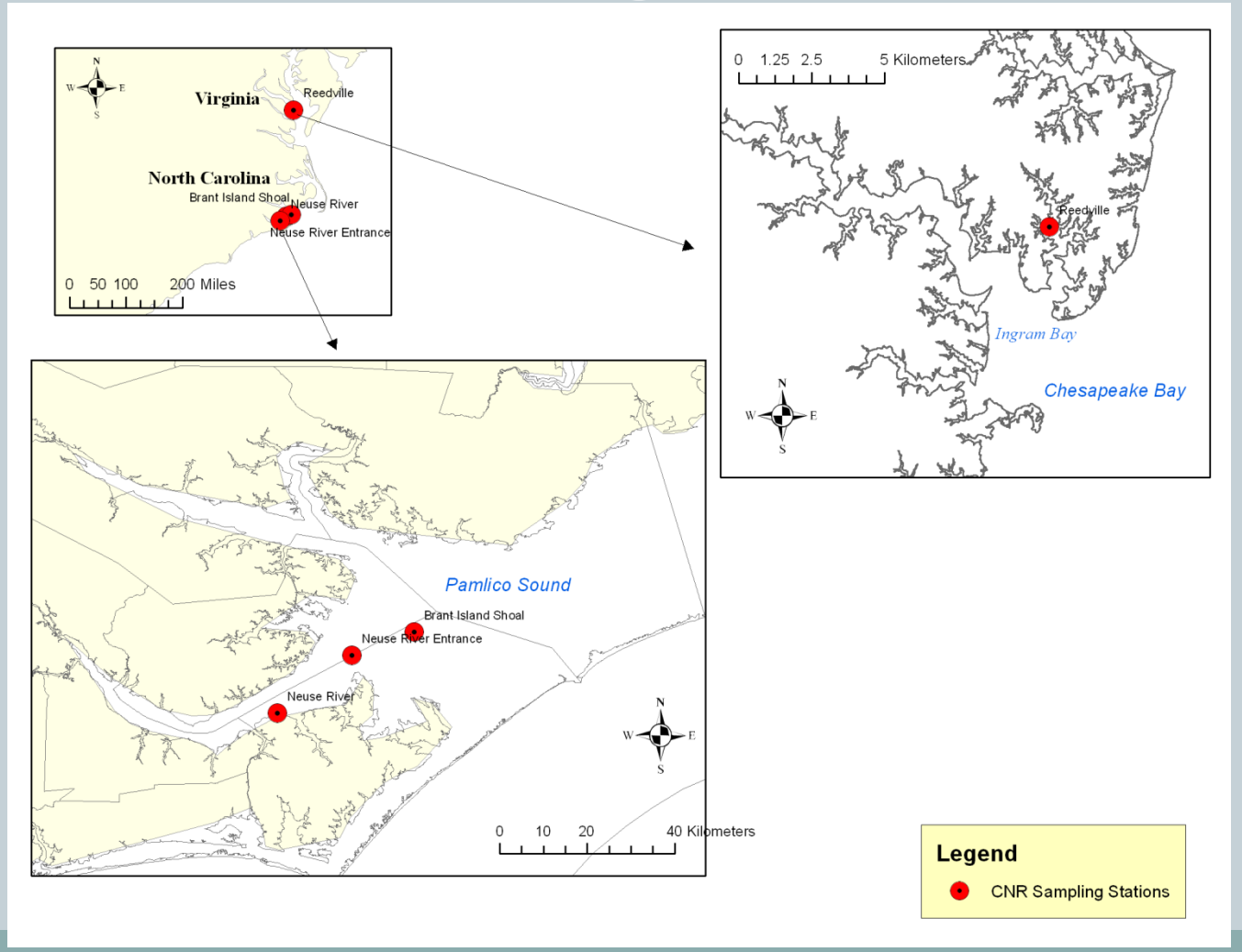
- Sex, disc width, length, tissue sample
- Dissections

Collection Sites

N = 33

NC = 8

VA = 25



Cownose Rays Collected

Table of cownose rays collected and used in this study, divided by location caught (state) and gear type.

