MATERNAL INPUT OF STRIPED BASS (*MORONE SAXATILIS*): DETERMINING A MOTHER'S LIFE HISTORY FROM ITS PROGENY IN COASTAL NORTH CAROLINA RIVERS

by

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Masters of Science Biology Program

Abstract

Otolith studies have become more prevalent in recent years as use has expanded from ageing to examination of migration patterns and fidelity to natal habitats, and more recently examining otoliths for possible maternal contribution to progeny otoliths. The otoliths of larval Striped Bass, *Morone saxatilis* (Walbaum; 1792), were used to determine the presence of maternal contribution through three experiments. The first experiment was to examine the formation of larval otoliths to determine if otoliths formed prior to/ during the yolk sac stage when maternal influences are present, and thus if maternal contribution is possible. Two of the three otolith pairs (sagitta and lapillus) are formed during the embryo stage (sagitta) or post hatch (lapillus). The sagittal otoliths are the most often used otolith in microchemical studies; therefore its formation during the embryo stage suggests maternal contribution to progeny otoliths is possible. The second experiment used microchemical analysis of adult Striped Bass

soft tissue (muscle, liver, kidney, and gonads) to determine whether adult Striped Bass develop trace elemental signatures similar to the adult otoliths. The gonadal tissues (ovaries and testes) were found to have similar signatures to adult otoliths utilizing a linear discriminate function analysis. As the two previous experiments support the hypothesis of maternal contribution the final step was to run a discriminate function analysis between the progeny and maternal otoliths. Embryo sagittal otoliths correctly identified the maternal clusters 91.67% of the time (n = 12), yolk sac larvae 66.67% of the time (n = 15), and non-yolk sac larvae only 60.94% of the time (n = 64). Progeny otoliths were also able to identify maternal river (Neuse, Roanoke or Tar); embryos classified the river 83.33% of the time (n = 12), yolk sac larvae classified 93.33% of the time (n = 15), and non-yolk sac larvae classified with 44.44% (n = 72) accuracy. Results of this study validate the hypothesis of maternal contribution and support the hypothesis of maternal life history determination from progeny.

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PREFACE

Statement of the problem

Striped Bass (*Morone saxatilis;* Walbaum 1792) is a species that consumes a number of different prey species, can live in a wide range of temperatures, and thrive in marine, freshwater, and estuarine environments (Fay et al. 1983). Despite the understanding that not all Striped Bass are anadromous, they are often classified as anadromous for management purposes (Bain and Bain 1982). Anadromous fish live in saltwater as adults and spawn in freshwater (Bain and Bain 1982; Secor et al. 2001). Striped Bass is an important species economically in North Carolina as it supports both commercial and recreational fisheries (Bain and Bain 1982). To help support these fisheries, North Carolina has had a widespread long-term stocking program for Striped Bass in the Cape Fear, Neuse, Roanoke, and Tar Rivers (Woodroffe 2011).

Fish can be classified to a particular river through the use of otolith (fish earbone) microchemistry of trace elements (Halden and Friedrich 2008; Mohan et al. 2012). Since otoliths accrete daily layers, the chemical signature of the ambient water is incorporated into the otolith, and thus the fish's migration patterns can be observed over time (Campana 1999; Secor and Piccoli 2007). Morris et al. (2003) were able to correctly classify Striped Bass to three different rivers: the Neuse and Roanoke rivers in North Carolina, USA and the Stewiacke River in Nova Scotia, Canada using the otolith elemental signatures. The Neuse had the highest classification with 88%, the Stewiacke had 79%, but the Roanoke was only 47%. It was hypothesized that young of year fish in Albemarle Sound used a number of different watersheds for nurseries, and so there were many 'signals' in the Ablemarle Sound; Mohan et al. (2012) confirmed this hypothesis of the Ablemarle Sound having many signals. Often times migrations

of anadromous fishes between fresh and saltwater are examined utilizing the Sr:Ca (strontium to calcium) ratio. Higher ratios correspond to saltwater, while lower ratios typically correspond with freshwater (Halden and Friedrich 2008).

Overall, the method of using otolith microchemistry to trace the migration pattern and juvenile habitat of fish is still expanding. Volk et al. (2000) used salmonid species to determine that progeny from mothers that matured in saltwater had higher Sr:Ca concentrations in the otoliths than those from freshwater mothers. The salmonid species were raised in captivity in either fresh or saltwater, and thus the life history of the mother was known for the duration of vitellogenesis (yolk deposition). The mothers for my study were determined to be either anadromous or resident though otolith microchemistry. This knowledge is important as the yolk sac is derived from maternal sources and is the nutrition source for Striped Bass larvae until first feeding at approximately 5 dph (Hardy 1978).

While it has been shown that maternal contribution can be passed on to otoliths of the offspring, not much is known about the formation of the otolith itself. In the European Anchovy (*Engraulis encrasicolus*) otolith formation occurs prior to hatching (Alanondo et al. 2008). The hatching left a mark on the otolith that could be seen both at 0 and 29 days post hatch (dph), and may be observed later in life if the microscope has a high enough resolution (Alanondo et al. 2008). The daily rings for the European Anchovy begin forming the day after hatching (Aldanondo et al. 2008).

It has been determined that Striped Bass otoliths incorporate the ‘watershed signature’ when residing in coastal streams for two weeks or longer (Mohan et al. 2012). Therefore Striped Bass age 0 can be assigned watersheds, and relative abundance in the adult population by

watershed should reflect quality of Striped Bass nursery habitat since the fish survived to adulthood. However, it remains unclear about the trace elemental concentrations occurring in the otolith just after formation.

The goals of my study were: 1) to determine if maternal contribution is possible based upon the timing of otolith formation; 2) to determine if maternal contribution is possible by comparing adult tissue elemental signatures to adult otolith elemental signatures; 3) to examine the existence of maternal contribution by comparing maternal otolith elemental signatures to their progeny. A series of objectives were utilized to meet each goal: 1.1) to examine of formation and timing of all three otolith pairs; 1.2) to determine the relationship between larval fish length and age to total otolith size; 1.3) to use the results from the two previous objectives to determine if maternal contribution is possible from the larval angle; 2.1) to group similar adult otolith elemental signatures; 2.2) to compare adult otolith group signatures to adult tissue signatures; 3) to use the results from the two previous objectives to determine if maternal contribution is possible from the adult angle; 3.1) to compare progeny otolith elemental signatures of the three stages of larval Striped Bass; 3.2) to compare adult otolith groups to progeny otoliths; 3.3) to compare larval otoliths to maternal river; and 3.4) to use the results of the objectives to determine if maternal contribution exists in Striped Bass.

Description of thesis chapters

This thesis is divided into four chapters. Chapter 1 examines the prospect of maternal contribution from the larval angle by determining if otoliths form when maternal influences exist (during the embryo or yolk sac stage). Chapter 2 determine if maternal contribution is possible from the adult angle by comparing adult otolith elemental signatures to the microchemical

signature of four different tissue types (muscle, liver, kidney, and gonads). Chapter 3 determines if there is any maternal contribution to progeny otoliths by seeing if the progeny's otoliths can be used to correctly classify adult otolith group and maternal river. Chapter 4 summarizes the findings of the first three chapters, explains the implications to management and suggests further research avenues.

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Chapter 1

STRIPED BASS (*MORONE SAXATILIS*) OTOLITH FORMATION AND DEVELOPMENT

Abstract

Otolith studies have become more prevalent in recent years as use has expanded from only ageing to examination of migration patterns and fidelity to natal habitats. The otolith of Striped Bass, *Morone saxatilis* (Walbaum; 1792), is used for all of these purposes, yet its formation and early development have not been documented. I was able to identify the timing and formation of the three otolith pairs during late pre-hatch embryo, post-hatch larva, and metamorphosis. The sagittal otoliths were first to appear, forming shortly before hatch and were observed growing larger throughout the larval stage. The lapilli otoliths formed within the first 24 hours post hatch. The asterisci otoliths were difficult to locate, but formed between 4 and 15 days post hatch (dph). At hatch the sagittal otoliths appeared circular, and by 5 dph gained some dimensionality. At 15 dph the sagittal otoliths began to elongate along the anterior/ posterior axis. This knowledge of when otoliths form and change to adult shape will affect any microchemical analysis done in the first year of life, especially as the asterisci otoliths form around first feeding, and should be taken into account when choosing an otolith for analysis of elemental chemistry and ageing.

Introduction

Otoliths are fish earstones that are used for hearing and maintaining equilibrium (Secor et al. 1991a). Each fish has three pairs of otoliths: the sagitta, lapillus and asteriscus. In most teleost fish the sagittae are the largest, and thus used for both ageing and microchemical analysis

studies (Secor 1991a). Otoliths are used to determine natal habitats through microchemical analysis (Thorrold et al. 1998; Hobbs et al. 2007; Dobbs 2013). This has led to a need for an understanding about when and how otoliths form as the formation and timing might have impact upon microchemical analysis, especially for natal origin studies. This chapter of my study focuses on the growth and formation of the sagittal otolith of Striped Bass, *Morone saxatilis* (Walbaum; 1792), but will also examine the timing of the lapillus and asteriscus formation using hatchery fish.

Since 1980, North Carolina in the United States has had a widespread long-term stocking program for Striped Bass in the Neuse, Roanoke, and Tar Rivers (Woodroffe 2011) and more recently the Cape Fear River. This is due to the economic importance of Striped Bass, both commercially and recreationally (Bain and Bain 1982). North Carolina currently has two hatcheries that raise fish for stocking into coastal watersheds: the Edenton National Fish Hatchery (ENFH) and the Watha State Fish Hatchery (WSFH) (Figure 1). The hatcheries collect adult fish from the wild population, breed them, and then raise the young for stocking. This use of hatcheries to artificially increase the population has led to billions of fry and fingerlings being stocked throughout the United States (Woodroffe 2011). Striped Bass have been stocked as far west as San Francisco as early as 1876 (Fay et al. 1983). Today, the hatchery programs use endemic broodstock when stocking the rivers. For example, progeny from Neuse River mothers are stocked in the Neuse River as phase I (5 cm/ 2 in) or phase II (15 cm/ 6 in) fingerlings.

As the larval fish used for this study are hatchery-raised it is important to understand hatchery protocol. Hatchery personnel collect adult broodfish on the spawning grounds of local coastal rivers, and then inject both the males and females with human chorionic gonadotropin (HCG) hormone to induce spawning within 20-36 hours of capture (Harrell et al. 1990). The

fertilized eggs are then placed into MacDonald jars with a slow current to circulate oxygenated water to all of the eggs. Once hatched, the larvae flow out of the McDonald jars and into five gallon aquaria filled with groundwater: the Castle Hayne Aquifer for ENFH, and a mix of Peedee and Black aquifers for WSFH. At 4 days post hatch (dph), fish are offered brine shrimp, though hatchery managers note that the majority do not feed until 5 dph (Steve Jackson, US Fish and Wildlife Service, ENFH, personal communication).

At 5 to 7 dph the larvae are transferred to outdoor ponds filled with surface water from a nearby creek (ENFH) or groundwater (WSFH). Once the larvae reach about 5 cm (1 to 2 months, "Phase I" fish) they are brought into the holding house where they are grown to a uniform size. This is done by altering the amount of food offered and water temperature between the different fish sizes. Once a uniform size for the cohort has been reached, they are placed back into the outdoor ponds now filled with groundwater (both hatcheries). In late October-November the fish (now about 15 cm in size and considered "Phase II fish") are removed from the ponds and stocked back into the parental natal stream (Harrell at al. 1990; Steve Jackson, ENFH, personal communication).

There are many questions concerning the formation of the otolith, specifically at which point in the life history they form and if different otoliths form at different times. The goal of my research is to determine if Striped Bass otoliths form while influenced by maternal material. The objectives are to determine the timing of formation for all three Striped Bass otolith pairs, particularly the sagittae, and document their growth relative to fish total length and age under near optimal hatchery conditions. We needed this information in order to accurately assess adult otolith microchemistry results within the primordium and first summer of life. I hypothesize that

the sagittal otolith will form first followed by the lapillus and then the asteriscus. I predict a change in relationship between otolith size and fish length after the early-larval/ yolk sac stage.

Methods

Adult collection

Adult broodfish were collected during March and April in 2012 and 2013 from the Cape Fear, Neuse, Roanoke and Tar rivers by electroshocking, then transported to either ENFH or WSFH for spawning (Figure 1.1). Fish were placed into circular spawning tanks. Each tank contained 1 female and 2 or 3 males depending upon the catch, and then all fish were injected with HGC to induce spawning. After injection, fish spawn naturally within the tanks.

Fish collection and measurements

At each hatchery fertilized eggs and larvae were collected daily from the time of spawn until 5 dph when the yolk was absorbed. At this time, the post yolk sac fish were moved to the outdoor ponds. Specimen collections in the outdoor ponds were then conducted weekly until approximately 40 dph when the fish metamorphosed to the juvenile phase. Fish were collected using a 500-µm mesh zooplankton net, which had been sterilized with vinegar for at least 8 hours to minimize cross-contamination between ponds, and to prevent accidental introduction of fish from other ponds.

Eggs and larval fish were euthanized using electro-narcosis and immediately preserved in 95% ethanol to ensure minimal shrinkage (Radtake 1989). Preserved specimens were then taken back to the lab where they were photographed using an Olympus SZX 16 dissection scope. For larvae (free of the egg sac), total length (TL), and total otolith length (TOL) were measured using

ImagePro 6.2 software. For eggs (embryos within the egg sac), the egg diameter (TL) and TOL (if present) were photographed and measured using the microscope. Otoliths were identified microscopically using high power magnification (up to 184x), then removed from the surrounding tissue by bleach dissolution as recommended in Secor et al. (1991b), or by dissection if 15 dph or older. The sagittal otoliths were measured for otolith length (TOL) along the longest axis. These measurements were then averaged each day to determine the average size for each age group by river.

Statistical analysis

All statistical analyses were done in JMP Pro 10. ANOVA was used to determine if TOL was different between rivers and years. If there was a difference between rivers and or years, all analyses were conducted by river and or year. Regressions were performed between TOL and TL, and TOL and age. Based upon the findings in Radtke (1989) and Bystydzienska et al. (2010) each of these regressions was performed three times, because as the fish grow the relationship between fish length and otolith size changes. Radtke (1989) found that the relationship switched from quadratic to linear while Bystydzienska et al. (2010) found a significant and conspicuous change in the slope. The three regressions were yolk sac larvae (egg to 5 dph), non-yolk sac larvae ($\geq$ 15 dph), and an all-inclusive regression. Results were considered significant at $\alpha = 0.05$.

Results and discussion

Timing of otolith formation

The Striped Bass ear canal starts development within the first 24 hours post hatch. There is a definitive area behind the eye and below the head case that is indented and in which the

otoliths are found. This indentation grows larger and rounder and by 2 dph the vestibular structure can be seen forming (Figure 1.2).

The structures in question were determined to be otoliths based upon their calcification, location and distance between each other. The otoliths were located in the dorso-posterior cranium directly behind the eyes, and were the first body structures to calcify. Under the assumption that the largest otolith would form first, the order of appearance was the sagitta, lapillus, and then the asteriscus. By 24 hours post hatch, the eyes were beginning to differentiate from the surrounding tissue as the lapillus formed (Table 1.1 and Figure 1.3). Just prior to hatch, one pair of otoliths formed and they were about 12.63µm ±2.06 µm (mean ± standard deviation) and were assumed to be the sagittal otoliths (Table 1.2). At 15 dph when the asteriscus formed, the larvae had developed fins, skin, scales, and the eyes were easily visible (Table 1.1 and Figure 1.3).

The formation of the sagittal otolith prior to hatch has also been documented in European Anchovy, *Engraulis encrasicolus* (Linnaeus; 1758) (Aldanondo et al. 2008). Though the otolith size prior to hatch is not known, its size is 4.07 ± 0.66 µm (Aldanondo et al. 2008), which is much smaller compared to the Striped Bass otolith within the first 24 hours post hatch, 19.00 ± 4.40 µm (Table 1.2).

Any disparity between the number of samples of TL and TOL in Table 1.2 are due to several reasons: 1) age 0 dph fish has the largest discrepancy since some eggs collected were less than 16 hours post spawn and embryos were not developed enough to have otoliths, 2) otoliths may have been difficult to locate due to wrinkles in the egg sac or formation of the vestibular

structure, and 3) for the very young larvae (< 1dph) it was hard to determine with a high level of certainty the otolith location and size.

Sagittal otolith growth

Otolith growth varied significantly by river, year, age, and the interactions of age**x**river, age**x**year, river**x**year, and age**x**river**x**year (Table 1.3). For example, the otolith size at less than 24 hours post hatch (0.5 dph) for the Neuse River was 23.43 ± 2.45 µm in 2012, but only 16.52 ±4.11 µm in 2013 (Appendix A). Possible hypotheses for this difference are mother size, water temperature at spawning, water temperature during larval growth, or other unmeasured parameters. When a discriminant function analysis was performed in an attempt to use otolith size to discriminate maternal length, high levels of misclassification (>75%) were found. Unfortunately, water temperature was not measured during spawning, but as the water is pumped directly from the aquifer the temperature should be similar between years. Water temperature during larval growth was not measured, but could have a small impact due to surrounding air temperatures differing between the years, and thus increasing the water temperature different amounts. Both of these year groups were hatched at Watha before being transported for grow-out at Edenton at 5 dph as they initiated feeding.

Striped Bass appear to have a quadratic relationship with the sagittal otolith between TL and TOL throughout the larval stage (Figure 1.4a; Table1.4). By 15 dph the quadratic relationship becomes linear, and when the entire larval stage is analyzed the relationship between TOL and TL is linear (Table 1.4; Figures 1.4b and c). This is consistent with Radtke's (1989) findings, but not Aldanondo et al. (2008) nor Bystydzienska et al. (2010). Radtke (1989) found a quadratic relationship for the first otolith stage. Radtke (1989) described three stages of otolith

growth for Atlantic Cod, *Gadus morhua* (Linnaeus; 1758): spherical, oblong and crenulated. For the first stage, spherical, the relationship between fish TL and TOL was quadratic, but once the sagittal otolith began to elongate and became convex (>25 mm) the relationship was linear (Radtke 1989), which is very similar to the sagittal otolith growth in Striped Bass.

Aldanondo et al. (2008) suggested that though an exponential model between European Anchovy otolith radius and standard length best fits their larval data, in the late larval/ early juvenile stage the relationship changed to linear between otolith radius and fish length (Aldanondo et al. 2011). While the 'All' relationship in Striped Bass for the four rivers appears to be linear, without data in the 6 to 12 mm TL size category it is hard to conclusively state this is the case. This data gap exists due to inability to collect these sizes from the outdoor ponds and cross contamination concerns. For future studies, it would be beneficial to consider tank raising fish for the duration of the study. Bystydzienska et al. (2010) had a similar issue where they were missing data in the middle fish size range for their work on the Blue Lanternfish, *Tarletonbeania crenularis* (Jordan and Gilbert, 1880).

A linear relationship was found between sagittal TOL and age for the three sample groups across all rivers (Table 1.5; Figure 1.5). It is possible that this linear relationship is due to the fish being hatchery-reared rather than wild-caught. Aldanondo et al. (2008) found that the growth rates between reared and wild European Anchovy differed, and reared fish had larger otoliths than wild fish. They hypothesized that this was due to the slower growth rates of the reared fish relative to wild fish (Aldanondo et al. 2008). This is something for fishery managers to bear in mind when using fish age or length to predict otolith size, and may not hold true across species.

Sagittal otolith shape

Similar to Radatke (1989), I found the growth of the sagittal otolith in Striped Bass larvae can be divided into multiple stages. Stage 1 is when the otolith is spherical and seems to grow consistently along the circumference (1 to 5 days). A portion of the otoliths exhibited a first feeding mark at 5 dph (Figure 1.6). This was assumed to be a first feeding mark as the yolk sac had been absorbed by day 4, but only a few fish at age 5 dph had this feature. Stage 2 occurs when the otolith begins to elongate along the anterior/posterior axis and the otolith becomes more oblong (15 days to 45 days post-hatch). Within the samples collected from egg to approximately 45 dph the otolith did not form into the adult shape which may mean the slope/ relationship changes again during the juvenile stage (Figure 1.7a). It is hypothesized that this change occurs during the juvenile stage. The sulcus appears to form between 5 and 15 dph (Figure 1.7a).

Implications

The sagittal otolith forms in the embryo during the egg stage and grows as the fish does in a quadratic pattern. The lapillus and asteriscus form later in development, 0.5 days post hatch and between 4 and 15 days post-hatch, respectively. As a result of the different timing of formation, the microchemical signatures should be different between the three pairs with the asteriscus being the outlier compared to the other two otoliths. The asteriscus forms in the range at which first feeding occurs, between 4 to 10 days, with wild fish typically being at the latter end of the spectrum (Hardy 1978). This will serve to further separate the asteriscus from the lapillus and sagitta. Since the asteriscus forms later than the other two otolith pairs, it may not contain the spawning river signal but a downstream or Sound signal instead.

Also, it is possible that both the sagitta and the lapillus contain a portion of the mother's microchemical signature as both pairs form before the gills and while the yolk-sac is present. Hobbs et al. (2012) has shown that when ripe mothers of Delta Smelt (*Hypomesus transpacificus*, McAllister; 1963) are injected with high levels of strontium the progeny contain a high strontium signature. Thus the larval sagittal and lapillus otoliths may contain some maternal input, while the asteriscus likely does not due to the late formation. This should be taken into account when planning otolith microchemical studies as a maternal signal may help to reduce confusion associated with wandering signals.

