ABSTRACT

KOWALCHYK, CARA KATHERINE. Influence of Genetic and Environmental Adaptation on Striped bass (*Morone saxatilis)* Egg Characteristics, (Under the direction of Dr. Benjamin Reading and Dr. Jesse Fischer).

Understanding recruitment and the ecology of early fish life history are increasingly important to managing fisheries and restoring populations due to changing and more variable environmental conditions. Striped bass (*Morone saxatilis*) is an important recreational and commercial fish species popular along the Atlantic Coast. Striped bass are native to North Carolina, however, there is an observed lack of natural recruitment in the Tar, Neuse, and Cape Fear River systems. Eggs are one of the most vulnerable life stages, but little is known about the extent of their adaptability to aquatic conditions. It is unclear whether adaptations are a fixed genetic effect or a plastic physiological response. To understand if physical egg characteristics may contribute to recruitment failure of migratory Striped bass populations, fertilized eggs were collected from female Striped bass during hatchery propagation from all four major river systems in North Carolina. Fertilized eggs collected from different developmental stages were measured for size, specific gravity, and proportion of yolk proteins. Differences in mean neutral buoyancy of eggs were observed such that freshwater systems produced eggs with greater buoyancy than salinity systems with the exception of the Cape Fear River. A link between size and buoyancy measurements was observed indicating more buoyant eggs have a larger outer diameter due to a potential hydration effect. Egg yolk composition of three key vitellogenin proteins (VtgAa, VtgAb, VtgC), that have been shown to determine egg buoyancy and critically timed nutrient delivery to embryos, were analyzed from ovarian biopsies with tandem mass spectrometry for protein discovery and quantification. Differences were observed between Rivers, for example, more VtgAa occurred in eggs from freshwater systems which contributes to more buoyant eggs. Egg characteristic results observed from wild-caught Striped bass populations were confirmed

with an experimental evaluation of domestic Striped bass reared in high or low water salinity treatments. The results from these concurrent analyses were used to determine egg characteristics of Striped bass in North Carolina are an environmental-physiological response to water salinity rather than a genetic contribution.

Influence of Genetic and Environmental Adaptation on Striped bass (*Morone saxatilis)* Egg Characteristics

by
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DEDICATION

Dedicated to Alan and Katherine Kowalchyk for encouraging my pursuits in science from a young age. Also dedicated to faculty from Wake Forest University department of Biology and North Carolina State University department of Applied Ecology. Notable mention to the North Carolina State University Student Fisheries Society for providing a network of students who encouraged valuable outreach opportunities and support.

BIOGRAPHY

My awakening to the wonder of aquatic ecology stems from growing up in Minnesota, the land of 10,000 lakes. I was surrounded with endless opportunities to learn from each lake, stream, and wetland I encountered, and each place I visited further instilled in me the passion that has taken me all over the world studying these beautiful, and fragile ecosystems. As a young child, one of my first projects was to research a local ecosystem, and rather than simply relying on the Internet to find answers, I went to my local lake and spent hours fishing with my dad. We took photos and learned the identities each of the various species that we pulled onto the dock. Although I did not know it at the time, a simple poster project became my first taste of the type of work that has proven to be my life's aspiration.

Throughout high school and during my undergraduate career at Wake Forest University, I had the opportunity to be involved in a variety of research projects. I was awarded the Minneapolis Scholar of Distinction in Science award, United States Army Award (TCRSF), United States Department of Agricultural Research award for the work I accomplished during high school. Additionally, I received the Stockholm Water Prize for the state of Minnesota in 2011 and had the opportunity to present my research on a national level. The result of our collaborative efforts at St. Thomas University was published in the Journal of Applied and Environmental Microbiology (2012).

Following this intensive microbiology lab experience, I set out to continue my education regarding aquatic ecosystems by broadening my experiences out-of-state. I have had the opportunity to examine, research, document, and run tests on a variety of local flora and fauna within aquatic ecosystems of vastly different regions. These travels instilled in me a greater understanding and appreciation for research regarding international ecology. As a collective ecosystem, pollutants from one country pose significant risk to other countries around the world

yet the differences in regulations often hinder progress towards improving environmental problems.

While conducting fieldwork at Lighthouse Reef in Belize, I found that water use such as recreation, fishing, resource extraction, and protected areas are subject to different regulations, which directly correlated to differences in ecosystem health. During this field study we analyzed a variety of habitats including marine protected areas, open-water fishing zones, numerous reefs (heavily trafficked and remote), and mangroves. We collaborated with Reef Environmental Education Foundation (REEF) and CoralWatch which led to me to have a great appreciation for citizen science.

After examining various aspects of water quality ranging from a microbial level to whole ecosystem dynamics, I ultimately decided to turn my focus to North Carolina anadromous fishes. Throughout my Masters program, I had the unique opportunity to partner with both the federal and state level field biologists from the NC Division of Marine Fisheries and NC Wildlife Resource Commission. I thoroughly enjoyed the rigorous fieldwork experiencing a variety of North Carolina coastal Rivers. I have presented my work at various stages at many professional American Fisheries Society meetings, most notably winning the Best Student Paper award at the 2019 NC chapter meeting and being selected as a finalist for Best Student Paper at the 149th National meeting. I am an active member of the NCSU Student Fisheries Society that provides a network of students who encouraged valuable outreach opportunities and support.