			Catch Demographics per Gear Type					
Location	Gear Type	Number	Female	Male	Unknown	Age	Avg DW (cm)	Tissue Storage Method
NC	Hook & Line	5	3	2	0	A	91.6	Ethanol
	Nets	3	0	3	0	YOY	48.5	Ethanol/Cold
VA	Bowfishing	19	11	7	1	A	96.5	Cold
	Haul Seine	6	6	0	0	A	101.9	Cold
	Totals	33	20	12	1			

Methods: Digestive Tract Contents

Visual investigation of stomach & spiral valve contents

Visual Analysis

Samples & Collection:

- Bivalve tissue
- Unidentified tissue (boluses)
- Well-digested “goo” (chyme)
- Fluid
- Hard parts from fish, crustaceans, shells
- Homogenate

Visual Diet Analysis

Stomach Contents:

Avg % by Wet Weight:

Unknown Tissue:	80.8%
Fish parts:	10.2%
Hard parts (shells):	4.5%
Bivalve tissue:	3.8%
Detritus:	0.08%

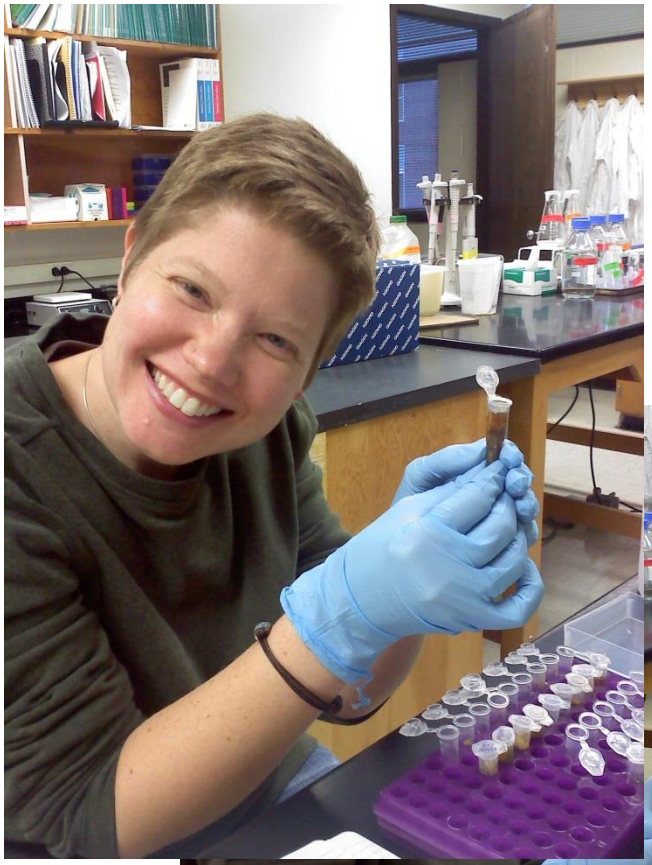
- 4 of 23 individuals with tissue identifiable as bivalve in stomachs; caught by bowfishing