Despite knowing when the otoliths form, using a back-calculation to determine date of spawning is inadvisable. Aldanondo et al. (2008) examined otolith growth in larval European Anchovy under different temperatures and found that though the relationship between otolith length and fish length remained the same, the slope was significantly different between the two temperatures. Aldanondo et al. (2008) also found that reared larvae had larger otoliths than wild larvae, which would make the determination of spawn date using my graphs a rough estimate at best. To improve this estimate, fish could be laboratory raised at different temperatures; the slopes can be determined and used for back-calculations. The otolith shape, however, should remain same regardless of slight temperature changes and could be used for an estimation of wild larval fish age, and spawning date could be back-calculated from the fish age.

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Table 1.1 Fish length/ egg diameter (mm), total sagittal otolith length (μm) by fish age, and physiological and otolith development by age. Photos correspond to the noted letters in Figure 3.

Age	Photos	Fish total length (mm)	Otolith development	Larval development	Sagitta otolith length (μm)
10 hr egg	A	2.08	no otoliths	Blastoderm with granular appearance.	N/A
2 day egg	B	2.56	a few hours before hatch 1 pair sagittae	Larvae mostly hatched but still curved around the yolk sac.	12.63
Less 24hr larvae	C	3.96	2 pairs sagittae and lapilli	Fully removed from egg case and straightened; eyes differentiating	19.00
1 dph	D	4.57		Myomeres becoming defined; eyes with slight pigmentation; yolk sac present.	23.63
2 dph	E	5.20		Myomeres defined; eyes pigmented; yolk sac smaller.	28.81
3 dph	F	5.35		Yolk sac nearly absent; lower jaw defined.	34.01
4 dph	G	5.48	formation of 3rd pair asterisci	Yolk sac absent; oil globule present; gut forming.	38.13
5 dph	H	5.54		Transparent; oil globule present; caudal fin and stomach forming.	43.79
15 dph	I	12.39	3 otoliths pairs	Fins developed; eye developed; scales and skin formed.	299.85
19 dph	J	16.71		Deeper bodied; operculum defined; upper mandible defined.	564.07
21 dph	K	18.72		2 distinct dorsal fins; snout pointed; defined lateral line.	658.90
30 dph	L	23.87		Starting to resemble a juvenile fish.	1096.58

Table 1.2 Growth of Striped Bass larvae in total length (TL-mm) and corresponding otolith size (TOL-µm) by fish age. Average TL, TOL and corresponding standard deviations (SD), and sample size (N) are across all rivers. Age 0 days is the egg stage and 0.5 days is larvae that were collected when the cohort was hatching. See Appendix A for separation by river and year.

	Total length (mm)			Total sagittal otolith length (µm)		
Age (dph)	N	Mean	1 SD	N	Mean	1 SD
0	320	1.90	0.443	39	12.63	2.063
0.5	213	3.96	0.425	189	19.00	4.400
1	259	4.57	0.530	230	23.63	4.145
2	210	5.20	0.466	186	28.81	3.673
3	224	5.35	0.449	207	34.01	5.061
4	200	5.48	0.484	180	38.13	5.209
5	200	5.54	0.481	191	43.79	4.865
15	5	12.39	0.938	5	299.85	40.443
19	5	14.36	2.030	5	429.52	100.962
21	8	17.49	2.628	8	581.61	118.132
22	8	17.73	3.613	8	619.24	151.684
23	4	25.62	0.860	4	958.25	89.405
25	5	25.19	3.253	5	947.92	131.111
26	5	16.75	1.688	5	565.43	80.921
27	9	23.80	7.668	9	910.32	351.464
29	5	22.73	2.609	5	1011.50	69.217
30	12	25.27	4.568	11	1125.90	109.730
31	2	19.56	0.007	2	901.30	327.861
32	3	30.01	5.347	3	1313.79	341.444
33	4	29.79	9.726	4	1304.38	325.631
34	5	22.94	1.206	5	894.19	129.986
35	6	26.48	6.806	6	1088.60	244.675
36	7	25.00	1.547	7	1177.16	70.369
37	3	26.73	2.193	3	1197.23	75.864
40	3	33.39	7.535	3	1506.60	367.083
41	3	37.93	8.778	3	1902.86	81.518
42	4	29.49	5.356	4	1297.81	248.335
45	12	31.43	4.136	12	1267.00	169.409
46	3	35.70	2.957	3	1379.51	104.625
47	9	38.54	4.321	9	1421.44	179.044
58	4	38.67	2.807	4	1819.57	84.287

Table 1.3 3-way ANOVA of river, age, and year differences in total otolith length (TOL-µm) and total length (TL-mm). * denotes significance.

	Source of Variability	DF	F-ratio	p-value
TOL	Age	1	2771.66	<0.0001*
	River	3	30.68	<0.0001*
	Age**x**River	3	111.23	<0.0001*
	Year	1	18.39	<0.0001*
	Age**x**Year	1	97.37	<0.0001*
	River**x**Year	1	48.69	<0.0001*
	Age**x**River**x**Year	1	124.08	<0.0001*
TL	Age	1	31531.11	<0.0001*
	River	3	13.59	<0.0001*
	Age**x**River	3	73.25	<0.0001*
	Year	1	0.17	0.6801
	Age**x**Year	1	25.11	<0.0001*
	River**x**Year	1	23.17	<0.0001*
	Age**x**River**x**Year	1	338.49	<0.0001*

Table 1.4 The regression between TOL and TL by year and river with corresponding sample sizes (n) and r^2 and p-values. Yolk sac larvae are age 0 to 5 dph, non-yolk sac larvae are 15 dph, and older while all includes both groups. Regressions were either quadratic (Q) or linear (L). * significant at $\alpha = 0.05$

Group	River	Year	Regression	N	r^2	Intercept	Slope	Quadratic	p-value
Yolk	Cape								
sac	Fear	2013	Q	158	0.44	-21.765	10.088	$0.731(TL-5.517)^2$	<0.0001*
Larvae		2012	Q	379	0.50	-24.122	11.706	$1.612(TL-4.720)^2$	<0.0001*
	Neuse	2013	Q	217	0.69	-31.826	12.036	$2.102(TL-4.936)^2$	<0.0001*
	Roanoke	2013	Q	333	0.70	-27.633	10.930	$2.223(TL-5.095)^2$	<0.0001*
		2012	Q	29	0.06	16.773	-0.936	$-1.441(TL-3.030)^2$	0.4664
	Tar	2013	Q	104	0.73	-31.846	12.775	$4.088(TL-4.668)^2$	<0.0001*
Non-	Cape								
yolk	Fear	2013	L	9	0.93	13.122	38.127		<0.0001*
sac		2012	L	75	0.92	-398.783	59.279		<0.0001*
Larvae	Neuse	2013	L	5	0.85	61.368	35.123		0.0247*
	Roanoke	2013	L	15	0.73	464.698	25.730		<0.0001*
		2012	L	22	0.73	28.226	42.683		<0.0001*
	Tar	2013	L	7	0.90	-563.992	59.695		0.0012*
All	Cape								
	Fear	2013	L	167	0.99	-210.141	44.458		<0.0001*
	Neuse	2012	L	454	0.98	-209.062	50.593		<0.0001*
		2013	L	222	0.96	-175.732	41.712		<0.0001*
	Roanoke	2013	L	348	0.98	-187.929	43.048		<0.0001*
	Tar	2012	L	51	0.97	-122.663	47.341		<0.0001*
		2013	L	111	0.99	-202.392	49.456		<0.0001*

Table 1.5 Linear regressions between TOL and age by river with corresponding sample sizes and r^2 and p-values. Yolk sac larvae are age 0 to 5 dph, Non-yolk sac larvae are ≥ 15 dph while All includes both groups. * significant at $\alpha = 0.05$; † denotes a r^2 value ≥0.69.

Group	River	Year	N	r^2	Intercept	Slope	p-value
Yolk sac Larvae	Cape Fear	2013	158	0.792†	19.588	5.437	<0.0001*
	Neuse	2012	379	0.752†	19.675	5.118	<0.0001*
		2013	217	0.846†	15.911	5.224	<0.0001*
	Roanoke	2013	333	0.850†	15.390	5.768	<0.0001*
	Tar	2012	30	0.217	10.943	3.479	0.0095*
		2013	104	0.834†	17.982	4.311	<0.0001*
Non-yolk sac Larvae	Cape Fear	2013	9	0.199	-6567.8	172.766	0.2288
	Neuse	2012	75	0.735†	-236.693	37.819	<0.0001*
		2013	5	0.895†	-12.582	42.210	0.0148*
	Roanoke	2013	15	0.093	-791.931	47.093	0.2681
	Tar	2012	22	0.691†	-328.519	52.152	<0.0001*
		2013	7	0.788†	214.875	27.605	0.0076*
All	Cape Fear	2013	167	0.962†	-40.613	28.278	<0.0001*
	Neuse	2012	454	0.939†	-41.710	30.959	<0.0001*
		2013	222	0.852†	-61.363	37.554	<0.0001*
	Roanoke	2013	348	0.967†	-44.333	30.195	<0.0001*
	Tar	2012	52	0.961†	-22.308	42.764	<0.0001*
		2013	112	0.966†	-52.192	32.411	<0.0001*

Fig. 1.1 a Map of North Carolina, USA with the Edenton National Fish Hatchery (ENFH) and Watha State Fish Hatchery (WSFH) marked with yellow stars, and the 4 rivers (Cape Fear, Neuse, Roanoke and Tar) the mothers are from marked with green drop pins. **b** is a map of the ENFH ponds with the ponds from which fish were collected marked with red drop pins for 2012 collections and yellow diamonds for 2013 collections.

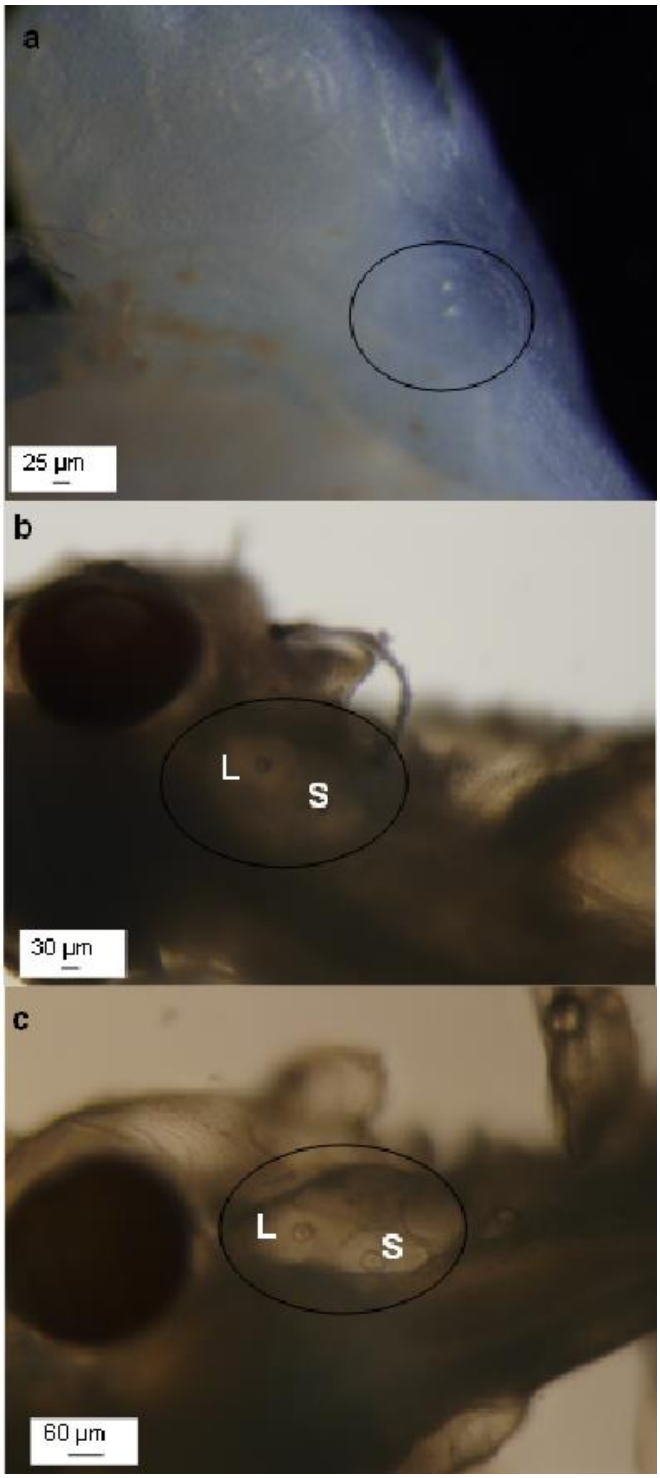

Fig 1.2 A vestibular structure in fish age 0.5 dph (**a**), 2 days (**b**), and 5 days (**c**). The vestibular structure is within the black circle. Photographs **b** and **c** were taken at 128x and **a** was taken at 184x magnification; the lapilli (L) and sagittae (S) are visible.

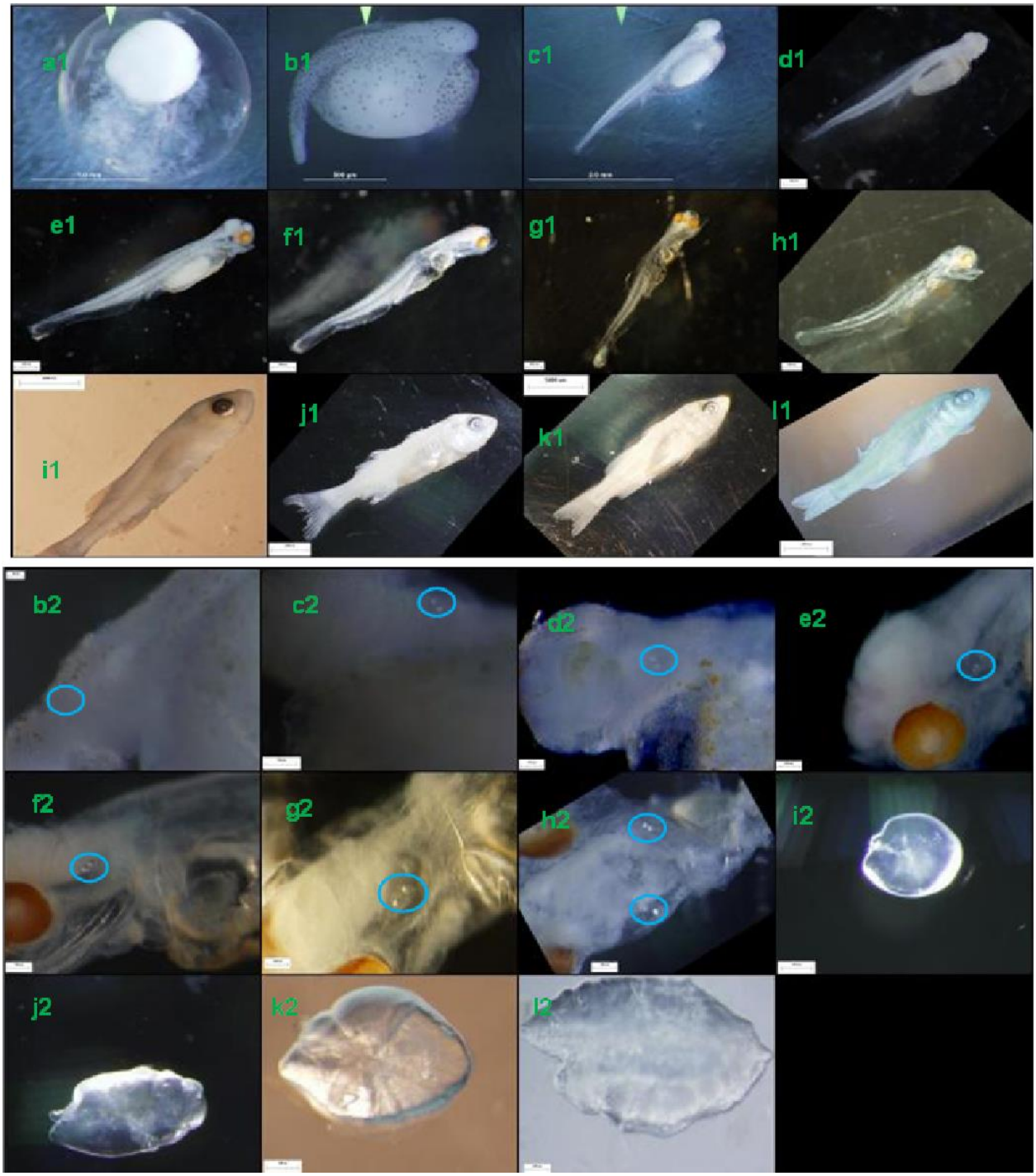

Fig 1.3 Images of larval fish taken over time. The letter corresponds with the table above. **a**1 is a 10 hr egg, **b1** is a 2 day egg / hatchling and **b2** is the corresponding otolith, **c1** is less than 24 hr old fish and **c2** its otolith, **d1** and **d2** are 1 dph, **e1** and **2** are 2 dph, **f1** and **2** are 3 dph, **g1** and **2** are 4 dph, **h1** and **2** are 5 dph, **i1** and **2** are 15 dph, **j1** and **2** are 19 dph, **k1** and **2** are 21 dph and **l1** and **2** are 30 dph. The blue circles indicate where the otoliths are for fish ≤ 5 dph.

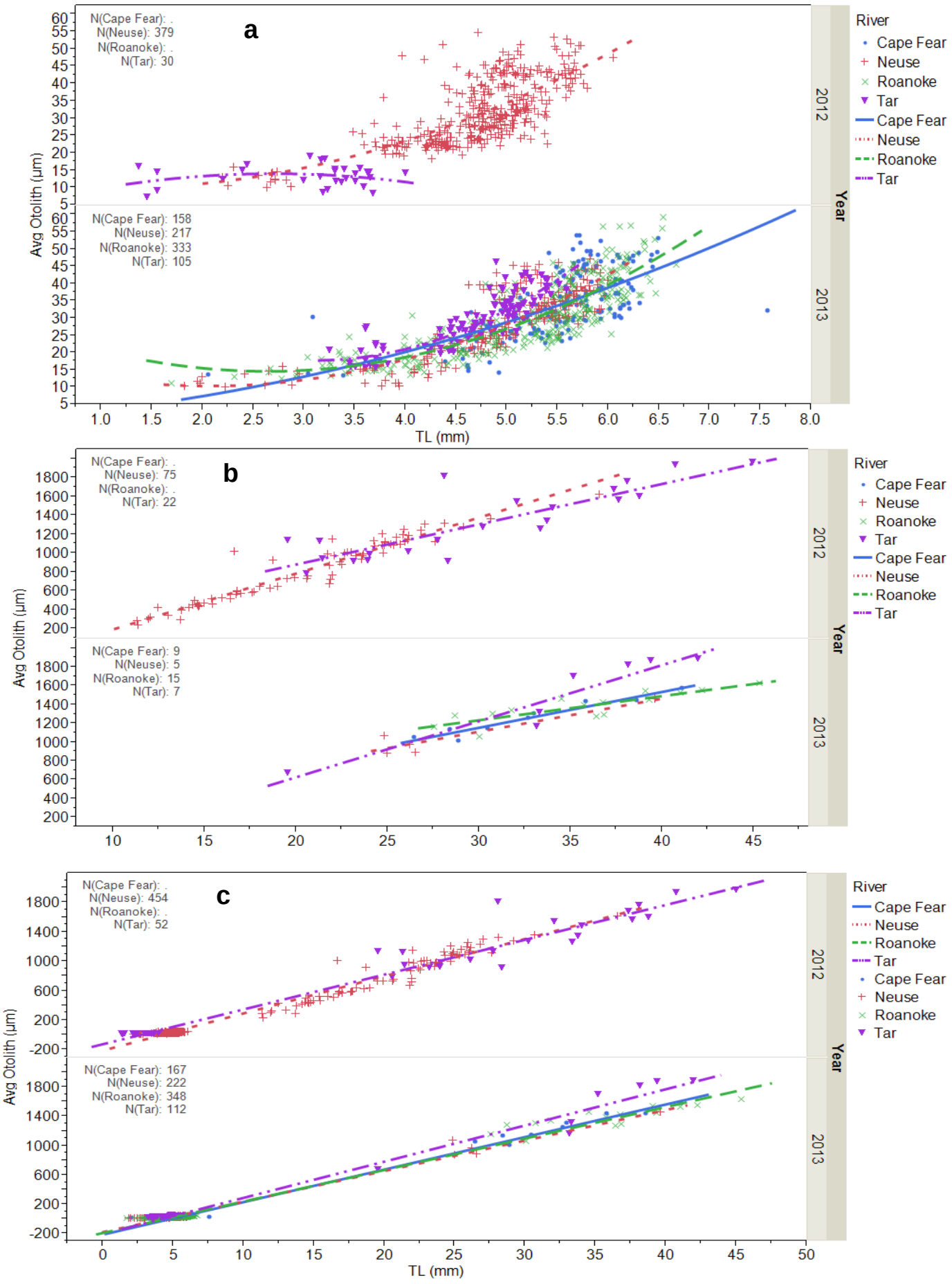

Fig 1.4 a 'Yolk Sac' (0 to 5 dph) graph of average otolith (TOL- µm) by fish total length (TL- mm) by river and year with quadratic lines of best fit. **b** 'Non-Yolk Sac' graphs of TOL vs TL by river and year with linear lines of best fit. **b** 'All' graph of TOL vs TL by river and year with linear lines of best fit. See Table 1.4 for r^2 and p-values.