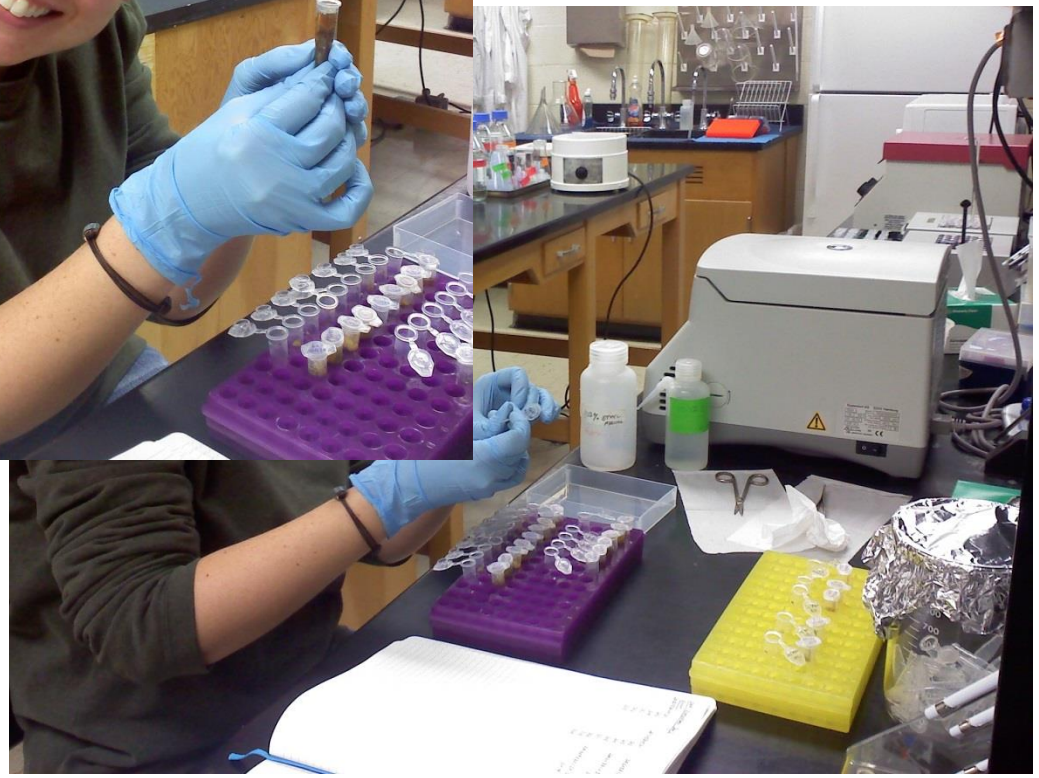
Spiral Valve Contents:

Avg % by Wet Weight:

Unknown Tissue:	94.5%
Fish parts:	2.5%
Hard parts (shells):	3.0%
Bivalve tissue:	0%
Detritus:	0.02%

Genetics Methods





Genetics Methods Overview

DNA

Amplify COI

Clean PCR

Sequence

Design Primers

Optimize

Apply test

1. Sample collection from locally acquired specimens of known species
2. DNA Extraction of tissue samples
3. Sequence COI gene using universal primers
4. Align sequences of bivalve and cownose ray species
5. Design specific primers for each bivalve species
6. Test primers
7. Multiplex PCR test designed & optimized
8. Process digestive tract contents with PCR test

Species-Specific Primers

DNA

Amplify COI

Clean PCR

Sequence

Design Primers

Optimize

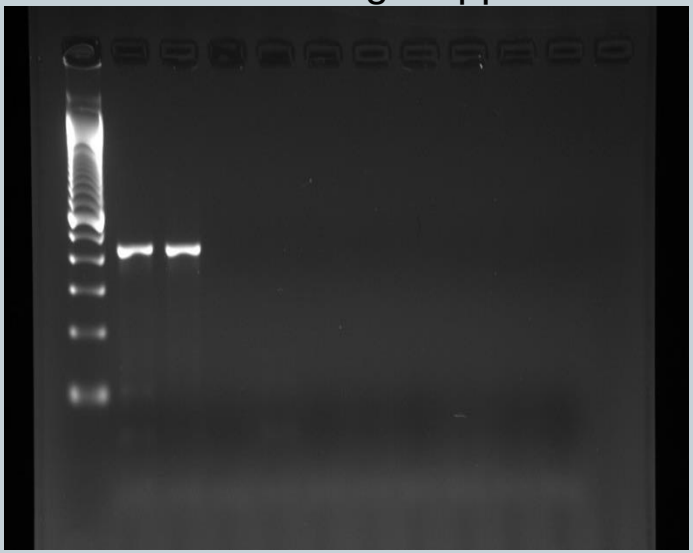
Apply test

- Forward and reverse primer for each species
- Species-specific primers; goal to not amplify the non-target species
- Primers amplify a portion of the COI region during PCR
- Goal to amplify different sized PCR products so can be distinguished on a gel
- Goal to multiplex (multiple primers in one PCR reaction) so conditions kept consistent