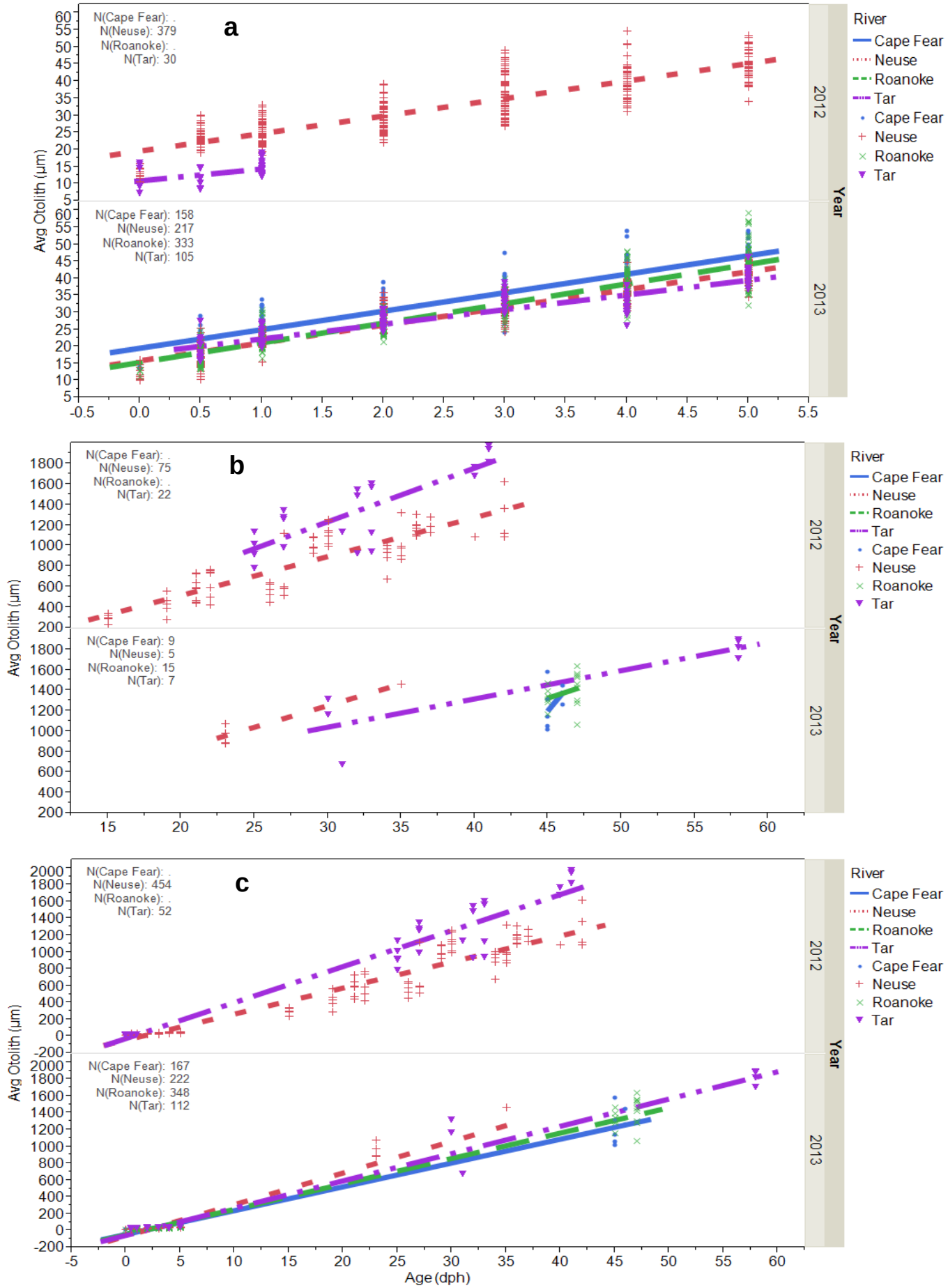

Fig 1.5 'Yolk Sac' (0 to 5 dph) graph of average otolith (TOL, µm) by fish age in days with a linear line of best fit. **b** 'Non-Yolk Sac' (15 to 58 dph) graph of TOL versus age separated by river and year with linear lines of best fit. **c** 'All' (0 to 58 dph) graph depicting the linear relationship between TOL and age by river and year. See Table 1.4 for r^2 and p-values.

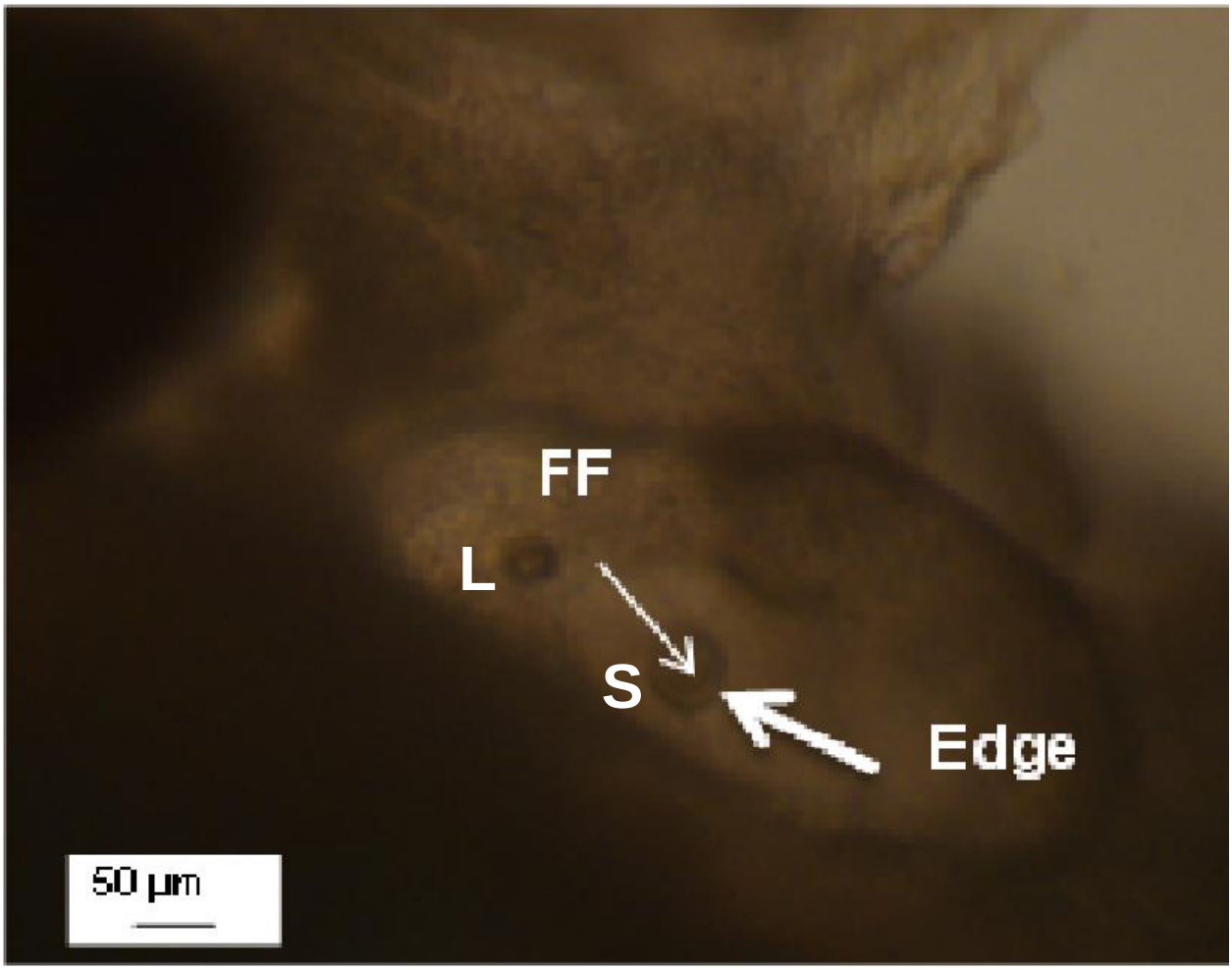

Fig 1.6 A photograph of a first feeding mark. FF denotes the first feeding mark while the edge is marked by the thick arrow. S is the sagittal otolith which has a TOL of 41.25 µm. L is the lapillus.

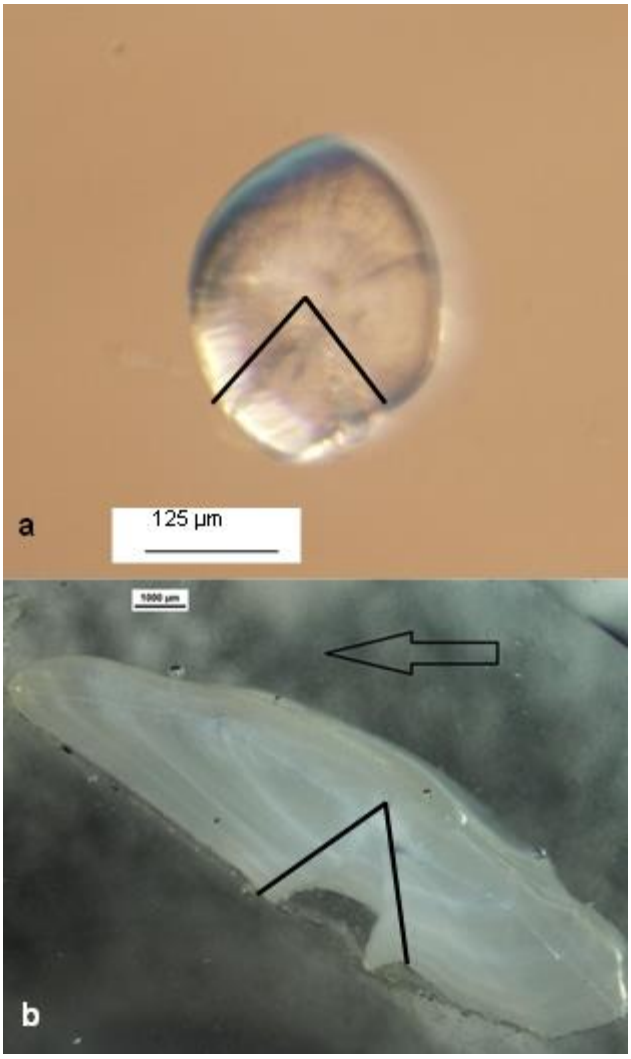

Fig 1.7 a 15 day old otolith from the Neuse River showing the forming sulcus (black angle). The TOL for this otolith is 309.46 µm. **b** A 5 year old adult female from the Neuse River (mother of **a**) with the sulcus outlined with a black angle, the TOL is 6953.24 µm.

Appendix A The growth of Striped Bass larvae, both otolith and physiologically by age. Average fish total lengths (TL-mm) and total sagittal otolith lengths (TOL-µm) and corresponding standard deviations (SD) are separated by river and year. Age 0 days is the egg stage and 0.5 days is larvae that were collected when their cohorts were hatching (less than 24 hrs old).

Age			Total length (mm)			Total sagittal otolith length (µm)		
(dph)	River	Year	N	Mean	1 SD	N	Mean	1 SD
0	Cape Fear	2013	20	2.36	0.435	3	13.59	0.297
0	Neuse	2012	80	1.97	0.433	13	12.72	1.740
0	Neuse	2013	80	1.87	0.402	12	12.57	1.989
0	Roanoke	2013	80	1.81	0.431	5	12.82	1.139
0	Tar	2012	20	1.92	0.696	6	11.90	3.747
0	Tar	2013	40	1.75	0.169	0		
0.5	Cape Fear	2013	20	4.62	0.159	17	20.33	4.219
0.5	Neuse	2012	53	4.07	0.270	53	23.43	2.455
0.5	Neuse	2013	40	4.08	0.255	33	16.52	4.110
0.5	Roanoke	2013	70	3.78	0.455	61	17.18	2.375
0.5	Tar	2012	9	3.47	0.175	8	11.25	2.379
0.5	Tar	2013	21	3.64	0.146	17	18.84	3.252
1	Cape Fear	2013	30	5.12	0.300	28	27.05	2.983
1	Neuse	2012	80	4.53	0.252	80	25.22	3.508
1	Neuse	2013	40	4.80	0.144	33	22.67	2.201
1	Roanoke	2013	70	4.66	0.366	56	22.98	3.130
1	Tar	2012	19	3.16	0.484	16	14.78	2.160
1	Tar	2013	20	4.42	0.130	17	22.78	2.612
1	Cape Fear	2013	30	5.61	0.724	26	31.35	3.320
2	Neuse	2012	60	5.02	0.199	60	29.25	4.094
2	Neuse	2013	40	5.20	0.283	31	30.25	3.251
2	Roanoke	2013	60	5.37	0.370	50	26.65	2.611
2	Tar	2013	20	4.63	0.275	19	27.33	1.845
3	Cape Fear	2013	30	5.95	0.283	28	34.65	5.021
3	Neuse	2012	74	4.97	0.237	74	36.22	6.150
3	Neuse	2013	40	5.38	0.268	36	30.96	3.400
3	Roanoke	2013	60	5.64	0.334	53	32.83	3.238
3	Tar	2013	20	5.00	0.143	16	33.43	2.531
4	Cape Fear	2013	30	5.73	0.174	27	41.89	5.509
4	Neuse	2012	50	5.10	0.475	49	39.66	5.025
4	Neuse	2013	40	5.46	0.398	35	36.17	3.224

Appendix A Continued

Age	River	Year	Total length (mm)			Total sagittal otolith length (µm)		
			N	Mean	1 SD	N	Mean	1 SD
4	Tar	2013	20	5.11	0.106	18	32.14	2.787
5	Cape Fear	2013	30	5.94	0.322	29	46.49	4.057
5	Neuse	2012	51	5.16	0.394	50	44.65	4.078
5	Neuse	2013	40	5.50	0.309	37	41.48	3.169
5	Roanoke	2013	60	5.80	0.466	57	44.24	6.130
5	Tar	2013	19	5.23	0.204	18	40.39	2.289
15	Neuse	2012	5	12.39	0.938	5	299.85	40.443
19	Neuse	2012	5	14.36	2.030	5	429.52	100.962
21	Neuse	2012	8	17.49	2.628	8	581.61	118.132
22	Neuse	2012	8	17.73	3.613	8	619.24	151.684
23	Neuse	2013	4	25.62	0.860	4	958.25	89.405
25	Tar	2012	5	25.19	3.253	5	947.92	131.111
26	Neuse	2012	5	16.75	1.688	5	565.43	80.921
27	Neuse	2012	5	18.58	5.080	5	669.81	256.500
27	Tar	2012	4	30.33	4.493	4	1210.95	157.406
29	Neuse	2012	5	22.73	2.609	5	1011.50	69.217
30	Neuse	2012	10	23.68	2.929	9	1101.33	99.425
30	Tar	2013	2	33.24	0.099	2	1236.46	106.989
32	Tar	2012	3	30.01	5.347	3	1313.79	341.444
33	Tar	2012	4	29.79	9.726	4	1304.38	325.631
34	Neuse	2012	5	22.94	1.206	5	894.19	129.986
35	Neuse	2012	5	23.85	2.479	5	1013.36	179.943
36	Neuse	2012	7	25.00	1.547	7	1177.16	70.369
37	Neuse	2012	3	26.73	2.193	3	1197.23	75.864
40	Tar	2012	2	37.74	0.530	2	1717.07	60.928
41	Tar	2012	3	37.93	8.778	3	1902.86	81.518
42	Neuse	2012	4	29.49	5.356	4	1297.81	248.335
45	Cape Fear	2013	6	31.37	5.230	6	1206.74	209.027
45	Roanoke	2013	6	31.50	3.203	6	1327.26	103.593
46	Cape Fear	2013	3	35.70	2.957	3	1379.51	104.625
47	Roanoke	2013	9	38.54	4.321	9	1421.44	179.044
58	Tar	2013	4	38.67	2.807	4	1819.57	84.287

Chapter 2

TRACE ELEMENT UPTAKE IN TISSUES OF ADULT STRIPED BASS: POTENTIAL FOR MATERAL CONTRIBUTION TO PROGENY OTOLITHS

Abstract

Microchemical analysis of fish soft tissue can be used to determine the levels of heavy metals and trace elements in a fish. In this study, soft tissue analysis was used to determine whether adult Striped Bass tissues develop trace elemental signatures, and to explore the likely pathways of maternal contribution to progeny otoliths. Muscle, liver, kidney, and gonadal (ovaries and testes) tissues were taken from 37 Striped Bass adults from 4 rivers (Roanoke (n = 12), Neuse (n = 11), Tar (n = 7), and Cape Fear (n = 5)) and the ocean (n = 2) for analysis. Adult coastal river fish were discriminated from ocean fish based upon the soft tissue types with high levels of classification: kidneys were correctly classified to either river or ocean 91.89% (n = 37) of the time; liver 97.22% (n = 36) of the time; muscle 88.89% (n = 36); and ovaries only 75% of the time (n = 20); and no ocean males were caught. As muscle tissue has a slow elemental turnover rate (several months) it could be used as a nonlethal way of determining the percentage of the population that is anadromous. Adult coastal river fish were discriminated from each other based upon ovary and testes soft tissue microchemical analysis 77.78% (n = 18) and 66.67% (n = 12) of the time, respectively. Liver and muscle tissue were less successful at classifying between the rivers, doing so only 52.94% (n = 34) of the time for both tissues. The ovaries and testes had the best discrimination between adult otolith clusters 61.11% (n = 18) and 58.33% (n = 12) of the time, respectively. Therefore we conclude that gonadal tissues should be major contributors of trace elements to progeny otoliths.

Introduction

The study of the elemental chemistry of fish otoliths (earbones) and fluids is relatively common, but microchemical analysis of soft tissues is less so (Engström et al. 2004). Trace elements can make their way into fish tissue and organs from the ambient environment or the diet. Campana (1999) examined this absorption from the ambient waters as it pertains to otolith elemental analysis, but the same processes also occur in the organs. Ions in the ambient water come into contact with the gills, which allow some of the ions to diffuse into the fish blood. Once in the gills the ions are passed into the bloodstream. Since the bloodstream feeds all of the organs, some ions also diffuse into the organs along with the necessary oxygen. Some ions including Sr, Ba, and Ca enter mainly through the ambient water. Other elements, such as Ba and Mn, may enter through the diet. If the ions are being received from the diet, then the ions will diffuse from the intestinal tract into the bloodstream and to the other organs and eventually be incorporated into the otolith (Campana 1999).

Most of the studies examining soft tissues of fish look at heavy metals for contamination, bioaccumulation, and human health concerns (Uysal et al. 2008; Alhashemi et al. 2012). For example, Uysal et al. (2008) examined gill, skin, and muscle tissue for heavy elements in Turkish fish species. They found that while the amounts of metals differed between fish species, the relative amounts did not, which means this study's findings may be applicable to species other than Striped Bass. The elemental order of accumulation was Mg>Zn>Fe>Cu>Co>Mn. The tissue type with the highest accumulation was the gills, likely because it actively transports ions unlike muscle tissue (Usyal et al. 2008).

This result is similar to what Engström et al. (2004) found when working with European Perch, *Perca fluviatilis* (Linnaeus 1758). There was little variation between liver and muscle concentrations of most major elements, such as Mg, P, K, S, and Ca among others, between European Perch, rabbits, and pigs. Accumulation of non-major elements, such as Mn, Fe, Zn, and Cu, was lower in the muscle tissue than the liver (Engström et al. 2004).

Other factors can also have an impact on the accumulation of trace elements: gender, gonadosomatic index (GSI), species and even other trace elements. Alhashemi et al. (2012) examined the microchemistry of three fish species in Iran and found Mn was higher in the liver than the muscle, kidney, gills, and gonads. There was higher element accumulation in females for *Barbus grypus* (Heckel 1843) and *Barbus sharpeyi* (Gunther 1874), while *Cyprinus carpio* (Linnaeus 1758) had higher accumulation in the males for muscle and gonadal tissue. Female *Cyprinus carpio* had a positive relationship between GSI and elemental accumulation (Alhashemi et al. 2012). Externally, De Vries et al. (2005) found that surrounding levels of Sr in brackish water facilitated the uptake of Ba. This facilitation does not lead to a decrease in Sr uptake, nor does Sr ease Ba uptake in sea water (De Vries et al. 2005).

The goal of my study was to determine if maternal contribution to progeny otoliths was possible from the maternal angle. The objectives of this study are to determine if adult soft tissues (white muscle, liver, kidney, and gonads) can be used to determine a specific river origin; if soft tissues can be used to discriminate between different adult otolith groups; and finally to use this information to examine the possibility of maternal contribution. I hypothesized that soft tissues in fish from the same river will be similar, and that elemental concentrations in the various tissue types will be similar within an individual. I predict that the ocean tissues will be

easily discriminated from riverine tissues based upon higher levels of Sr:Ca as the Sr:Ca ratio is indicative of salinity level.

Methods

Adult tissue analysis

The adult female and male Striped Bass were collected by electrofishing on the spawning grounds of 4 different rivers (Roanoke (n = 12), Neuse (n = 11), Tar (n = 7), and Cape Fear (n = 5)), and transported to the Edenton National and Watha State Fish Hatcheries for spawning. Ocean fish were caught using hook and line (n = 2). All fish were transported back to East Carolina University where they were dissected and muscle tissue, liver, kidney, and gonads were removed. The tissues were stored in the freezer until they were digested in 70% nitric acid (Engström et al. 2004). The digested material was then diluted to 7% nitric acid. Due to the dilution of the nitric acid, some of the digested material precipitated out of solution. To remove the particulates, all samples were filtered through 42.5-mm circular Whatman glass microfiber filters. After being run on an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) for Ca, Ba, Mn, Mg, and Sr, the relationship of each trace element to calcium concentration was determined, and the ratio of each element was then used in the analyses.

Adult otolith collection and analysis

As described by Boyd (2011), sagittal otoliths from the adult fish were removed using plastic forceps, cleaned with distilled deionized water, and stored in 1.5-mL microcentrifuge polypropylene vials. The vials were left open for at least 12 hours to dry before being closed. One of the otoliths from each fish was randomly selected for shipping to the University of

Manitoba, Winnipeg, Canada for microchemical analysis. The other otolith was used for ageing the fish by the NC Wildlife Resources Commission.