Testing Primers for Specificity

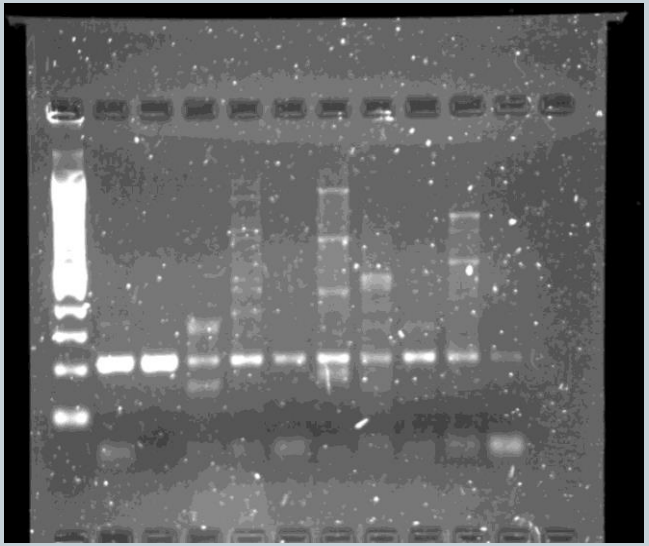
Good Result:
Soft-shell clam primer sets:

M *Mar* Non-Target spp Neg



Bad Results: Bay scallop primer sets:

M *Aic* Non-Target spp Neg



*Non-target species: all other bivalves (6 species) plus cownose ray DNA

Multiplex PCR Test

Chose species with different COI gene fragment sizes

- Based on design of primers

Used species with similar optimized PCR conditions

Allows for testing of multiple species in 1 reaction!