At the University of Manitoba, otoliths were embedded in epoxy resin (Buehler Epoxicure), and a 2-mm thick dorso-ventral transverse section was cut (including the core) utilizing a diamond blade Isomet saw (Buehler 646) at low speed. This revealed the annuli and allowed the laser beam to discern each annulus (Halden and Friedrich 2008). These cut sections were placed into 25-mm diameter Plexiglass ring mounts and embedded again. The orientation placement of each otolith within the ring was recorded for sample identification.

In order to access the core, these sections were sanded using 320, 400, and 600 grit wet sandpaper then ultrasonically cleaned for 2 minutes. To remove any scratches, the otoliths were polished using Buehler diamond polishing suspensions (9 μm and 0.05μm) on a polishing wheel to produce a smooth surface for laser ablation. After this polishing the otoliths were once again cleaned ultrasonically with ultrapure water and digitally photographed for reference for Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysis. These laser scans started at one end of the otolith, passed through the entire core and then along the longest axis to the outer edge of growth, so the entire otolith diameter was ablated. The intensity (counts per second) was converted into ppm for Sr, Ba, Mg, and Mn using a Macro in Microsoft Excel.

Statistical analyses

All statistical analyses were conducted using JMP Pro 10. An ANOVA determined if there was any difference in microchemical signatures between gonad types (ovaries and testes). If differences were found, ovaries and testes were then kept separate for further analyses. A 3-way ANOVA was run on river, year caught and tissue type to determine any differences and

interactions between river, year caught, and tissue types. Tukey HSD tests were run to determine which variable levels were significantly different. A hierarchical cluster analysis was performed on the Striped Bass adult otoliths to separate them into clusters for comparison to the tissue analysis. After running the ANOVAs, linear discriminant function analyses (DFA) were performed to determine the level of classification of habitat (river or ocean), coastal river caught, and adult otolith hierarchical clustering based upon the tissue concentrations.

Results and discussion

Tissue type and river

A 3-way ANOVA was performed between year caught, river, and tissue type for the 4 different elemental ratios (Table 2.1). For Sr:Ca, Ba:Ca, and Mg:Ca year caught, river, and tissue type were significant and there were no interactions between the three variables. Mn:Ca, however, had significant differences between year caught, tissue type, and river**x**tissue type. Since Sr:Ca, Ba:Ca, and Mg:Ca, had no interactions between the variables, one-way ANOVAs were run on river and tissue type. For river, only Mg:Ca was significant ($n = 133$; $F = 4.32$; $p = 0.0062$) and the Cape Fear and Neuse Rivers were separated from each other. For tissue type all three ratios were significantly different. For Sr:Ca ($n = 133$; $F = 3.04$; $p = 0.0198$) the liver and muscle were separated. Ba:Ca ($n = 133$; $F = 4.65$; $p = 0.0015$) had the ovaries and testes significantly different than the muscle. Despite Mg:Ca ($n = 133$; $F = 2.69$; $p = 0.0342$) being significant, the Tukey HSD test did not separate any variable levels.

When a DFA was run using the 4 ratios to discriminate between ocean and riverine fish by tissue type, there were high levels of classification, $\geq 75\%$ for all tissues (Table 2.2). Liver tissues had the best discrimination between ocean and riverine fish with 97.22% correctly

classified. Kidney classified the habitat correctly 91.89% of the time, muscle 88.89% of the time, and finally ovaries with 75% of the time. The ability to separate ocean fish from resident coastal river fish is not surprising and supports the hypothesis that the ocean could be discriminated from the coastal rivers. This is likely due to the higher level of salinity, which is strongly correlated with Sr:Ca ratios and negatively correlated with Ba:Ca ratios (De Vries et al. 2005; Halden and Friedrich 2008; Brown and Severin 2009). The high levels of classification may also be due to the low sample size of ocean fish (n = 2) to the larger riverine sample size (n = 34).

When discriminating between coastal rivers, the ovaries and testes had the highest classification percentages, 77.78% (n = 18), and 66.67% (n =12) respectively (Table 2.3). Both the liver and kidney elemental ratios were able to correctly discriminate between rivers 52.94% of the time. Over 20% of river misclassification from the liver was due to similar elemental signatures in the Neuse and Tar Rivers. Almost 15% of the river misclassification in white muscle tissue was due to similarity between the Cape Fear and Tar Rivers. The kidney was only able to discriminate between rivers with 37.14% accuracy.

Adult otolith clustering

A hierarchical cluster analysis based upon the otolith microchemical analysis on the last year of life produced six groups. Group 1 had 6 female fish from all 4 rivers, group 2 was comprised of 15 fish (11 female and 4 male) from all 4 rivers, group 3 contained 4 male fish from the Cape Fear (2) and Neuse (2) Rivers, group 4 held 1 Roanoke female fish, and group 5 had 4 male fish from the Roanoke (3) and Tar (1) Rivers, and group 6 contained 5 Roanoke female fish 8 years and older for a total of 35 fish analyzed (Figure 2.6). While the split was not

between rivers, most of the groups separated out by gender. This agrees with the ANOVAs as gender was significant for the 4 elemental ratios (Sr:Ca, Ba:Ca, Mg:Ca, and Mn:Ca). The gender difference might be due to slightly different habitat use, genetics, or differences in elemental needs for spawning males and females.

A DFA was performed to determine if the clusters could be related to the elemental concentrations in the different tissue types. The only tissues that had a classification greater than 50% were the testes and ovaries (Table 2.4). The ovaries (n = 18) correctly identified adult otolith cluster 61.11% of the time, while testes (n = 12) elemental ratios could only discriminate between adult otolith clusters 58.33% of the time. Results indicate that ovaries and testes are better at discriminating between rivers than between otolith groups. One possible explanation may be that otoliths grouped based upon the elemental ratios of the last year of life, but gonads only contain an elemental signal for a couple of months (Paramore and Rulifson 2001). Another possibility is that wandering may mask the average riverine signal over the course of the year.

Regardless of the reason behind the better discrimination of river of origin than adult otolith clusters, otolith microchemistry is related to gonadal microchemistry. This gives credence to the hypothesis for maternal contribution to offspring otolith development. There is the possibility of determining maternal river, as both the ovaries and testes can discriminate between the coastal rivers with >65% accuracy. More research is needed to determine the reasons for the similarities between otolith and gonadal microchemistry and the dissimilarities between otolith and liver, kidney, and muscle tissues.

Implications

Though white muscle tissue microchemistry does not have the highest classification for determining river from ocean fish, it would be an accurate tool for determining the percentage of a population that is anadromous. The reasons that muscle tissue is better than liver or kidney for anadromy or residency are the length of turnover time for trace elements, and that muscle can be sampled without killing the fish. The trace element turnover rate of white muscle is several months, which is longer than the liver, which is longer than the kidney (Bucher and Hofer 1993; Kojadinovic et al. 2007; Madigan et al. 2012). This means that even if the individual is caught when it is leaving the spawning grounds it will still have an ocean signal in the white muscle but perhaps not in the kidney. Also, muscle tissue has been shown to have less variability than other tissues (Pinnegar and Polunin 1999). As muscle tissue can be removed without killing the fish it could be a useful method for determining anadromy in endangered or threatened populations.

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Table 2.1 3-way ANOVA of soft tissue microchemistry by elemental ratio. Tukey HSD was not performed due to non-estimable values.

Elemental Ratio	Source of Variability	DF	F-ratio	p-value
Sr:Ca	Year	1	15.59	0.0001*
	River	3	4.58	0.0047*
	Year**x**River	1	0.08	0.7851
	Tissue Type	4	3.36	0.0125*
	Year**x**Tissue Type	4	1.90	0.1161
	River**x**Tissue Type	12	1.38	0.1871
	Year**x**River**x**Tissue Type	2	0.06	0.9449
Mg:Ca	Year	1	55.58	<0.0001*
	River	3	4.78	0.0037*
	Year**x**River	1	3.42	0.0674
	Tissue Type	4	3.73	0.0070*
	Year**x**Tissue Type	4	0.58	0.6811
	River**x**Tissue Type	12	1.16	0.3253
	Year**x**River**x**Tissue Type	2	0.82	0.4444
Mn:Ca	Year	1	25.44	<0.0001*
	River	3	1.14	0.3349
	Year**x**River	1	2.95	0.0889
	Tissue Type	4	30.00	<0.0001*
	Year**x**Tissue Type	4	1.74	0.1468
	River**x**Tissue Type	12	3.11	0.0008*
	Year**x**River**x**Tissue Type	2	0.31	0.7328
Ba:Ca	Year	1	47.05	<0.0001*
	River	3	3.98	0.0099*
	Year**x**River	1	1.44	0.2329
	Tissue Type	4	5.10	0.0009*
	Year**x**Tissue Type	4	0.33	0.8604
	River**x**Tissue Type	12	0.60	0.8413
	Year**x**River**x**Tissue Type	2	1.11	0.3343

Table 2.2 Classification table for linear DFA between ocean and riverine fish by tissue type; numbers represent number of fish predicted by actual habitat. Kidney has 91.89% classification; Liver 97.22%; Muscle 88.89%; and Ovaries 75%. No male ocean fish were caught so testes could not be discriminated.

			Predicted habitat	
Tissue type	Actual habitat	N	Ocean	River
Kidney	Ocean	2	1	1
	River	35	2	33
Liver	Ocean	2	2	0
	River	34	1	33
Muscle	Ocean	2	2	0
	River	34	4	30
Ovaries	Ocean	2	2	0
	River	18	5	13

Table 2.3 Classification tables for linear DFA between coastal rivers by tissue type; numbers represent number of fish predicted by actual river. Kidney has a classification of 37.14% (n = 35); Liver 52.94% (n = 34); Muscle 52.94% (n = 34); Ovaries 77.78% (n = 18); and Testes 66.67% (n = 12).

			Predicted coastal river			
Tissue type	Actual coastal river	N	Cape Fear	Neuse	Roanoke	Tar
Kidney	Cape Fear	5	4	1	0	0
	Neuse	11	5	2	0	4
	Roanoke	12	3	2	6	1
	Tar	7	3	3	0	1
Liver	Cape Fear	5	4	0	0	1
	Neuse	11	1	5	1	4
	Roanoke	11	1	3	7	0
	Tar	7	2	3	0	2
Muscle	Cape Fear	5	2	1	0	2
	Neuse	11	0	9	1	1
	Roanoke	11	1	4	4	2
	Tar	7	3	1	0	3
Ovaries	Cape Fear	3	2	0	0	1
	Neuse	5	0	4	1	0
	Roanoke	8	0	1	7	0
	Tar	2	1	0	0	1
Testes	Cape Fear	2	2	0	0	0
	Neuse	4	1	2	0	1
	Roanoke	4	0	0	3	1
	Tar	2	1	0	0	1

Table 2.4 Classification tables for linear DFA between adult otolith clusters by tissue type; numbers represent number of fish predicted by actual adult otolith cluster. Kidney has a classification of 45.71% (n = 35); Liver 47.06% (n = 34); Muscle 50.00% (n = 34); Ovaries 61.11% (n = 18); and Testes 58.33% (n = 12). Columns are left blank if tissue did not contain that cluster.

			Predicted adult otolith cluster					
Tissue type	Actual adult otolith cluster	N	1	2	3	4	5	6
Kidney	1	7	6	0	1	0	0	0
	2	14	4	2	2	0	6	0
	3	4	1	0	2	0	1	0
	4	1	0	0	0	1	0	0
	5	4	0	0	0	0	4	0
	6	5	0	0	1	3	0	1
Liver	1	7	4	1	2	0	0	0
	2	13	3	6	0	0	5	0
	3	4	0	1	1	0	1	1
	4	1	0	0	0	1	0	0
	5	4	1	1	0	0	2	0
	6	4	0	0	2	0	0	2
Muscle	1	8	4	1	2	0	1	0
	2	13	3	5	1	0	4	0
	3	3	0	1	2	0	1	0
	4	1	0	0	0	1	0	0
	5	4	1	0	0	0	3	0
	6	4	0	1	0	1	0	2
Ovaries	1	3	2	1		0		0
	2	9	3	6		0		0
	4	1	0	0		1		0
	6	5	1	0		2		2
Testes	2	6		2	2		2	
	3	4		2	2		0	
	5	4		1	0		3	

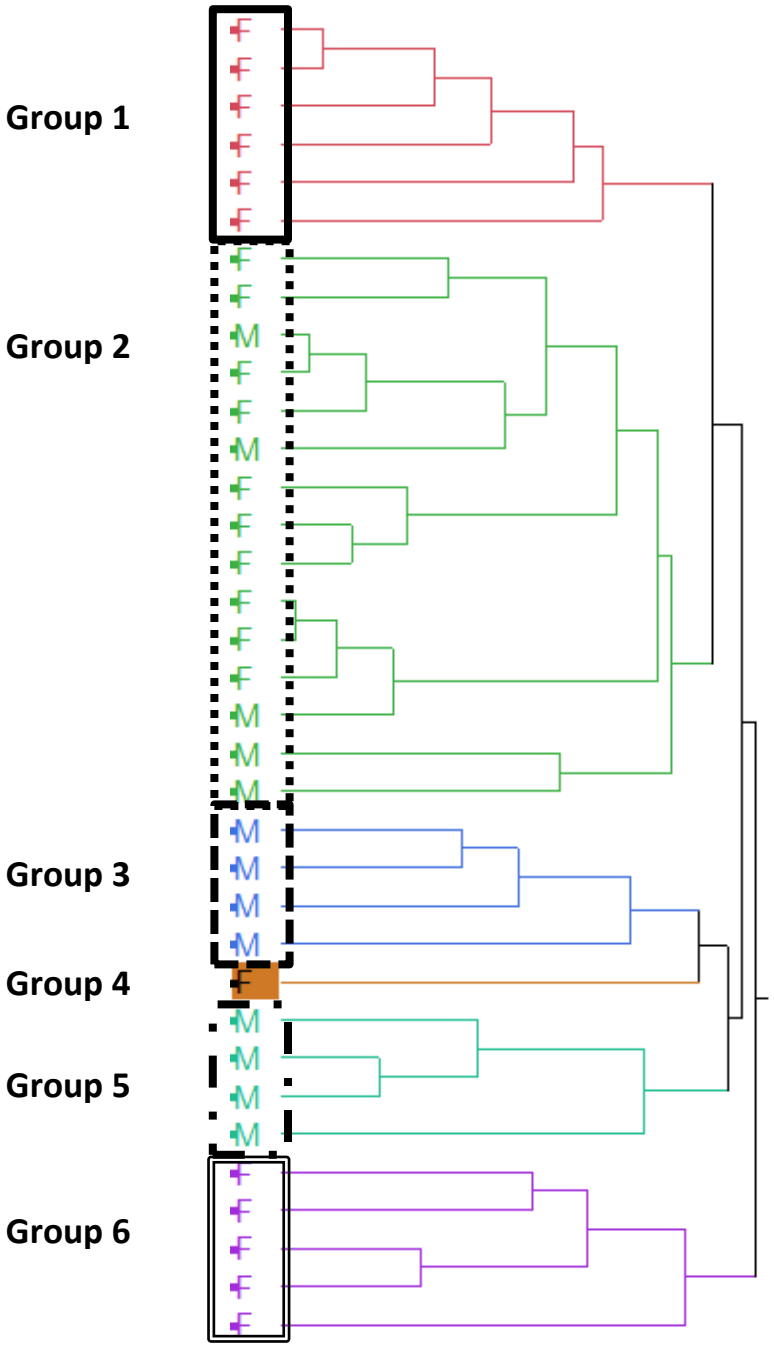

Fig 2.6 Hierarchical cluster analysis of the adult otoliths averaged over the last year of life with the groups marked. Group 1 is the solid box, group 2 is the dot box, group 3 is the dashed box, group 4 is highlighted, group 5 is the dot and dash box, and group 6 is the double lined box.

Chapter 3

MATERNAL CONTRIBUTION OF TRACE ELEMENTS IN PROGENY OF ADULT STRIPED BASS

Abstract

Further understanding of otolith microchemistry has hinted at the possibility of trans-generational markers and maternal input. Hatchery raised Striped Bass (*Morone saxatilis*) progeny from known mothers were examined to determine the extent/ existence of maternal contribution in progeny sagittal otoliths of three life stages (embryo, yolk sac, and non-yolk sac larvae). Maternal otoliths were grouped using hierarchical cluster analysis on the otolith area representing the last year of life. Embryo sagittal otoliths correctly identified the maternal clusters 91.67% of the time (n = 12), yolk sac larvae 66.67% of the time (n = 15), and non-yolk sac larvae only 60.94% of the time (n = 64). Progeny otoliths were also able to identify maternal river (Neuse, Roanoke or Tar); embryos classified the river 83.33% of the time (n = 12), yolk sac larvae classified 93.33% of the time (n = 15), and non-yolk sac larvae had a classification rate of 44.44% (n = 72) of the time. The high levels of classification support the hypothesis of maternal contribution and allow the determination of maternal river. This will allow fishery managers to better identify critical nursery habitat and reduce confusion associated with wandering. Results of my study validate the hypothesis of maternal contribution and support the hypothesis of trans-generational markers for anadromous and resident fish.

Introduction

Fishery managers and scientists have found that otoliths (earstones) can be used for ageing and also tracking the life history and migration patterns of several fish species including

Striped Bass (*Morone saxatilis*; Walbaum 1792), Red Snapper (*Lutjanus campechanus*; Poey 1860), Threespine Stickleback (*Gasterosteus aculeatus*; Linnaeus 1758), Sockeye Salmon (*Oncorhynchus nerka*; Walbaum 1792), and the Humpback Whitefish (*Coregonus pidschian*; J.F. Gmelin 1789) (Campana 1999; Brown and Severin 2009; Gibson et al. 2010). Since the otolith accretes daily layers, the chemical signature of the water is incorporated to the otolith, and thus the fish's migration patterns can be observed over time provided the habitat signature is known (Campana 1999).

One of the most important elemental ratios for anadromous fish is the Sr:Ca (strontium/calcium) ratio. Higher ratios correspond to saltwater, while lower ratios typically correspond with freshwater (Halden and Friedrich 2008). The elemental signatures of the otoliths can often be traced back to a specific river based upon water chemistry and so the migration of these fish between freshwater and the ocean can be plotted over time (Secor and Piccoli 2007). For example, Morris et al. (2003) were able to correctly classify Striped Bass to three different rivers: the Neuse and Roanoke rivers in North Carolina, USA and the Stewiacke River in Nova Scotia, Canada using the otolith elemental signatures. The Neuse had the highest classification with 88 %, the Stewiacke had 79%, but the Roanoke only 47%. Morris et al. (2003) hypothesized that the misclassified fish from the Roanoke was caused by either wandering to multiple coastal watersheds, or environmental variability of the system. Overall, this method of using otolith microchemistry to trace the migration pattern and juvenile habitat of fish is still expanding.

It has even been suggested that instead of tracing fish back to a specific river, that they could be traced to a hatchery (Gibson et al. 2010). Juvenile wild Red Snapper from the Gulf of Mexico and those from hatcheries were distinguished with 100% accuracy. Two methods of

otolith signature testing were used to separate wild from hatchery juveniles. One method was the dissolution of the whole otolith before it was analyzed with a sector-field inductively-coupled plasma mass spectrometer (SF-ICP-MS). For the other method, the otolith was ground and analyzed for ^{13}C and ^{18}O stable isotopes (Gibson et al. 2010). The hatchery otoliths analyzed using the SF-ICP-MS had values that were consistently lower than the wild fish except for magnesium (Mg), which was higher. The otoliths tested with stable isotope analysis had very different $\delta^{13}C$ values between wild and hatchery fish, while $\delta^{18}O$ values overlapped between wild and hatchery fish (Gibson et al. 2010). Both methods worked, though the stable isotope analysis worked better for Red Snapper, and could be considered as natural tags (Gibson et al. 2010).

Another possible natural tag are trans-generational markers based upon maternal contribution. Hobbs et al. (2012) examined the viability of using Sr as a trans-generational mark in Delta Smelt (*Hypomesus transpacificus*; McAllister 1963) by using two concentrations of Sr injections into the peritoneal cavity of the mother and examining the progeny otolith microchemistry and response to elevated Sr levels. High levels of Sr did elevate levels in the otolith relative to non-marked fish, but had a physiological impact upon the progeny: reduced yolk and oil globule diameters, and slower growth rates. Hobbs et al. (2012) recommended trans-generational marking for endangered fish, but suggested further research into the concentrations needed as excess trace elements can have adverse effects on the progeny development and growth.

Volk et al. (2000) tested sea water as a possible trans-generational marker using salmonid species. They determined that progeny from salmonid mothers that matured in saltwater had higher Sr:Ca concentrations in the otoliths than those from freshwater mothers. The salmonid species were raised in captivity in either fresh or saltwater, and thus the life history of the mother

was known for the duration of vitellogenesis (yolk deposition). This is important as the yolk sac is the nutrition source for the larvae until first feeding. Raising salmonid mothers in captivity accounted for most of the factors regulating otolith Sr uptake when progeny otoliths were analyzed, thus allowing the connection between maternal contribution and progeny otoliths to be observed.

Veinott et al. (2014) examined the effects different life histories (anadromous and non-anadromous) had on Brown Trout (*Salmo trutta*; Linnaeus 1758) progeny. Otoliths of progeny from anadromous mothers had higher levels of Sr:Ca than those progeny from non-anadromous mothers. It took between 3 to 5 weeks post emergence (defined as free swimming and yolk-sac absorption) for the Sr:Ca ratios to be non-significantly different between the two life histories using a t-test. Veinott et al. (2014) also examined the temporal stability of elements in the core (defined as the pre-emergence to pre-feeding time period) located by the high spike of Mn prior to reaching the core. Zn, Sr, and Ba were found to be temporally stable, while Mg and Mn changed concentrations as the fish grew (Veinott et al. 2014).

The goal of my study was to determine the existence of maternal contribution to Striped Bass otoliths. The objectives to meet this goal were: 1) to compare the elemental ratios of the three larval life stages (embryo, yolk sac larvae, and non-yolk sac larvae); 2) to compare the elemental ratios of the different life stages to adult otolith clusters; 3) to examine the ability of the different life stages to discriminate between maternal rivers; and 4) to use the information from the previous objectives to determine the existence of maternal contribution. I hypothesized that elemental ratios will vary between the three different life stages, and that the earlier life stages (embryo and yolk sac) will be able to discriminate between adult otolith clusters and maternal river better than non-yolk sac larvae.

Methods

Adult collection and tissue analysis

Adult Striped Bass were collected using electroshocking from the wild and transported to either the Edenton National Fish Hatchery in Edenton, NC or the Watha State Fish Hatchery in Watha, NC. Once they had spawned, mothers were sacrificed and then transported back to the laboratory where they were dissected. Muscle tissue, liver, and kidney were removed and digested as detailed by Elking (2014, Chapter 2). The tissues were stored in the freezer until they were digested in 70% nitric acid and then diluted to 7% to be analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Any particulate matter was removed by filtration through 42.5-mm circular Whatman glass microfiber filters.

Adult otolith collection and analysis

Sagittal otoliths from the mothers were removed through dissection with a hand saw and sent off for analysis at the University of Manitoba as described by Elking (2014, Chapter 2). Plastic forceps were used for otolith removal after collection; otoliths were cleaned with distilled deionized water, and stored in 1.5-mL microcentrifuge polypropylene vials. Vials were left in a fume hood for at least 12 hours to dry before being shipped. Once at the University of Manitoba, the otoliths were digitally photographed for reference for Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysis. These laser scans started at one end of the otolith, passed through the entire core and then along the longest axis to the outer edge of growth so the whole otolith diameter was ablated.

Larval otolith collection

Larval otoliths were collected in the same manner as discussed in Elking (2014, Chapter 1). Adult Striped Bass were caught in the wild and transported to either the Edenton National Fish Hatchery or the Watha State Fish Hatchery. Once at the hatchery fish were injected with human chorionic gonadotropin (HCG) hormone to induce spawning in circular tanks (Harrell et al. 1990). Typically the eggs were collected into McDonald Jars for hatching, and once hatched the larvae drop into five gallon aquaria (Harrell et al. 1990). Samples were collected from McDonald Jars and then daily from the aquaria until 5 dph. At 5 dph, larvae were released into outdoor ponds where they were sampled weekly until they reached approximately 40 dph and became juveniles rather than larvae (Hardy 1978).

Larval otolith dissolution

After collection, otoliths and embryos (where otoliths could not be collected) were dissolved in 50% nitric acid (Dove et al. 1996). After digestion, the samples were diluted to 7 % nitric acid and run on the ICP-OES for Ca, Mg, Mn, Sr, and Ba. Multiple embryos and otoliths from the same mother were analyzed in one sample in an attempt to keep the elements within detectable range and limit the dilution factor. This should not affect the results as all the embryos or larvae were taken from the same mother and kept together since spawning. The amount of otolith digested per sample was between 0.25 and 1.25 mg depending upon individual otolith sizes.

Statistical analyses

Progeny otoliths were grouped for three different analyses based upon life stage (embryo, yolk sac larvae and non-yolk sac larvae) for analysis. Elemental ratios were plotted by larval

stage and visually examined for differences. A 3-way ANOVA was used to compare year spawned, maternal river, and stage and the interactions. While results from maternal tissues and larval otoliths could not be compared directly as a result of the use of Ca as an internal versus external standard, a hierarchical cluster analysis was used to group adult otoliths for comparison through a linear discriminate function analysis (DFA). A second DFA was run to determine the ability of progeny otoliths to classify maternal river by stage.

Results and discussion

Larval microchemistry

No results were obtained from Cape Fear River progeny, and some of the Roanoke River progeny. There were several possible reasons: 1) not enough material in the sample; 2) there was 0 ppm of the elements in the progeny otolith; or 3) there were small amounts at or below detection limit of the ICP-OES. Due to this, Cape Fear embryo and yolk sac stages were excluded from the analysis.

A visual examination of the elemental concentrations shows a difference between the yolk sac and non-yolk sac larvae (Figures 3.1-3.4). For example, Sr in 2012 in Tar River progeny the embryo is high, the yolk stage moderate, and the non-yolk larvae lower than the yolk stage. This could be indicative of maternal contribution that is decreasing as the yolk-sac is absorbed.

The 3-way ANOVA for all 4 elements was significant for river, stage and year**x**stage (Table 3.1). Since the ratios of all four elements changed between the yolk-sac and non-yolk-sac stage it is likely that there is some maternal contribution. Veinott et al. (2014) proposed that fish with large eggs/ yolk-sacs would be more likely to have maternal input than species with smaller

eggs. As Striped Bass have large embryos and yolk-sacs that do not absorb until 5dph, this agrees with their hypothesis.

Progeny and maternal otoliths

Since it was likely that there was some maternal contribution to the progeny, a discriminant function analysis was performed using progeny otolith signatures. Progeny signatures were used to classify the adult otolith clusters (Elking 2014, Chapter 2) by life stage. Using Striped Bass embryo microchemical signatures, 91.67% of the adult otoliths were placed into the correct cluster (Table 3.2; n=12). Otoliths from yolk-sac stage had less discrimination ability with 66.67% of the mothers being correctly classified into 4 different clusters (Table 3.2; n = 15). As predicted, otoliths from the non-yolk sac larvae were the least accurate with 60.94 % of the mothers correctly classified into 5 different clusters (Table 3.2; n = 64). The probable reasoning behind these results is that the embryo otoliths have the highest maternal classification because they developed surrounded by maternal material, while yolk sac larval otoliths are influenced by maternal and ambient sources, and the oil globule. Non-yolk sac larval otoliths are only influenced by ambient sources as both the yolk sac and oil globule have been absorbed.

The microchemical signatures of the progeny can also be used to identify the river from which their mothers originated (Table 3.3). The embryo stage classified the river 83.33% of the time (n = 12), while the yolk-sac stage had 93.33% classification (n = 15). Non-yolk sac larvae identified the river 44.44% of the time (n = 72). It is possible that the yolk-sac stage better identified the river than the embryo stage due to less contribution from the egg sac and oil globule.

Implications

The results of this research will allow fishery managers to make better informed decisions about critical areas such as nursery habitats because data can be gathered from two generations on a single otolith. Using knowledge of the maternal river (and likely maternal life history strategy: anadromous or resident) managers should be able to determine which rivers produce the most offspring that survive to reproduce based not only upon natal river and nursery habitat but maternal river (and life history strategy) as well. This research also reduces the confusion associated with wandering when determining natal origin, as the maternal river can be discriminated at the primordium, and is the river where the fish were hatched.

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Table 3.1 3-way ANOVA of progeny otolith microchemistry variables year, river, and stage. * significant at α = 0.05 level. Tukey HSD tests could not be performed as data was non-estimable.

Elemental Ratio	Source of Variability	DF	F-ratio	p-value
Sr:Ca	Year	1	7.66	0.0069*
	River	3	8.17	<0.0001*
	Year**x**River	1	4.98	0.0283*
	Stage	2	90.32	<0.0001*
	Year**x**Stage	2	26.34	<0.0001*
	River**x**Stage	4	0.30	0.8781
	Year**x**River**x**Stage	1	4.21	0.0433*
Ba:Ca				
	Year	1	1.27	0.2625
	River	3	37.53	<0.0001*
	Year**x**River	1	0.03	0.8585
	Stage	2	122.77	<0.0001*
	Year**x**Stage	2	19.12	<0.0001*
	River**x**Stage	4	7.79	<0.0001*
	Year**x**River**x**Stage	1	0.59	0.4460
Mg:Ca				
	Year	1	3.86	0.0527
	River	3	43.98	<0.0001*
	Year**x**River	1	13.57	0.0004*
	Stage	2	215.79	<0.0001*
	Year**x**Stage	2	49.82	<0.0001*
	River**x**Stage	4	0.46	0.7667
	Year**x**River**x**Stage	1	0.76	0.3863
Mn:Ca				
	Year	1	224.14	<0.0001*
	River	3	22.51	<0.0001*
	Year**x**River	1	14.74	0.0002*
	Stage	2	437.70	<0.0001*
	Year**x**Stage	2	96.00	<0.0001*
	River**x**Stage	4	0.69	0.5588
	Year**x**River**x**Stage	1	1.98	0.1630

Table 3.2 Classification table of progeny otolith microchemistry predicting maternal otolith cluster by larval life stage; numbers represent number of fish predicted by actual adult otolith cluster. Embryo is still in the egg case (n = 12), Yolk Sac is ≤ 5dph (n = 15), and Non-Yolk Sac Larvae are ≥ 15 dph (n = 64). Embryo classification is 91.67%, Yolk Sac is 66.67; and Non-Yolk Sac is 60.94% accurate. Blank cells had no progeny caught from that adult otolith cluster. Clusters 3 and 5 do not contain mothers, and thus could not be predicted.

			Predicted adult otolith cluster			
Larval stage	Actual adult otolith cluster	N	1	2	4	6
Embryo	1	3	3	0		0
	2	8	0	7		1
	6	1	0	0		1
Yolk-Sac Larvae	1	11	7	4		0
	2	3	1	2		0
	6	1	0	0		1
Non-Yolk Sac Larvae	1	14	5	0	9	0
	2	40	0	26	2	12
	4	2	0	0	2	0
	6	8	0	0	2	6

Table 3.3 Classification table of progeny otolith microchemistry predicting maternal river by larval life stage; numbers represent number of fish predicted by actual maternal river. Embryo is still in the egg case (n = 12), Yolk Sac is ≤ 5dph (n = 15), and Non-Yolk Sac Larvae are ≥ 15 dph (n = 72). Embryo classification is 83.33%, Yolk Sac is 93.33%; and Non-Yolk Sac is 44.44% accurate. Blank cells had no progeny caught from that adult otolith cluster.

			Predicted maternal river			
Larval stage	Actual maternal river	N	Cape Fear	Neuse	Roanoke	Tar
Embryo	Neuse	5		4	1	1
	Roanoke	1		0	1	0
	Tar	5		0	0	5
Yolk-Sac Larvae	Neuse	12		11	0	1
	Roanoke	1		0	1	0
	Tar	2		0	0	2
Non-Yolk Sac Larvae	Cape Fear	6	4	0	2	0
	Neuse	29	7	5	5	12
	Roanoke	10	4	0	6	0
	Tar	27	0	2	8	17

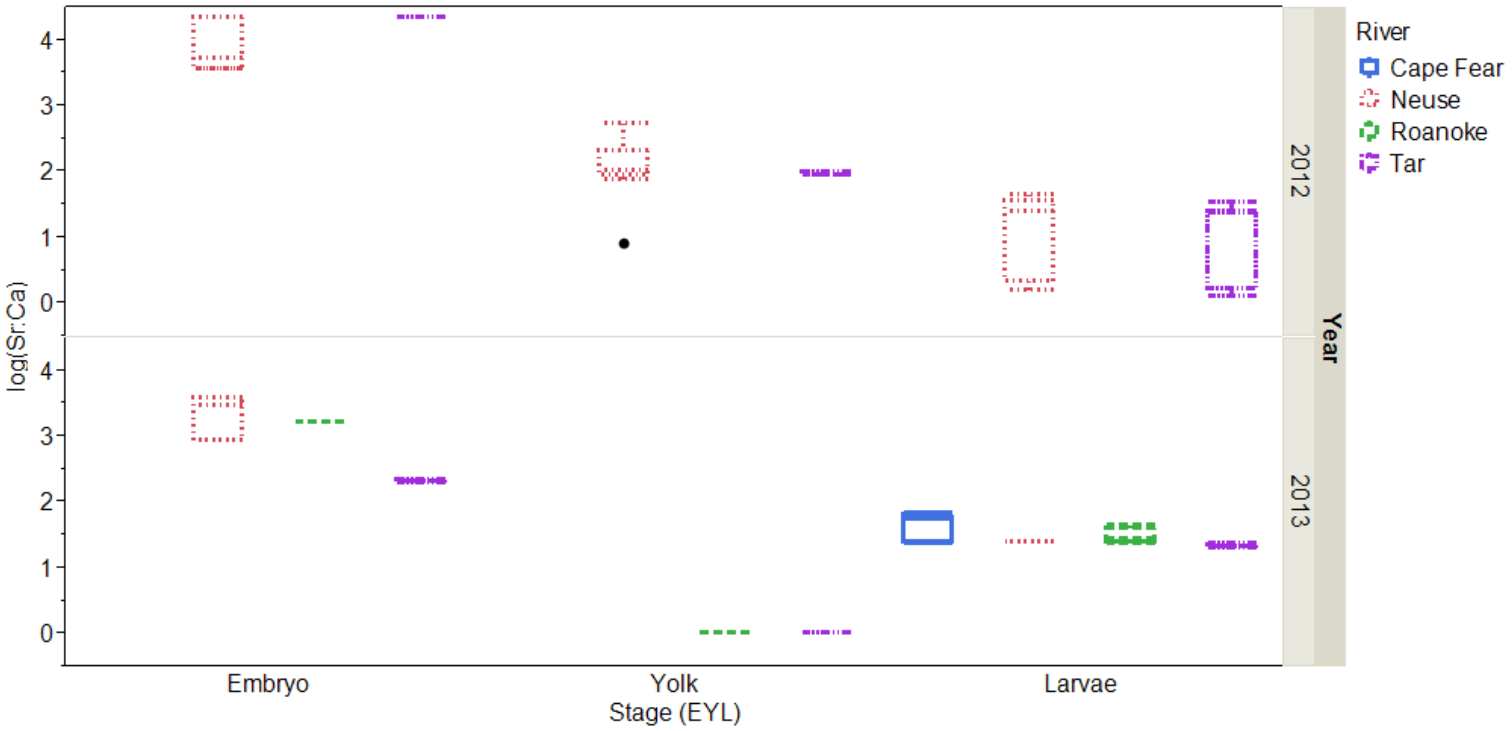

Fig 3.1 Boxplot of change in log(Sr:Ca) ratio by larval life stage otoliths of progeny. Embryo is 0 dph or fish that are still within the egg case. Yolk sac stage fish are ≤ 5 dph. Larvae are non-yolk sac fish that are ≥ 15 dph. Boxes contain the 25th and 75th quartiles; the line within the box represents the median. Extensions from the box represent 10th and 90th quartiles. Dots outside the boxes are possible outliers.

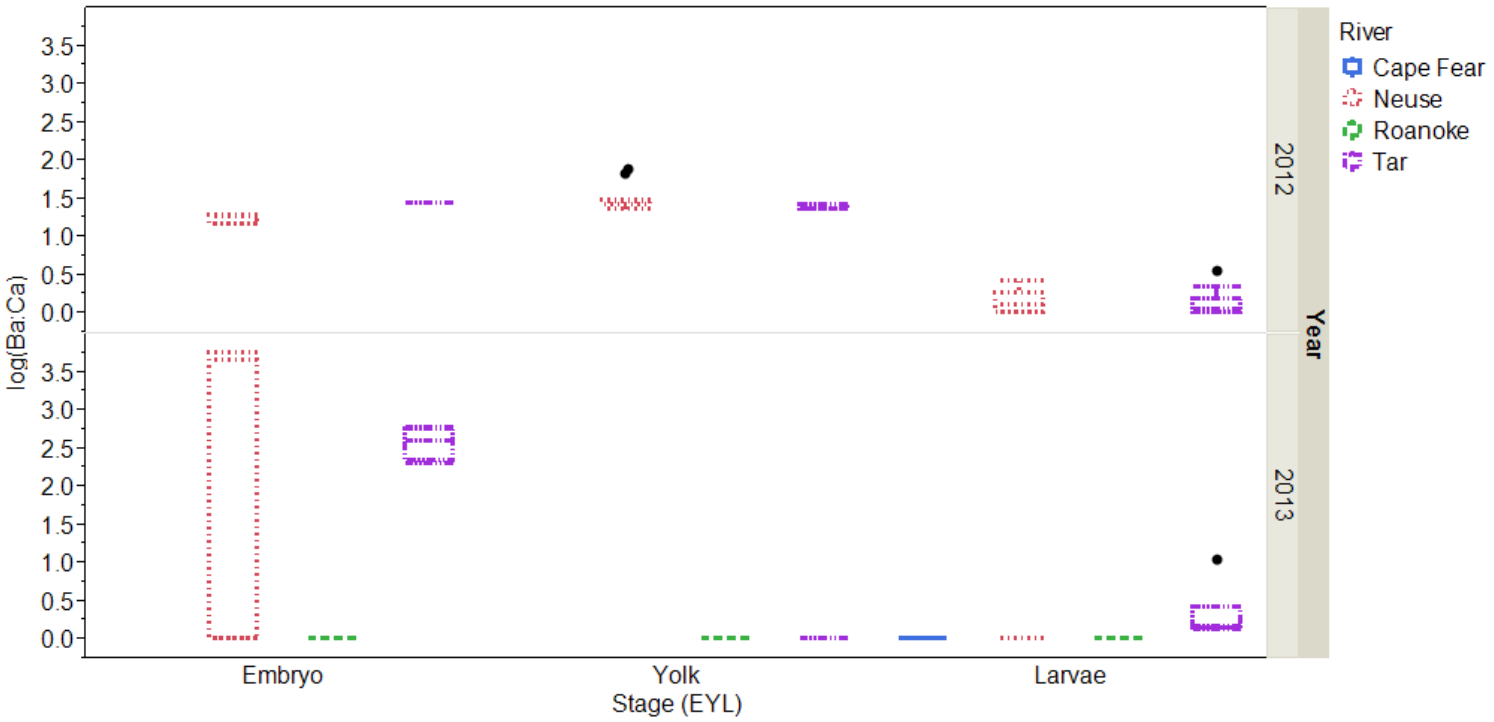

Fig 3.2 Boxplot of change in log(Ba:Ca) ratio by larval life stage otoliths of progeny. Embryo is 0 dph or fish that are still within the egg case. Yolk sac stage fish are ≤ 5 dph. Larvae are non-yolk sac fish that are ≥ 15 dph. Boxes contain the 25th and 75th quartiles; the line within the box represents the median. Extensions from the box represent 10th and 90th quartiles. Dots outside the boxes are possible outliers.

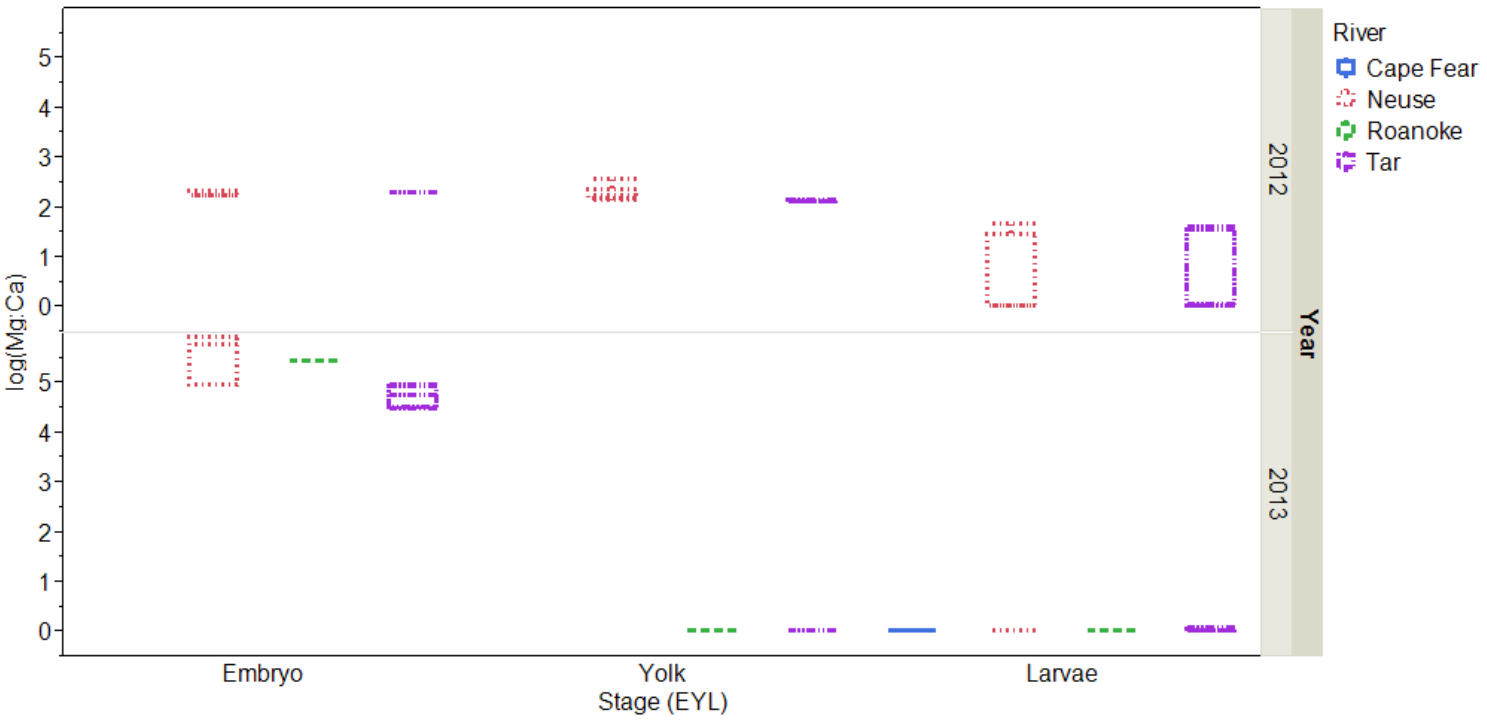

Fig 3.3 Boxplot of change in log(Mg:Ca) ratio by larval life stage otoliths of progeny. Embryo is 0 dph or fish that are still within the egg case. Yolk sac stage fish are ≤ 5 dph. Larvae are non-yolk sac fish that are ≥ 15 dph. Boxes contain the 25th and 75th quartiles; the line within the box represents the median. Extensions from the box represent 10th and 90th quartiles. Dots outside the boxes are possible outliers.