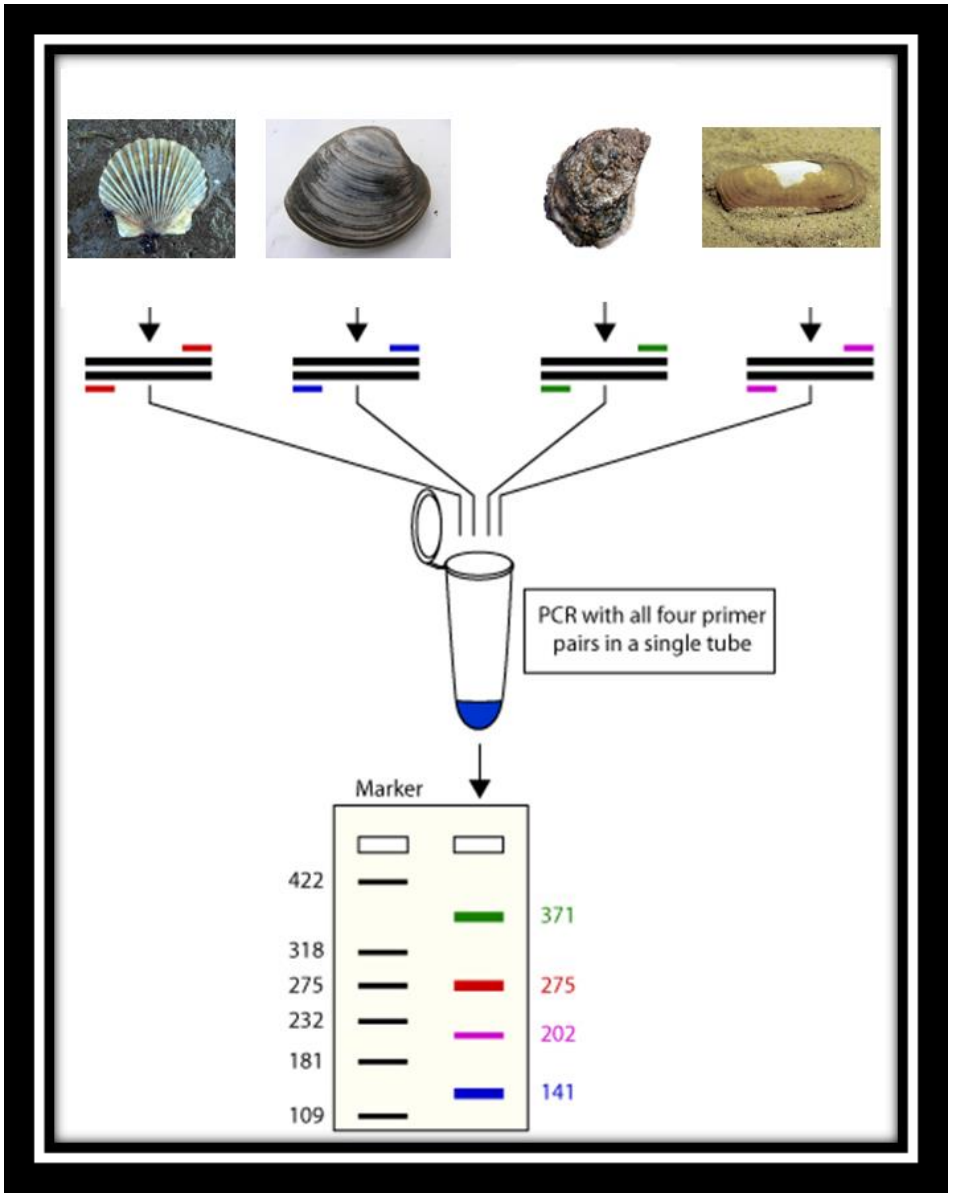


Image adapted from Premier Biosoft

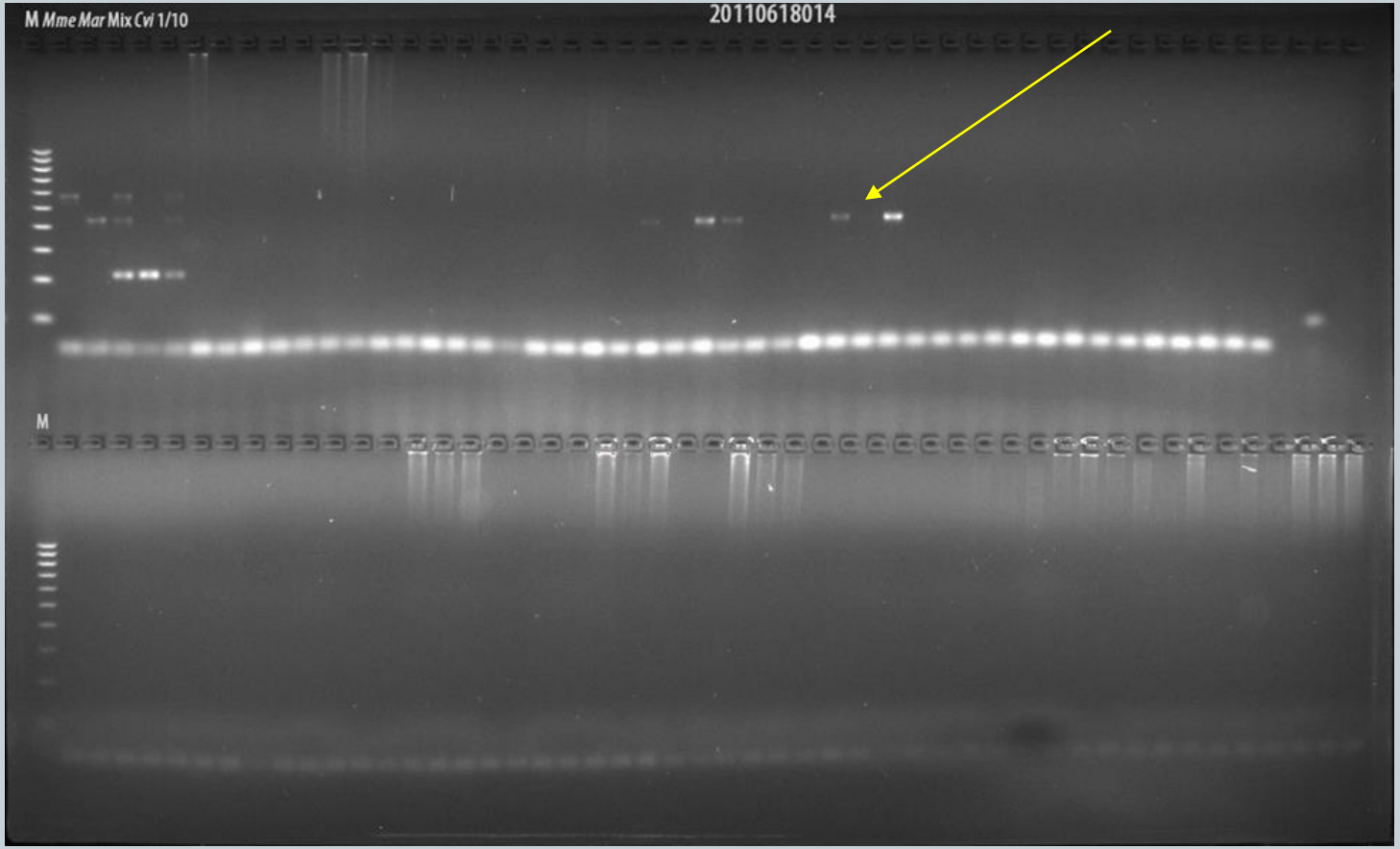
Species-Specific Primers & Multiplexed PCR reactions