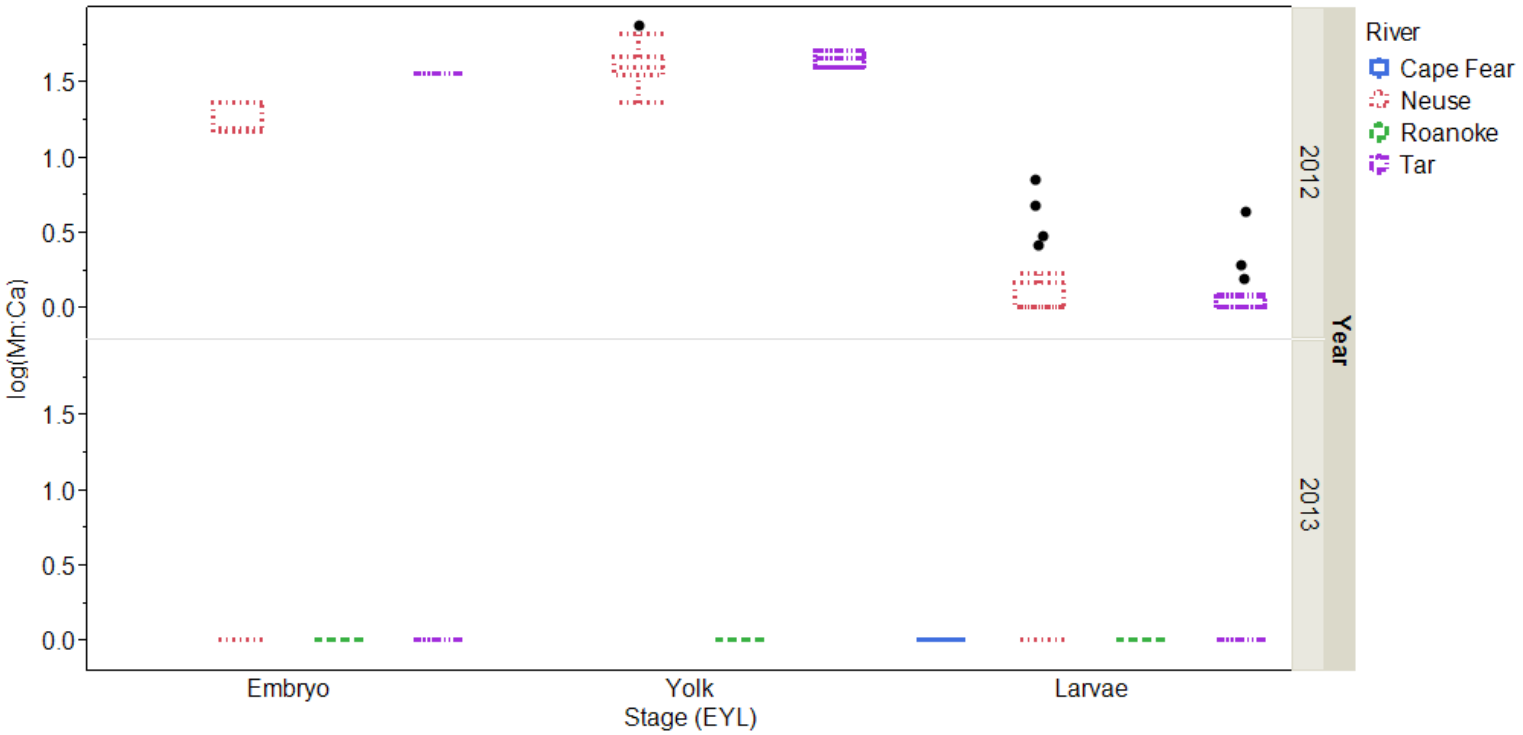

Fig 3.4 Boxplot of change in log(Mn:Ca) ratio by larval life stage otoliths of progeny. Embryo is 0 dph or fish that are still within the egg case. Yolk sac stage fish are ≤ 5 dph. Larvae are non-yolk sac fish that are ≥ 15 dph. Boxes contain the 25th and 75th quartiles; the line within the box represents the median. Extensions from the box represent 10th and 90th quartiles. Dots outside the boxes are possible outliers.

Chapter 4

CONCLUSIONS AND IMPLICATIONS

Summary of chapters

After determining that Striped Bass (*Morone saxatilis*; Walbaum 1792) otoliths take on the 'watershed signature', age 0 Striped Bass can be assigned watersheds where they resided in nursery habitats, and abundance in the adult population should reflect quality of Striped Bass habitat (Mohan et al. 2012). However, the origins of trace elemental concentrations occurring in the otolith just after formation within the primordium remain unclear (maternal or ambient waters). The goals of my study were 1) to determine if maternal contribution is possible based upon otolith formation and timing; 2) to examine the possibility of maternal contribution by comparing adult soft tissue and otolith elemental signatures; and 3) to determine the existence of maternal contribution to progeny otoliths.

Otolith formation

Otoliths are fish earstones that are used for hearing and maintaining equilibrium (Secor 1991a). Each fish has three pairs of otoliths: the sagitta, lapillus and asteriscus. In most teleost fish the sagittae are the largest, and thus used for both ageing and microchemical analysis studies (Secor 1991). In recent years there has been an increase in the use of otoliths to determine natal habitats through microchemical analysis (Thorrold et al. 1998; Hobbs et al. 2007). This has led to a need for an understanding of when and how otoliths form. The otoliths of Striped Bass are used for all of these purposes, but its formation and early development have not been documented.

Adult Striped Bass were collected through electroshocking on the spawning grounds of four coastal rivers (Cape Fear, Neuse, Roanoke, and Tar) in 2012 and 2013 and transported to either the Edenton National Fish Hatchery or the Watha State Fish Hatchery. Progeny from known mothers were collected daily during the egg and yolk sac phase (5 dph) and weekly during the non-yolk sac stage. All fish were photographed and measured for total length (TL-mm) and total sagittal otolith length (TOL-µm).

After photographing and measuring these fish we were able to identify the timing and formation of the three otolith pairs during late pre-hatch embryo, yolk sac larvae, and non-yolk sac larvae. The sagittal otoliths were first to appear, forming shortly before hatch (0 dph) and were observed growing larger throughout the larval stage. The lapilli otoliths formed within the first 24 hours post hatch (0.5 dph). The asterisci otoliths were difficult to locate, but seemed to form between 4 and 15 days post hatch (dph). At hatch the sagittal otoliths appeared circular, and by 5 dph seemed to gain some dimensionality. At 15 dph the sagittal otolith began to elongate along the anterior/ posterior axis. This knowledge of when otoliths form will affect any microchemical analysis done in the first year of life, especially as the asterisci otoliths form around first feeding, and should be taken into account when choosing an otolith for analysis of elemental chemistry and ageing. Despite knowing the growth rate of each river, using this information to back-calculate spawn date is inadvisable as the growth rate can be significantly impacted by temperature and has been shown to be different between wild and reared fish (Aldanondo et al. 2008). Otolith shape, however, might be able to be used for an estimate of age which could then be back-calculated for spawn date.

Adult signatures

Fish soft tissue microchemical analysis can be used to determine the levels of heavy metals and trace elements in a fish for pollution studies and fish consumption advisories. Trace elements can make their way into fish tissue and organs from the ambient environment or the diet. Some ions that enter mainly through the ambient water are Sr, Ba and Ca (Campana 1999). Other elements, such as Ba and Mn, may enter through the diet. Other factors can also have an impact on the accumulation of trace elements include gender, degree of gonad development (reflected in the gonadosomatic index), species and even other trace elements (Alhashemi et al. 2012). In this study, soft tissue analysis was used to determine river signatures and likely pathways of maternal contribution to development of progeny otoliths.

Adult fish from four coastal rivers (Cape Fear, Neuse, Roanoke and Tar) and the ocean were dissected and muscle, liver, kidney, gonadal tissues and otoliths were removed from 37 Striped Bass. The tissues were dissolved in 70% nitric acid before being diluted to 7% and analyzed on an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) for Ba, Ca, Sr, Mg, and Mn concentrations. Each concentration was then put into a ratio against Ca for further analysis and to allow comparisons between tissues. Otoliths were analyzed using a Laser Ablation Inductively Coupled Plasma Mass Spectrometer (LA-ICP-MS), which were then grouped in a hierarchical cluster analysis before being compared to the tissues in discriminate function analyses.

In a discriminant function analysis the ocean could be separated from the coastal rivers by all tissues with over 75% accuracy. The coastal rivers could be discriminated between each other using ovaries and testes. The otoliths were able to be discriminated from each other using

the testes and ovaries with 61.11% and 58.33% classification, respectively. This provides a mechanism for maternal contribution to progeny otoliths, and gives credence to the determination of maternal river from progeny otoliths. The next step was to test for the existence of maternal contribution in progeny otoliths using fish from known mothers.

Maternal contribution

Fishery managers and scientists have found that the otoliths can be used for ageing and also tracking the life history and migration patterns of several fish species including Striped Bass (*Morone saxatilis*; Walbaum 1792), Red Snapper (*Lutjanus campechanus*; Poey 1860), Threespine Stickleback (*Gasterosteus aculeatus*; Linnaeus 1758), Sockeye Salmon (*Oncorhynchus nerka*; Walbaum 1792), and the Humpback Whitefish (*Coregonus pidschian*; J.F. Gmelin 1789) (Campana 1999; Brown and Severin 2009; Gibson et al. 2010). Since the otolith accretes daily layers, the chemical signature of the water is incorporated to the otolith, and thus the fish's migration patterns can be observed over time provided the habitat signature is known (Campana 1999). Further understanding of otolith microchemistry has hinted at the possibility of trans-generational markers and maternal input. Veinott et al. (2014) examined the effects different life histories (anadromous and non-anadromous) had on Brown Trout (*Salmo trutta*; Linnaeus 1758) progeny. Anadromous progeny had higher levels of Sr:Ca than non-anadromous progeny.

Edenton National Fish Hatchery and Watha State Fish Hatchery raised Striped Bass progeny from known mothers were examined to determine the extent/ existence of maternal contribution in progeny sagittal otoliths in three life stages (embryo, yolk sac, and non-yolk sac larvae). Progeny otoliths were removed from the larvae through dissection (>15 days post hatch) or

bleach dissolution (<5 days post hatch), dissolved in 50% nitric acid, sampled were then diluted to 7% nitric acid before being analyzed on an ICP-OES. Maternal otoliths were grouped using microchemical data (analyzed using a LA-ICP-MS) from their last year of life by hierarchical cluster analysis. Discriminate function analyses were then used to examine the ability of progeny microchemical data to be placed in the same group as the mother and the maternal river.

Embryo otoliths correctly identified the maternal clusters 91.67% of the time (n = 12), yolk sac larvae otoliths 66.67% of the time (n = 15), and non-yolk sac larvae otoliths only 60.94% of the time (n = 64) using a DFA. Progeny otoliths also were able to identify maternal river (Neuse, Roanoke or Tar). Embryo otoliths classified the river 83.33% of the time (n = 12), yolk sac larvae 93.33% of the time (n = 15), and non-yolk sac larvae 44.44% of the time (n = 72). This validates the hypothesis of maternal contribution and supports the hypothesis of trans-generational markers for anadromous and resident fish, however more sampling should be done to ensure this result is not a factor of small sample size.

Adult to egg: The maternal contribution process

Striped Bass sagittal otoliths form during the egg stage and grow quadratically relative to fish length during the yolk sac stage (5 dph). By 15 dph, the relationship between fish length and otolith diameter is linear. While the growth relationships are the same for each river, there are significant differences on the slopes of the regressions for each river. Since the sagittal otolith is growing quickly until at least 5 dph, the embryo and yolk-sac stages are likely reflective of any maternal contribution to progeny otoliths occurs.

The next step to determining if any maternal contribution was occurring during the yolk sac stage was to examine the adult tissues (particularly the gonads) for similar elemental ratios to

adult sagittal otoliths. Adult fish tissues from the ocean and four coastal rivers were analyzed to determine how closely they resembled each other between and within systems. Adult otoliths were separated using a hierarchical cluster analysis to group similar adult otoliths together. A discriminant function analysis was then used to classify each organ type back to otolith clusters. The highest classification rate were the ovaries (61.11%; n=18) and the testes (58.33%; n = 12). The lowest classification rate was the kidney (37.14%; n = 35). Since the ovaries are where the eggs develop this increases the likelihood of maternal contribution to progeny otoliths as the elemental ratios of the ovaries can be used to classify otolith elemental ratios with some accuracy.

With this understanding, the progeny otoliths' elemental signatures were grouped into three larval life stages (egg, yolk sac, and non-yolk sac larvae), and a discriminant function analysis was run to determine the maternal grouping based upon the hierarchical cluster analysis. The eggs classified back to the correct maternal group with 91.67% accuracy (n = 12), the yolk sac with 66.67% accuracy (n = 15), and the non-yolk sac larvae with 60.94% accuracy (n = 64). These progeny were also classified to maternal river with 83.33% (n = 12), 93.33% (n = 15), and 44.44% (n = 72) accuracy for the egg, yolk sac, and non-yolk sac larvae respectively. With each successive digestion, more of the otolith being digested consisted of ambient rather than maternal material.

There are two possible reasons for why the yolk sac larvae have a higher maternal river classification than the embryos. The first is that since the whole egg was digested rather than the otolith that there was some chemical interference from the egg sac and oil globule. The second hypothesis is that the yolk sac larvae had more time to incorporate the maternal river signature from the adult into their otolith, and thus discriminates between rivers with higher accuracy then

the embryo stage. Embryos, however, had higher maternal classification than yolk sac larvae. This is likely because the otolith grew surrounded by maternal material, while yolk sac larvae otoliths were influenced by both maternal and ambient sources. Regardless of the mechanism behind this discrepancy, the data lend credence to the hypothesis of maternal contribution.

Implications and future research

The knowledge of otolith formation is important for determining where in the otolith maternal contribution could occur. Not only that, but it allows researchers to reach more informed decisions as to which otoliths they are choosing for microchemical analysis. With the knowledge that female Striped Bass pass on a riverine signature to progeny otoliths, data on two generations of fish can be obtained from a single individual. This better enables fishery managers to identify successful nursery habitats and residency to anadromous ratios.

Future research should be done to examine the microchemical signature of progeny from anadromous mothers and compare the signature of progeny from different maternal rivers. Research should also be done to determine if the lapillus contains a maternal signature similar to the sagittal otolith. While it is likely that the microchemical signature is being passed to the progeny though maternal contribution from the ovaries, more research should be done to determine the exact mechanism.

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East Carolina University

Animal Care and
Use Committee
[illegible]
[illegible]
[illegible]
Greenville, NC 27834
[illegible]
252-744-2355 fax

October 5, 2012

Fishery Resources Grant Program
N.C. Sea Grant
NC State University, Box 8605
Raleigh, NC 27695-8605

Dear Sir or Madam:

The vertebrate animal use described in the following application submitted to the Fishery Resources Grant Program was reviewed and approved by this institution's Animal Care and Use Committee:

Title of Application: "What Determines High Strontium Levels in the Primordium of Some Striped Bass Otoliths?"

Name of Principal Investigator: Roger A. Rulifson, Ph.D.

Name of Institution: East Carolina University

Date of Approval: October 5, 2012

This institution is fully accredited by AAALAC and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The Assurance Number is A3469-01.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

APPENDIX B

East Carolina University
Animal Use Protocol (AUP) Form
Latest Revision, July, 2010

Project Title:	Strontium levels in the primordium of striped bass otoliths

1. Personnel

1.1. Principal investigator and email:	Dr. Roger Rulifson; rulifsonr@ecu.edu
1.2. Department,	
office phone:	ISCP/Biology 252-328-9400

1.3. Emergency numbers:

	Principal Investigator	Other (Co-I, technician, PhD. student)
Name:	Dr. Roger Rulifson	Coley Hughes
Cell:	252-412-4411	252-702-1400
Pager:	n/a	n/a
Home:	252-355-7632	n/a

FOR IACUC USE ONLY

AUP #

New/renewal:

Date received:

Full Review and date: Designated Reviewer and date:

Approval date:

Study type:

Pain/Distress category:

Surgery: Survival: Multiple:

Prolonged restraint:

Food/fluid restriction:

Hazard approval/dates: Rad: IBC: EH&S:

OHP enrollment/mandatory animal training completed: Coley Hughes, Brie Elking

Amendments approved:

1.4. Co-Investigators if any:

1.5. List all personnel (PI, Co-I, technicians, students) that will be performing procedures on live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name	Required ECU Training	Other Relevant Animal Experience/ Training
PI: Dr. Roger Rulifson	IACUC	Completed IACUC Humane Animal Care and Use Test – 30 years of experience in fish research. PI will be training students on fish collection, proper care and use techniques and tissue collection protocol.
Others: Coley Hughes	IACUC	Aseptic Surgery Course and Animal Handling Course – 3 years working in fish research implanting acoustic receivers. Student will be collecting dead fish and working up fish for otoliths and tissue samples in the lab.

Brie Elking	IACUC	2 yrs. working in fish research. Will be trained by PI. Student will be collecting eggs, larvae, and adult fish from hatcheries. Student will be working up fish for otolith collection and tissue samples in the lab.
Dan Zurlo	IACUC	Student will be assisting with tissue sample collection.
Evan Knight	IACUC	Student will be assisting with tissue sample collection.

2. Regulatory Compliance

2.1 Non-Technical Summary

Using language a non-scientist would understand, please provide a 6 to 8 sentence summary explaining the overall study objectives and benefits of proposed research or teaching activity, and a brief overview of all procedures involving live animals (more detailed procedures are requested later in the AUP). Do **not** cut and paste the grant abstract.

The study aims to assess the birthplace of striped bass residing in the North Carolina estuaries. This study is a two part research project. Phase one consists of adult fish being collected by a commercial fisherman Captain Aaron Kelly through hook and line. Fish will be sacrificed immediately upon collection by the commercial fishermen. The fish will be dead when collected by the research personnel. Their earbones (otoliths), liver, kidney, muscle tissue, blood, and gonads will be removed for the study. The earbones will be analyzed for their trace elemental concentrations. These concentrations will be matched to the elemental concentrations of the watersheds they are residing in to determine their natal origin.

The second phase of this research involves the collection of fish larvae at the Watha and Edenton Hatcheries. This deals with the maternal input in the earbone itself and discovering how they form. Striped bass egg, larvae, and adults will be collected from these two hatcheries. All adults and eggs will be collected post mortem from hatchery personnel. Larvae will be caught by zooplankton net and sacrificed using electronarcosis. The earbones of the mothers will be compared against those of the progeny to discover the amount of maternal input to the center of the earbone (otolith).

2.2. Duplication

Does this study duplicate existing research? Yes ☐ No ☒

If yes, why is it necessary? (note: teaching by definition is duplicative)

2.3 Alternatives to the Use of Live Animals

Are there less invasive procedures, other species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? Yes ☐ No ☒

If yes, please explain why you cannot use these alternatives.

2.4 Literature Search to ensure that there are no alternatives to all potentially painful and/or distressful procedures

List the following information for each search (please do not submit search results but retain them for your records):

Date Search was performed: March 2012 to August 2012

Database searched: Google Scholar, Web of Science, American Fisheries Society, various journals

Period of years covered in the search: 1985 to present Keywords used and strategy: otolith (fish earbone) removal, natal origin, trace elemental analysis Other sources consulted: Other researchers conducting similar studies in other areas and on other fish, experts in the field, state and federal managing agencies.
Narrative indicating the results of the search (2-3 sentences) and explaining why there are no alternatives to your proposed procedures that have the potential to cause pain and/or distress. If alternatives exist, describe why they are not adequate. Please use the concept of the 3 R's when considering alternatives (reducing the number of animals to what is necessary to obtain scientifically sound results; refining techniques to minimize pain and discomfort to animals; and replacing animal models with non-animal models whenever possible): Otoliths (fish earbones) are the only calcified structure in fish that can be used as a temporal record of the fishes movements. There is no way to remove the otoliths from the fish without sacrificing the animal because they are essential for the fish's orientation and position and their removal would lead to death. The number of animals collected is what realistically could be collected to ensure a statistically significant sample size. Phase 1 of the project will only involve 32 adult fish and be dead upon collection. Phase 2 of the project will be the collection of adults, eggs, and larvae from hatcheries. The adults and eggs will be collected post mortem from hatchery personnel. Larvae will immediately be euthanized by immersion in a portable electroanesthesia system to minimize pain and discomfort. It is necessary for the project to use actual otoliths in order test strontium because models do not exist for this research.

2.5 Hazardous agents

2.5a. Protocol related hazards

Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix 1 Completed?
Radioisotopes	Radiation	No	
Ionizing radiation	Radiation	No	
Infectious agents (bacteria, viruses, rickettsia, prions)	IBC	No	
Toxins of biological origins (venoms, plant toxins, etc.)	IBC	No	
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	IBC	No	
Human tissues, cells, body fluids, cell lines	IBC	No	
Viral/ Plasmid Vectors/ Recombinant DNA or recombinant techniques	IBC	No	
Oncogenic/toxic/mutagenic chemical agents	EH&S	No	
Nanoparticles	EH&S	No	
Cell lines injected or implanted in animals (MAP test)	DCM	No	
Other agents		No	

2.5b. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

- Fish spines; All handlers will be taught appropriate ways to handle fish and minimize risk
- Electrocution; Everyone will be trained on the electroanethesia unit and no one will reach into the unit while it is on to minimize the risk

3. Animals and Housing

3.1. Species and strains:

Striped Bass *Morone saxatilis*

3.2. Weight, sex and/or age:

Both male and female in a range of ages and sizes

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project
600	+ 0	= 600

3.3. Justify the species and number (use statistical justification when applicable) of animals requested:

Phase 1: Adult samples will be collected from 4 regions: the Croatan Sound, Roanoke Sound, Oregon Inlet, and nearshore coastal environments by a commercial fisherman. These sites will be sampled twice monthly for 4 months (n=32), from November 2012 to February 2013. This number is what realistically could be collected by the commercial fisherman to ensure a statistically significant sample size.

Phase 2: Adults, eggs and larvae will be collected from the Watha State Fish Hatchery and the Edenton National Fish Hatchery. The sample size of the younger fish will depend upon otolith size and weight. About 2 mg of otoliths will be used to keep the elemental concentrations above detection limit. The youngest fish have otoliths that are approximately 25 microns, so the number of specimens required will vary between ages.

3.4. Justify the number and use of any additional animals needed for this study (i.e. breeder animals, inappropriate genotype/phenotype, extra animals due to problems that may arise, etc.):

N/A

3.5. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health behavioral, or physical abnormalities? Yes ☐ No ☒ (if yes, describe)

3.6. Are there any unusual husbandry and environmental conditions required? Yes ☐ No ☒ If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, feed on cage floor, prolonged weaning times, wire-bottom cages, no enrichment, social isolation, etc.):

3.7. If wild animals will be captured or used, provide permissions (collection permit # or other required information):

North Carolina Division of Marine Fisheries Collecting Permit #706671

3.8. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

Phase 1: Deceased fish will be collected at the dock in Oregon Inlet. Phase 2: Fish will be collected at the Watha and Edenton fish hatcheries. Samples will be worked up in the necropsy lab in Flanagan room 384 and the lab in 388.

4. Animal Procedures

4.1. Will procedures other than euthanasia and tissue collection be performed? Yes ☐ No ☒

If animals will be used exclusively for tissue collection following euthanasia (answer "no" above), then skip to Question 5 (Euthanasia).

4.2. Outline the Experimental Design including all treatment and control groups and the number of animals in each. If this is a breeding protocol, please describe the breeding

strategy (pairs, trios, etc.) and method and age of genotyping (if applicable). Tables or flow charts are particularly useful to communicate your design.

The larval fish will be caught at the Edenton National Fish Hatchery ponds with a zooplankton net before being sacrificed with electronarcosis and stored in ethanol until analysis.

In sections 4.3-4.19 below, please respond to all items relating to your proposed animal procedures. If a section does not apply to

your experimental plans, please leave it blank.

Note: Procedures covered by DCM and IACUC guidelines and policies are indicated by asterisk (*). Please refer to these and justify any departures.

4.3. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management (for procedures other than surgery)

Adequate records describing anesthetic monitoring and recovery must be maintained for all species.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling

scientific justification as to why this is necessary.

Describe the pre-procedural preparation of the animals:

1a. Food restricted for hours | |

1b. Food restriction is not recommended for rodents and rabbits and must be justified:

2a. Water restricted for hours

2b. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

	Agent	Concentration	Dose (mg/kg)	Volume	Route	Frequency	Duration
Pre-emptive analgesic							
Pre-anesthetic							
Anesthetic							
Analgesic Post procedure							
Other							

a. Reason for administering agent(s):

b. For which procedure(s):

c. Method of monitoring anesthetic depth:

d. Methods of physiologic support during anesthesia and recovery:

e. Duration of recovery:

f. Frequency of recovery monitoring:

g. Specifically what will be monitored?

h. When will animals be returned to their home environment?

i. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:

4.4 Use of Paralytics

Will paralyzing drugs be used?

For what purpose:

Please provide scientific justification for paralytic use:

Paralytic drug:

Dose:

Method of ensuring appropriate analgesia during paralysis:

4.5. Blood or Body Fluid Withdrawal/Tissue Collection/Injections/Tail Snip*/Gavage

Please fill out appropriate sections of the chart below:

	Location on animal	Needle/ catheter/ gavage tube size	Route of administration	Biopsy size	Volume collected	Compound and volume administered (include concentration and/or dose)	Frequency of procedure
Body Fluid Withdrawal	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tissue Collection	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Injection/Infusion	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tail snip*	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Gavage	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Other	N/A	N/A	N/A	N/A	N/A	N/A	N/A

4.6. Prolonged restraint with mechanical devices

*Restraint in this context means **beyond routine care and use procedures** for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes **any** use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.*

a. For what procedure(s):

b. Restraint device(s):

c. Duration of restraint:

d. Frequency of observations during restraint/person responsible

e. Frequency and total number of restraints:

f. Conditioning procedures:

g. Steps to assure comfort and well-being:

h. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.7 Tumor* and Disease Models/Toxicity Testing

a. Describe methodology:

b. Expected model and/or clinical/pathological manifestations:

c. Signs of pain/discomfort:

d. Frequency of observations:

e. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.8 Treadmills/Swimming/Forced Exercise

a. Describe aversive stimulus (if used):

b. Conditioning:

c. Safeguards to protect animal:

d. Duration:

e. Frequency:

f. Total number of sessions:

g. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.9 Projects Involving Food and Water Deprivation or Dietary Manipulation

(Routine pre-surgical fasting not relevant for this section)

a. Food Restriction

i. Amount restricted and rationale:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented (weight, etc):

iv. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

b. Fluid Restriction

i. Amount restricted and rationale:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented:

iv. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

c. Dietary Manipulations

i. Compound supplemented/deleted and amount:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented:

iv. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.10 Endoscopy/Fluroscopy/X-Ray/Ultrasound/MRI/CT/PET/Other Imaging

a. Describe animal methodology:

b. Duration of procedure:

c. Frequency of observations during procedure:

d. Frequency/total number of procedures:

e. Method of transport to/from procedure area:

e. Please provide or attach appropriate permissions/procedures for animal use on human equipment:

4.11 Polyclonal Antibody Production*

a. Antigen/adjuvant used:

b. Needle size:

c. Route of injection:

d. Site of injection:

e. Volume of injection:

f. Total number of injection sites:

g. Frequency and total number of boosts:

h. What will be done to minimize pain/distress:

i. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.12 Monoclonal Antibody Production

a. Describe methodology:

b. Is pristane used: [ ] Yes [ ] No

- Volume of pristane:

c. Will ascites be generated: [ ] Yes [ ] No

d. Criteria/signs that will dictate ascites harvest:

e. Size of needle for taps:

f. Total number of taps:

g. How will animals be monitored/cared for following taps:

h. What will be done to minimize pain/distress:

j. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.13 Temperature/Light/Environmental Manipulations

a. Describe manipulation(s):

b. Duration:

c. Intensity:

d. Frequency:

e. Frequency of observations/parameters documented:

f. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.14 Behavioral Studies

a. Describe methodology/test(s) used:

b. If aversive stimulus used, frequency, intensity and duration:

c. Frequency of tests:

d. Length of time in test apparatus/test situation:

e. Frequency of observation/monitoring during test:

f. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.15 Capture with Mechanical Devices/Traps/Nets

a. Description of capture device/method:

A zooplankton net will be dragged around the corners of the pond then reeled in and fish removed and sacrificed by submersion in a portable electroanethesia system.

b. Maximum time animal will be in capture device:

Max time 5 minutes, probably closer to 2 minutes on average.

c. Frequency of checking capture device:

Fish will be removed from the net immediately.

d. Methods to ensure well-being of animals in capture device:

Research has shown zooplankton nets to be a safe method to catch larval fish.

e. Methods to avoid non-target species capture:

The only species in the hatchery ponds are the target species (striped bass).

f. Method of transport to laboratory/field station/processing site and duration of transport:

Larval fish will be immediately sacrificed with electronarcosis then be transported to the

lab in ethanol for analysis.

g. Methods to ensure animal well-being during transport:

Dead during transport.

h. Expected mortality rates:

All fish will be sacrificed.

i. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

All larval fish will be sacrificed via electronarcosis as quickly as possible to reduce stress.

4.16 Manipulation of Wild-Caught Animals in the Field or Laboratory

a. Parameters to be measured/collected:

All parameters will be measured post mortem.

b. Approximate time required for data collection per animal:

c. Method of restraint for data collection:

d. Methods to ensure animal well-being during processing:

e. Disposition of animals post-processing:

f. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.17 Wildlife Telemetry/Other Marking Methods

a. Describe methodology (including description of device):

b. Will telemetry device /tags/etc be removed? If so, describe:

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.18 Other Animal Manipulations

a. Describe methodology:

b. Describe methods to ensure animal comfort and well-being:

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

4.19 Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

A. Location of Surgery (Room #):

B. Type of Surgery:

[ ] Nonsurvival surgery (animals euthanized without regaining consciousness)

[ ] Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)

[ ] Minor survival surgery

[ ] Multiple survival surgery*

If yes, provide scientific justification for multiple survival surgical procedures:

C. Describe the pre-op preparation of the animals:

1a. Food restricted for hours

1b. Food restriction is not recommended for rodents and rabbits and must be justified:

2a. Water restricted for hours

2b. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

D. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

[ ] Sterile instruments

- How will instruments be sterilized:

- If serial surgeries are done, how will instruments be sterilized between surgeries:

[ ] Sterile gloves

[ ] Cap and mask

[ ] Sterile gown

[ ] Sanitized operating area

[ ] Clipping or plucking of hair or feathers

[ ] Skin preparation with a sterilant such as betadine

[ ] Practices to maintain sterility of instruments during surgery

[ ] Non-survival (clean gloves, clean instruments, etc.)

E. Describe all surgical procedures:

1. Skin incision size and site on the animal:

2. Describe surgery in detail (include size of implant if applicable):

3. Method of wound closure:

a. Number of layers

b. Type of wound closure and suture pattern:

c. Suture type/size / wound clips/tissue glue:

d. Plan for removal of skin sutures/wound clips/etc:

F. Anesthetic Protocol:

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary.

	Agent	Concentration	Dose (mg/kg)	Volume	Route	Frequency	Duration

Pre-emptive analgesic							
Pre-anesthetic							
Anesthetic							
Analgesic Post Op							
Other							

1. Criteria to monitor anesthetic depth, including paralyzing drugs:

2. Methods of physiologic support during anesthesia and immediate post-op period:

3. Duration of recovery from anesthesia (immediate post-op period):

4. Frequency/parameters monitored during immediate post-op period:

5. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period:

6. List criteria used to determine when animals are adequately recovered and when the animals can be returned to their home environment:

G. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

1. What parameters will be monitored:

2. How frequently will animals be monitored:

3. How long post-operatively will animals be monitored:

H.

Surgical Manipulations affecting animals

1. Describe any signs of pain/ discomfort/ functional deficits resulting from the surgical procedure:

2. What will be done to manage any signs of pain or discomfort/ (include pharmacologic and non-pharmacologic interventions):

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

5. Euthanasia

**Please refer to the 2007 AVMA Guidelines on Euthanasia and DCM Guidelines to determine appropriate euthanasia methods.*

5.1 Euthanasia Procedure. If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a **scientific justification** must be provided. All investigators, even those doing survival or field studies, must complete this section in case euthanasia is required for humane reasons.

Phase 1: Fish will be received dead from the commercial fishermen.

Phase 2: All adults and eggs will be received from the hatchery personnel post mortem. The larvae will be euthanized by being placed in a Smith-Root Portable Electroanethesia System (PES) until they become pale white (this happens upon death) at which point they will be preserved in ethanol until analysis.

5.2. Method of ensuring death (can be a physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Phase 1: Fish collected are dead upon receiving them from the commercial fishermen. If a fish does not show signs of rigor mortis when received it will be monitored for other signs of life such as twitching, fin movement, or opercular expansion. If signs of life are observed the specimen will immediately be decapitated to ensure death.

Phase 2: Adults are collected dead from hatchery personnel. If an adult fish does not show signs of rigor mortis, as above, it will be monitored for other signs of life and if necessary decapitated to ensure death. If the eggs appear to have life signs (beating heart, movement) they will be left in the unit until death. The larvae will be kept in PES until dead, if an individual is alive significantly longer than others it will be decapitated.

5.3. For field studies, describe disposition of carcass following euthanasia (If carcass will be kept for genetic/morphological/phylogenetic analysis, please include preservation, transportation, and storage technique):

Specimens will be stored frozen until processed.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using

ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: _____ Date:_____

Veterinarian:_____ Date:_____

IACUC Chair: _____Date:_____

Principal Investigator:	Campus Phone:		Home Phone:
IACUC Protocol Number:	Department:		E-Mail:
Secondary Contact: Department:	Campus Phone:	Home Phone:	E-Mail:
Chemical Agents Used:	Radioisotopes Used:		
Biohazardous Agents Used:	Animal Biosafety Level:	Infectious to humans?	
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion:			
Precautions for Handling Live or Dead Animals:			
Animal Disposal:			
Bedding / Waste Disposal:			
Cage Decontamination:			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:			
Initial Approval Safety/Subject Matter Expert Signature & Date _____			

Appendix C Average adult Striped Bass elemental otolith data. River caught from, year caught, gender and fish age are also included. Distance is the distance averaged in micrometers to get the average elemental concentration.

Fish	River	Year	Gender	Age	Distance (μm)	Otolith Cluster	Sr (ppm)	Ba (ppm)	Mg (ppm)	Log(Mn (ppm))
NSP12 162	Neuse	2012	F	5	368.42	1	2199.21	10.22	11.22	-0.97
NSP12 I001	Neuse	2012	F	6	315.79	2	2083.18	18.99	11.80	0.63
NSP12 I002	Neuse	2012	M	3	736.84	2	2044.96	12.33	12.08	1.90
NSP12 159	Neuse	2012	F	6	263.16	1	1601.50	9.51	11.82	-1.42
NSP12 I003	Neuse	2012	M	3	473.68	3	1600.43	16.21	16.01	0.94
SB101	Roanoke	2013	F	6	315.79	1	2236.64	9.29	11.08	-0.43
SB105	Roanoke	2013	F	6	263.16	2	2084.02	10.02	11.28	0.33
SB106	Roanoke	2013	M	3	526.32	5	1820.44	23.22	10.18	2.18
SB111	Neuse	2013	M	4	263.16	2	2240.96	9.30	12.39	0.42
SB113	Neuse	2013	M	3	789.47	2	2251.40	14.80	12.60	0.19
SB114	Neuse	2013	F	6	526.32	2	1994.38	10.99	11.43	0.53
SB120	Tar	2013	F	6	947.37	2	2186.23	14.19	12.03	-0.04
SB122	Tar	2013	M	3	789.47	2	2380.84	12.48	15.44	1.76
SB124	Tar	2013	F	8	210.53	2	2081.74	17.46	10.92	-0.26
SB126	Tar	2013	M	4	578.95	5	2066.57	27.27	13.97	0.36
SB128	Roanoke	2013	F	13	263.16	6	3080.56	2.47	6.47	-2.81
SB132	Roanoke	2013	F	8	210.53	4	1351.78	16.70	17.59	-1.54
SB133	Roanoke	2013	M	3	578.95	5	1627.86	22.67	12.78	2.15
SB136	Roanoke	2013	F	9	263.16	6	3042.27	7.05	10.38	0.40
SB139	Roanoke	2013	M	4	526.32	5	1655.76	24.98	11.42	1.66
SB140	Roanoke	2013	F	8	263.16	6	2779.09	2.56	6.05	-0.94
SB144	Cape Fear	2013	F	7	210.53	2	2159.64	11.50	10.95	0.12
SB147	Cape Fear	2013	M	3	894.74	3	1744.23	15.14	17.99	1.50
SB148	Cape Fear	2013	F	9	263.16	2	1791.33	16.93	11.42	-0.19
SB151	Neuse	2013	M	3	473.68	3	1795.11	11.04	15.00	1.03
SB152	Neuse	2013	F	7	315.79	2	2174.54	18.92	13.79	0.05
SB168	Cape Fear	2013	F	9	263.16	1	2189.48	12.86	11.87	-1.23
SB172	Cape Fear	2013	F	9	263.16	2	1889.91	14.94	11.96	-0.74
SB173	Cape Fear	2013	M	4	736.84	3	1159.24	21.28	15.58	1.62
SB180	Roanoke	2013	F	10	263.16	6	2885.01	4.13	8.89	-1.81
SB183	Roanoke	2013	M	3	526.32	2	2349.46	15.67	12.33	1.05
SB184	Roanoke	2013	F	11	263.16	6	2592.56	5.62	5.20	-1.40
TSP12 43	Tar	2012	F	7	263.16	2	2180.59	12.52	13.36	0.36
TSP12 34	Tar	2012	F	5	263.16	1	2260.88	11.55	10.11	-2.29
TSP13 30	Tar	2012	F	4	263.16	1	2276.20	11.07	13.51	-0.56

Appendix D Adult striped bass tissue chemistry. Gender M=Male; F=Female; I=Immature. Age in days post hacth (dph). Stage: T=testes; L=liver; K=kidney; O=ovaries; Embryo=progeny in egg case; Yolk=progeny ≤ 5 dph; Larvae=progeny ≥ 15 dph. Asterisk (*) = a lack of otolith data. Cross (†) = unknown mother.

Fish	Year	River	Gender	Age (dph)	Stage	Cluster	Sr:Ca	Mg:Ca	Mn:Ca	Ba:Ca
NSP12 162 54	2012	Neuse	F		T	1	7.04	199.16	199.16	165.87
NSP12 162 54	2012	Neuse	F		L	1	8.04	144.48	144.48	138.91
NSP12 162 54	2012	Neuse	F		K	1	8.07	97.11	97.11	206.51
NSP12 159 53	2012	Neuse	F		T	1	7.47	192.92	192.92	95.41
NSP12 159 53	2012	Neuse	F		L	1	8.44	188.57	188.57	156.01
NSP12 159 53	2012	Neuse	F		K	1	8.19	110.45	110.45	142.65
NSP12 159 53	2012	Neuse	F		O	1	8.35	112.11	112.11	469.78
NSP12 I001	2012	Neuse	F		T	2	7.28	214.94	214.94	197.43
NSP12 I001	2012	Neuse	F		L	2	7.53	172.80	172.80	198.46
NSP12 I001	2012	Neuse	F		K	2	5.29	21.00	21.00	47.55
TSP12 034 7	2012	Tar	F		T	1	5.75	192.17	192.17	71.23
TSP12 034 7	2012	Tar	F		L	1	9.36	178.12	178.12	460.42
TSP12 034 7	2012	Tar	F		K	1	8.19	88.01	88.01	246.62
TSP12 030 8	2012	Tar	F		T	1	6.77	196.51	196.51	180.41
TSP12 030 8	2012	Tar	F		L	1	10.16	132.20	132.20	545.01
TSP12 030 8	2012	Tar	F		K	1	8.86	88.66	88.66	350.36
TSP12 043	2012	Tar	F		T	2	5.41	234.06	234.06	122.43
TSP12 043	2012	Tar	F		L	2	8.38	196.25	196.25	204.59
TSP12 043	2012	Tar	F		K	2	7.70	61.85	61.85	232.41
NSP12 I002	2012	Neuse	M		T	2	5.81	198.40	198.40	143.36
NSP12 I002	2012	Neuse	M		L	2	4.46	49.01	49.01	97.11
NSP12 I002	2012	Neuse	M		K	2	10.36	88.48	88.48	366.40
NSP12 I002	2012	Neuse	M		TE	2	11.68	390.90	390.90	406.76
NSP12 I003	2012	Neuse	M		T	3	6.38	211.44	211.44	85.19
NSP12 I003	2012	Neuse	M		L	3	7.39	198.75	198.75	124.19
NSP12 I003	2012	Neuse	M		K	3	6.71	88.89	88.89	65.43
NSP12 I003	2012	Neuse	M		TE	3	0.00	258.46	258.46	217.81
NSP13 151	2013	Neuse	M		T	3	7.15	177.71	177.71	85.14
NSP13 151	2013	Neuse	M		L	3	7.08	191.98	191.98	88.36
NSP13 151	2013	Neuse	M		K	3	7.37	163.03	163.03	154.28
NSP13 151	2013	Neuse	M		TE	3	0.27	179.97	179.97	211.82
NSP13 113	2013	Neuse	M		T	2	4.74	487.35	487.35	1214.83
NSP13 113	2013	Neuse	M		L	2	33.33	650.47	650.47	3602.98
NSP13 113	2013	Neuse	M		K	2	28.60	283.00	283.00	3080.86
NSP13 113	2013	Neuse	M		TE	2	37.16	551.10	551.10	4130.82
NSP13 152	2013	Neuse	F		T	2	6.41	226.15	226.15	183.96
NSP13 152	2013	Neuse	F		L	2	20.04	528.34	528.34	2137.77
NSP13 152	2013	Neuse	F		K	2	15.45	316.30	316.30	1538.39
NSP13 152	2013	Neuse	F		O	2	11.11	227.97	227.97	660.99

Appendix D Continued.