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

Testing Digestive Tract Contents

- DNA
- Amplify COI
- Clean PCR
- Sequence
- Design Primers
- Optimize
- Apply test

Positive for *Mar*



Results:
Genetic Testing of Digestive Tract Samples

Table 7. 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*). Treating Baltic macoma clam results with caution.

			Bivalve Species						
Location	Gear Type	Number	*Aic*	*Tpl*	*Mme*	*Mar*	*Cvi*	*Mba*	*Cca*
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%)	

Table 5. Samples positive by multiplex PCR tests for any of the seven target bivalve species, divided by the stomach and spiral valve and type of sample taken from each. Samples are listed by cownose ray specimen identification code, location of capture, gear type, and method of sample storage (EtOH = 95% ethanol preservation, Cold = 4°C storage with same-day DNA extraction). Blank cells indicate no evidence of the target species in samples of that type, an x indicates that at least one of the target bivalve species was detected in that sample type, and a dotted line indicates that sample type was not available or taken for that cownose ray digestive tract.

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

Conclusions

- Baltic macoma and cross-barred venus primers to be redesigned
  - Uncertain samples were sequenced to determine accurate species identity
  - Baltic macoma primers were amplifying samples positive for stout tagelus
  - Baltic macoma positives were false positives, confirmed by sequencing
- COI-based species-specific genetic testing works on stomach and spiral valve samples!
- Differing degrees of digestion
- Tests are designed and ready for future use!

Future Research

- Application on digestive tracts from increased number of individuals:
  - Different locations in NC, Chesapeake Bay, FL
  - Increased number of individuals in different size classes
- Include more molluscan and crustacean species

- Testing of all other "unknown" samples (through direct sequencing) to see what the rays ate
- Quantify sampling and tissue storage techniques for diet samples

- Valuable tool for elucidating cownose ray impact on prey sources & trophic impact

Many Thanks To…

ECU Department of Biology
Institute for Coastal Science & Policy
Luczkovich & McRae labs

Dr. Roger Rulifson
Dr. Kevin O'Brien
Robert Fisher, VIMS
Dr. John Morrissey, Sweet Briar College

Dr. Dean Grubbs, FSU
Joseph Smith, NOAA
George & Anna Beckwith
Chuck Bangley, Andrew Thaler
Jason Rock, April Goodman Hall
UNC-IMS & NC DMF Morehead City
Dr. Joel Fodrie, Nathan Geraldi
Dr. Roger Newell & Dr. David Kimmel
Will Flannery
VA cownose ray team!

Dr. Erik Pilgrim, EPA
Dr. Ashley Egan
Denise Mayer
Lena Keller

Friends in the Luczkovich, McRae, and Rulifson labs
American Elasmobranch Society
AES friends & mentors:
Julie Neer, Matt Ajemian, Jimmy G, Neil Aschliman, Matthew Kolmann
Dr. Shona Paterson

And a huge thanks to our hosts and organizers!
NOAA
Chesapeake Bay Program
Shark Advocates International
The National Aquarium
Emilie Franke