Fish	Year	River	Gender	Age (dph)	Stage	Cluster	Sr:Ca	Mg:Ca	Mn:Ca	Ba:Ca
NSP13 148	2013	Neuse	F		T	2	15.99	1148.50	1148.50	1716.92
NSP13 148	2013	Neuse	F		L	2	26.50	539.09	539.09	2892.36
NSP13 148	2013	Neuse	F		K	2	15.71	264.68	264.68	1597.16
NSP13 148	2013	Neuse	F		O	2	16.53	531.24	531.24	1587.93
NSP13 114	2013	Neuse	F		T	2	14.31	1214.57	1214.57	950.32
NSP13 114	2013	Neuse	F		L	2	9.43	783.29	783.29	485.55
NSP13 114	2013	Neuse	F		K	2	9.77	483.66	483.66	534.08
NSP13 114	2013	Neuse	F		O	2	25.43	712.07	712.07	1698.79
NSP13 111	2013	Neuse	F		T	2	16.55	913.44	913.44	1065.53
NSP13 111	2013	Neuse	F		L	2	30.36	1854.73	1854.73	2195.40
NSP13 111	2013	Neuse	F		K	2	11.42	486.75	486.75	647.49
NSP13 111	2013	Neuse	F		O	2	34.56	640.42	640.42	2436.93
TSP13 126	2013	Tar	M		T	5	9.10	2221.22	2221.22	590.60
TSP13 126	2013	Tar	M		L	5	38.43	1820.05	1820.05	2678.97
TSP13 126	2013	Tar	M		K	5	15.83	629.76	629.76	949.98
TSP13 126	2013	Tar	M		TE	5	59.33	3807.12	3807.12	4402.88
TSP13 122	2013	Tar	M		T	2	6.49	1524.05	1524.05	391.56
TSP13 122	2013	Tar	M		L	2	24.10	1595.61	1595.61	1588.08
TSP13 122	2013	Tar	M		K	2	6.68	39.71	39.71	38.08
TSP13 122	2013	Tar	M		TE	2	27.38	1604.50	1604.50	1859.94
TSP13 124	2013	Tar	F		T	2	9.21	1733.93	1733.93	606.48
TSP13 124	2013	Tar	F		L	2	12.53	1657.91	1657.91	750.16
TSP13 124	2013	Tar	F		K	2	12.39	414.26	414.26	626.26
TSP13 124	2013	Tar	F		O	2	12.54	755.75	755.75	635.35
TSP13 120	2013	Tar	F		T	2	8.07	1751.45	1751.45	551.02
TSP13 120	2013	Tar	F		L	2	18.74	1720.63	1720.63	1321.46
TSP13 120	2013	Tar	F		K	2	9.95	524.33	524.33	497.05
TSP13 120	2013	Tar	F		O	2	15.13	676.43	676.43	939.66
CFSP13 147	2013	Cape Fear	M		T	3	10.28	1458.65	1458.65	713.63
CFSP13 147	2013	Cape Fear	M		L	3	17.25	953.90	953.90	1295.96
CFSP13 147	2013	Cape Fear	M		K	3	9.26	563.84	563.84	522.13
CFSP13 147	2013	Cape Fear	M		TE	3	18.95	1029.89	1029.89	1281.46
CFSP13 173	2013	Cape Fear	M		T	3	5.16	882.03	882.03	216.46
CFSP13 173	2013	Cape Fear	M		L	3	12.04	693.05	693.05	696.46
CFSP13 173	2013	Cape Fear	M		K	3	6.16	69.02	69.02	154.38
CFSP13 173	2013	Cape Fear	M		TE	3	28.66	976.85	976.85	2145.60
CFSP13 172	2013	Cape Fear	F		T	2	5.15	187.33	187.33	92.50
CFSP13 172	2013	Cape Fear	F		L	2	19.65	1013.92	1013.92	1411.72
CFSP13 172	2013	Cape Fear	F		K	2	15.18	594.29	594.29	985.99
CFSP13 172	2013	Cape Fear	F		O	2	11.04	798.69	798.69	658.49
CFSP13 168	2013	Cape Fear	F		T	1	21.63	2588.50	2588.50	1454.79

Appendix D Continued.

Fish	Year	River	Gender	Age (dph)	Stage	Cluster	Sr:Ca	Mg:Ca	Mn:Ca	Ba:Ca
CFSP13 168	2013	Cape Fear	F		L	1	9.73	598.08	598.08	455.36
CFSP13 168	2013	Cape Fear	F		K	1	55.58	374.99	374.99	4333.54
CFSP13 168	2013	Cape Fear	F		O	1	15.10	654.11	654.11	892.47
CFSP13 144	2013	Cape Fear	F		T	2	6.06	1296.69	1296.69	315.19
CFSP13 144	2013	Cape Fear	F		L	2	12.09	1286.19	1286.19	791.87
CFSP13 144	2013	Cape Fear	F		K	2	13.16	481.82	481.82	958.97
CFSP13 144	2013	Cape Fear	F		O	2	13.57	701.18	701.18	799.52
RSP13 183	2013	Roanoke	M		T	2	7.77	1363.89	1363.89	475.44
RSP13 183	2013	Roanoke	M		L	2	6.97	490.29	490.29	310.25
RSP13 183	2013	Roanoke	M		K	2	16.56	477.98	477.98	1208.88
RSP13 183	2013	Roanoke	M		TE	2	19.10	692.47	692.47	1324.39
RSP13 139	2013	Roanoke	M		T	5	6.02	1648.00	1648.00	408.57
RSP13 139	2013	Roanoke	M		L	5	14.20	1016.19	1016.19	986.73
RSP13 139	2013	Roanoke	M		K	5	11.95	492.06	492.06	792.10
RSP13 139	2013	Roanoke	M		TE	5	25.27	949.96	949.96	2065.51
RSP13 133	2013	Roanoke	M		T	5	6.30	1557.02	1557.02	388.93
RSP13 133	2013	Roanoke	M		L	5	10.45	1007.96	1007.96	641.15
RSP13 133	2013	Roanoke	M		K	5	16.67	645.01	645.01	1143.06
RSP13 133	2013	Roanoke	M		TE	5	15.73	1713.84	1713.84	1013.29
RSP13 106	2013	Roanoke	M		T	5	3.61	56.87	56.87	40.99
RSP13 106	2013	Roanoke	M		L	5	6.53	166.90	166.90	432.52
RSP13 106	2013	Roanoke	M		K	5	6.53	186.23	186.23	424.49
RSP13 106	2013	Roanoke	M		TE	5	0.82	15.63	15.63	204.99
RSP13 184	2013	Roanoke	F		K	6	1.33	181.36	181.36	60.80
RSP13 184	2013	Roanoke	F		O	6	11.47	832.99	832.99	627.05
RSP13 180	2013	Roanoke	F		T	6	4.87	108.72	108.72	153.86
RSP13 180	2013	Roanoke	F		L	6	9.29	284.29	284.29	177.72
RSP13 180	2013	Roanoke	F		K	6	10.70	1284.62	1284.62	701.38
RSP13 180	2013	Roanoke	F		O	6	8.69	488.83	488.83	328.49
RSP13 140	2013	Roanoke	F		T	6	10.84	401.31	401.31	560.40
RSP13 140	2013	Roanoke	F		L	6	16.17	538.54	538.54	1026.49
RSP13 140	2013	Roanoke	F		K	6	8.00	1528.20	1528.20	496.67
RSP13 140	2013	Roanoke	F		O	6	11.16	998.73	998.73	591.42
RSP13 136	2013	Roanoke	F		T	6	8.29	411.84	411.84	368.98
RSP13 136	2013	Roanoke	F		L	6	20.01	525.54	525.54	1335.38
RSP13 136	2013	Roanoke	F		K	6	6.31	1369.87	1369.87	506.84
RSP13 136	2013	Roanoke	F		O	6	15.44	791.49	791.49	854.17
RSP13 132	2013	Roanoke	F		T	4	10.87	447.26	447.26	574.07
RSP13 132	2013	Roanoke	F		L	4	27.36	773.94	773.94	1867.25
RSP13 132	2013	Roanoke	F		K	4	7.80	1249.52	1249.52	445.00
RSP13 132	2013	Roanoke	F		O	4	11.63	1012.08	1012.08	687.50

Appendix D Continued.

Fish	Year	River	Gender	Age (dph)	Stage	Cluster		Sr:Ca	Mg:Ca	Mn:Ca	Ba:Ca
RSP13 128	2013	Roanoke	F		T	6		8.71	415.53	415.53	527.46
RSP13 128	2013	Roanoke	F		L	6		7.17	238.04	238.04	167.54
RSP13 128	2013	Roanoke	F		K	6		6.35	201.49	201.49	147.33
RSP13 128	2013	Roanoke	F		O	6		10.02	139.87	139.87	660.53
RSP13 105	2013	Roanoke	F		T	2		8.28	277.89	277.89	410.67
RSP13 105	2013	Roanoke	F		L	2		7.49	207.51	207.51	208.62
RSP13 105	2013	Roanoke	F		K	2		6.33	99.13	99.13	172.07
RSP13 105	2013	Roanoke	F		O	2		8.28	83.66	83.66	305.15
RSP13 101	2013	Roanoke	F		T	1		7.44	237.58	237.58	310.62
RSP13 101	2013	Roanoke	F		L	1		8.84	206.40	206.40	468.15
RSP13 101	2013	Roanoke	F		K	1		8.05	132.20	132.20	429.87
RSP13 101	2013	Roanoke	F		O	1		9.07	103.84	103.84	491.57
NSP12 162 22d 2	2012	Neuse	I	22	Larvae	1		0.50	4.16	4.16	0.04
NSP12 162 37d 1	2012	Neuse	I	37	Larvae	1		3.89	0.00	0.00	0.00
NSP12 162 37d 2	2012	Neuse	I	37	Larvae	1		3.82	0.00	0.00	0.00
NSP12 162 37d 3	2012	Neuse	I	37	Larvae	1		3.93	0.00	0.00	0.00
NSP12 162 29d 1	2012	Neuse	I	29	Larvae	1		3.92	0.00	0.00	0.00
NSP12 162 29d 2	2012	Neuse	I	29	Larvae	1		3.76	0.00	0.00	0.00
NSP12 162 22d 1	2012	Neuse	I	22	Larvae	1		4.19	0.00	0.00	0.00
NSP12 162 22d 1	2012	Neuse	I	22	Larvae	1		4.19	0.00	0.00	0.00
NSP12 162 29d 1	2012	Neuse	I	29	Larvae	1		0.40	4.16	4.16	0.02
nsp12 162 10h 1	2012	Neuse	I	0	Egg	1		33.93	9.18	9.18	2.61
NSP12 162 0.5d 1	2012	Neuse	I	0.5	Yolk	1		6.84	9.53	9.53	3.32
NSP12 I001 40d 1	2012	Neuse	I	40	Larvae	1		0.39	3.55	3.55	0.18
TSP12 78 33d 3	2012	Tar	I	33	Larvae		†	0.44	3.96	3.96	0.23
TSP12 78 33d 2	2012	Tar	I	33	Larvae		†	0.13	3.70	3.70	0.03
TSP12 78 33d 1	2012	Tar	I	33	Larvae		†	3.39	0.00	0.00	0.03
TSP12 78 33d 1	2012	Tar	I	33	Larvae		†	3.39	0.00	0.00	0.03
TSP12 78 41d 1	2012	Tar	I	41	Larvae		†	3.56	0.00	0.00	0.03
TSP12 78 33d 4	2012	Tar	I	33	Larvae		†	0.16	3.84	3.84	0.10
TSP12 78 41d 3	2012	Tar	I	41	Larvae		†	0.12	3.88	3.88	0.06
NSP12 159 0.5d 1	2012	Neuse	I	0.5	Yolk	1		9.74	7.64	7.64	3.20
TSP12 78 41d 2	2012	Tar	I	41	Larvae		†	0.12	3.84	3.84	0.07
NSP12 159 36h 2	2012	Neuse	I	0	Egg	1		75.37	8.17	8.17	2.51
NSP12 159 1d 1	2012	Neuse	I	1	Yolk	1		5.51	7.67	7.67	3.26
NSP12 159 5d 1	2012	Neuse	I	5	Yolk	1		5.97	7.76	7.76	3.03
NSP12 I001 35d 1	2012	Neuse	I	35	Larvae	2		0.20	3.26	3.26	0.11
NSP12 I001 42d 1	2012	Neuse	I	42	Larvae	2		0.22	3.28	3.28	0.13
NSP12 159 3d 1	2012	Neuse	I	3	Yolk	1		1.46	7.38	7.38	3.31
TSP12 043 1d 1	2012	Tar	I	1	Yolk	2		6.25	7.29	7.29	2.85
TSP12 043 25d 1	2012	Tar	I	25	Larvae	2		0.31	3.48	3.48	0.19

Appendix D Continued.

Fish	Year	River	Gender	Age (dph)	Stage	Cluster	Sr:Ca	Mg:Ca	Mn:Ca	Ba:Ca
NSP12 53 4d 2	2012	Neuse	I	4	Yolk	1	6.08	7.71	7.71	2.87
TSP12 043 0.5d 1	2012	Tar	I	0.5	Yolk	2	6.01	7.52	7.52	3.08
TSP12 043 40d 1	2012	Tar	I	40	Larvae	2	0.11	2.92	2.92	0.05
TSP12 043 36h 1	2012	Tar	I	0	Egg	2	75.36	8.83	8.83	3.17
TSP12 043 27d 1	2012	Tar	I	27	Larvae	2	2.93	0.00	0.00	0.00
TSP12 043 27d 1	2012	Tar	I	27	Larvae	2	2.93	0.00	0.00	0.00
TSP12 043 27d 2	2012	Tar	I	27	Larvae	2	3.07	0.04	0.04	0.00
TSP12 043 31d 1	2012	Tar	I	31	Larvae	2	3.05	0.00	0.00	0.00
TSP12 043 31d 1	2012	Tar	I	31	Larvae	2	3.05	0.00	0.00	0.00
TSP12 043 32d 1	2012	Tar	I	32	Larvae	2	3.06	0.00	0.00	0.00
NSP12 I001 19d 1	2012	Neuse	I	19	Larvae	2	3.23	0.00	0.00	0.00
NSP12 I001 19d 1	2012	Neuse	I	19	Larvae	2	3.23	0.00	0.00	0.00
NSP12 I001 21d 1	2012	Neuse	I	21	Larvae	2	3.21	0.14	0.14	0.53
NSP12 I001 22d 1	2012	Neuse	I	22	Larvae	2	3.15	0.00	0.00	0.45
NSP12 I001 35d 2	2012	Neuse	I	35	Larvae	2	2.82	0.00	0.00	0.40
NSP12 I001 42d 2	2012	Neuse	I	42	Larvae	2	3.02	0.02	0.02	0.21
NSP12 I001 26d 1	2012	Neuse	I	26	Larvae	2	3.05	0.02	0.02	0.49
NSP12 I001 27d 1	2012	Neuse	I	27	Larvae	2	3.09	0.04	0.04	0.30
NSP12 I001 34d 1	2012	Neuse	I	34	Larvae	2	2.93	0.04	0.04	0.21
NSP12 I001 35d 3	2012	Neuse	I	35	Larvae	2	2.98	0.02	0.02	0.39
NSP12 I001 42d 3	2012	Neuse	I	42	Larvae	2	0.33	0.04	0.04	0.30
NSP12 I001 42d 4	2012	Neuse	I	42	Larvae	2	2.79	0.15	0.15	0.50
TSP12 043 25d 2	2012	Tar	I	25	Larvae	2	2.88	0.06	0.06	0.22
TSP12 043 27d 3	2012	Tar	I	27	Larvae	2	2.04	0.26	0.26	0.75
TSP12 043 27d 4	2012	Tar	I	27	Larvae	2	2.95	0.02	0.02	0.39
TSP12 043 32d 2	2012	Tar	I	32	Larvae	2	2.94	0.04	0.04	0.21
TSP12 043 40d 2	2012	Tar	I	40	Larvae	2	2.83	0.05	0.05	0.18
TSP13 120 36h 1	2013	Tar	I	0	Egg	2	9.38	139.84	139.84	14.84
TSP13 120 36h 2	2013	Tar	I	0	Egg	2	9.24	130.85	130.85	14.11
TSP13 120 0.5d 1	2013	Tar	I	0.5	Yolk	2	0.00	0.00	0.00	0.00
TSP13 120 30d 1	2013	Tar	I	30	Larvae	2	2.93	0.06	0.06	0.22
TSP13 124 36h 1	2013	Tar	I	0	Egg	2	8.89	87.78	87.78	8.98
TSP13 124 36h 2	2013	Tar	I	0	Egg	2	9.22	99.22	99.22	10.58
TSP13 124 31d 1	2013	Tar	I	31	Larvae	2	2.63	0.00	0.00	1.81
TSP13 124 58d 1	2013	Tar	I	58	Larvae	2	2.78	0.04	0.04	0.15
TSP13 124 58d 2	2013	Tar	I	58	Larvae	2	2.80	0.03	0.03	0.17
TSP13 124 58d 3	2013	Tar	I	58	Larvae	2	2.75	0.02	0.02	0.19
TSP13 124 58d 4	2013	Tar	I	58	Larvae	2	2.78	0.02	0.02	0.14
NSP13 111 36h 1	2013	Neuse	I	0	Egg	2	31.19	311.99	311.99	37.45
NSP13 111 36h 2	2013	Neuse	I	0	Egg	2	34.83	362.33	362.33	41.58
NSP13 111 35d 1	2013	Neuse	I	35	Larvae	2	2.97	0.00	0.00	0.00

Appendix D Continued.

Fish	Year	River	Gender	Age (dph)	Stage	Cluster		Sr:Ca	Mg:Ca	Mn:Ca	Ba:Ca
NSP13 148 23d 1	2013	Neuse	I	23	Larvae	2		3.00	0.00	0.00	0.00
NSP13 152 12h 1	2013	Neuse	I	0	Egg	2		17.60	139.33	139.33	0.00
CFSP13 73 45d 1	2013	Cape Fear	I	45	Larvae	1		5.20	0.00	0.00	0.00
CFSP13 73 45d 2	2013	Cape Fear	I	45	Larvae	1		4.64	0.00	0.00	0.00
CFSP13 74 45d 1	2013	Cape Fear	I	45	Larvae	2		5.10	0.00	0.00	0.00
CFSP13 74 45d 2	2013	Cape Fear	I	45	Larvae	2		4.65	0.00	0.00	0.00
CFSP13 46 46d 1	2013	Cape Fear	I	46	Larvae	2		3.06	0.00	0.00	0.00
CFSP13 46 46d 2	2013	Cape Fear	I	46	Larvae	2		2.90	0.00	0.00	0.00
RSP13 34 47d 1	2013	Roanoke	I	47	Larvae	4		4.12	0.00	0.00	0.00
RSP13 34 47d 2	2013	Roanoke	I	47	Larvae	4		3.95	0.00	0.00	0.00
RSP13 136 36h 1	2013	Roanoke	I	0	Egg	6		23.64	229.94	229.94	0.00
RSP13 136 47d 1	2013	Roanoke	I	47	Larvae	6		3.14	0.00	0.00	0.00
RSP13 136 47d 2	2013	Roanoke	I	47	Larvae	6		3.32	0.00	0.00	0.00
RSP13 140 47d 1	2013	Roanoke	I	47	Larvae	6		2.93	0.00	0.00	0.00
RSP13 140 47d 2	2013	Roanoke	I	47	Larvae	6		3.00	0.00	0.00	0.00
RSP13 136 2d 1	2013	Roanoke	I	2	Yolk	6		0.00	0.00	0.00	0.00
RSP13 180 45d 1	2013	Roanoke	I	45	Larvae	6		3.00	0.00	0.00	0.00
RSP13 180 45d 2	2013	Roanoke	I	45	Larvae	6		3.11	0.00	0.00	0.00
RSP13 184 45d 2	2013	Roanoke	I	45	Larvae	6		3.95	0.00	0.00	0.00
RSP13 184 45d 1	2013	Roanoke	I	45	Larvae	6		4.07	0.00	0.00	0.00
OWI14 1-41	2013	Ocean	F		T		*	19.18	1270.31	1270.31	2088.85
OWI14 1-41	2013	Ocean	F		L		*	36.12	441.69	441.69	3956.43
OWI14 1-41	2013	Ocean	F		K		*	45.16	1393.32	1393.32	5039.28
OWI14 1-41	2013	Ocean	F		O		*	10.23	417.86	417.86	872.51
OWI14 002	2013	Ocean	F		T		*	18.90	1489.61	1489.61	2119.81
OWI14 002	2013	Ocean	F		L		*	48.13	1642.21	1642.21	5192.76
OWI14 002	2013	Ocean	F		K		*	12.62	0.00	0.00	950.19
OWI14 002	2013	Ocean	F		O		*	20.80	1126.03	1126.03	1121.11
NSP12 162 36h 1	2012	Neuse	I		Egg	1		40.13	9.24	9.24	2.17
NSP12 162 1d 1	2012	Neuse	I	1	Yolk	1		6.95	9.55	9.55	3.26
NSP12 159 2d 1	2012	Neuse	I	2	Yolk	1		5.88	7.99	7.99	3.10
NSP12 162 2d 1	2012	Neuse	I	2	Yolk	1		7.04	9.35	9.35	3.33
NSP12 162 3d 1	2012	Neuse	I	3	Yolk	1		14.13	11.91	11.91	5.56
NSP12 I001 4d 1	2012	Neuse	I	4	Yolk	2		6.29	8.80	8.80	3.18
NSP12 162 5d 1	2012	Neuse	I	5	Yolk	1		9.76	9.90	9.90	5.17
NSP12 162 29d 3	2012	Neuse	I	29	Larvae	1		2.14	4.31	4.31	0.18
NSP12 162 30d 3	2012	Neuse	I	30	Larvae	1		0.30	4.05	4.05	0.09
NSP12 162 36d 3	2012	Neuse	I	36	Larvae	1		0.28	4.09	4.09	0.10