I am a person who really enjoys adventure and field work, especially in the water. My ultimate goal is working to find practical solutions to pressing environmental problems. Water is one of our most important resources, and it is my desire to work in a field that is likely to result in the improvement of water quality and future sustainability. I hope to pursue a career as a field

biologist with a focus on environmental assessment and protecting vulnerable species. In summation, my interest in science began at a young age and will continue to grow in the future as I explore new opportunities.

ACKNOWLEDGMENTS

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CHAPTER 1
INTRODUCTION

Understanding recruitment and the ecology of early fish life history are increasingly important to managing fisheries and restoring populations due to changing and more variable environmental conditions (e.g., temperature, salinity, Kucera et al. 1992, Moran and McAlister 2009, Sundby and Kristiansen 2015), increasing contaminants and pollution that can disproportional affect early life development and survival (i.e., eggs and larvae; Schilling et al. 2015, Moran and McAlister 2009), and the indirect genetic effects of overfishing and other population limitations (e.g., bottlenecks; Rice 2002, Rachels and Ricks 2018, Lewis and Bonner 1966). Plasticity and variation in early life history characteristics and strategies are assumed to benefit populations compared to those with more restricted environmental or genetic limitations and often occur for species with large geographic distributions. Fishes that commonly demonstrate wide variation include diadromous species that either spawn (i.e., anadromy) or mature (i.e., amphidromy and catadromy) in rivers that may vary widely in physical and chemical characteristics (Hardy 1978, Jenkins and Burkhead 1993, Engman et al. 2017). Furthermore, environmental conditions of staging areas for diadromous species may affect early life characteristics of larvae and juveniles spawned by migrating females (Kucera et al. 2002).

Striped bass (*Morone saxatillis*) are a widely distributed anadromous fish species native to the Atlantic Coast of the United States and Canada and are economically valuable. However, historically Striped bass populations have crashed and were re-established through fishing moratoriums (Bradley et al. 2018, ASMFC 2019) and intensive managements (i.e., stock enhancement). A 2018 stock assessment for Striped bass estimated spawning stock biomass along the Atlantic coast at approximately 151 million pounds which is below the threshold value of 202 million pounds (NOAA, 2018). This assessment indicated that at current fishing mortality

rates, the spawning stock biomass may continue to decline below the threshold for sustainability necessitating additional management action.

Striped bass fisheries in North Carolina are primarily supported by annual stocking efforts. Examining the unique early life history of Striped bass is the first step to understanding the mechanisms behind an observed lack of natural recruitment. Eggs are one of the most vulnerable life stages, but little is known about the extent of their adaptability. It is unclear whether adaptations are a fixed genetic effect or a plastic physiological response. Estuarine systems are stochastic and water conditions can vary from year to year based on rainfall/freshwater discharge, wind action, and other natural influences. Striped bass naturally migrate upstream and spawn in freshwater rivers after staging in estuaries.

Striped bass are broadcast spawners and eggs must remain neutrally buoyant in the water column for approximately 48 hours in order to have the best chance of survival to larvae. Free-drifting eggs are susceptible to small changes in buoyancy and may be particularly problematic for Striped bass that spawn in higher salinity water (Thorsen et al. 1996, Kjesbu et al. 1992). Previous studies have shown Spotted seatrout (*Cynoscion nebulosus*) are locally adapted to spawn eggs of the appropriate neutral buoyancy in the prevailing salinity of particular estuaries (Kucera et al. 2002). Egg buoyancy was shown to be a regulator of year class strength in Baltic cod (*Gadus morhua*) from both marine and brackish water due to a negative correlation of egg diameter and specific gravity (Thorsen et al. 1996). White perch (*Morone americana*) are closely related to Striped bass, however they spawn eggs that stick together in a mass and often attach to the surrounding environment, this illustrates that even fish in the same genus may have completely different early life history strategies (Schilling et al. 2015).

Unlike other species, little is known about the developmental process of eggs in Striped bass, beyond that variation in egg physical characteristics is known to exist (Bergey et al. 2003). Therefore, Striped bass is well-suited as a focal species to understand the potential effects of previous management legacies (e.g., stocking) and the plasticity of early life history characteristics associated with egg development.

Female Striped bass begin developing eggs, known as oocyte growth, in marine environments during fall and winter prior to upstream spring migration. Egg formation proceeds after a decline in temperature and daylight in the fall that triggers endocrine changes in the fish. During oocyte growth, the major egg yolk (vitellogenin) and lipid components are accumulated and stored in the ovary (Harrell 1997, Reading et al. 2009). Changes in lipid and vitellogenin protein deposition are key factors in this process that make long term contributions to egg characteristics that may influence buoyancy (Reading et al. 2009, Hiramatsu et al. 2015). Proteins are the heaviest component of the egg whereas lipids and water provide the egg with lift. The vitellogenin particles may be processed into free amino acids that drive osmosis and thus contributes to egg characteristics such as diameter and buoyancy. The ratio of these yolk components influences egg buoyancy in the water and could contribute to survivability of larvae. For example, eggs that are too dense for their specific system sink to the substrate, become anoxic, and die. Eggs that are too buoyant may float to the surface and become susceptible to the elements including ultraviolet light and predation from aquatic and terrestrial organisms. Increasing water temperatures and daylight cues migration of the fish upstream into freshwater rivers, where only minor adjustments to egg buoyancy can occur in ovulated eggs via hydration during ovarian maturation (Reading et al. 2017). The protein composition of the eggs developed

during oocyte growth also provide the foundations of energy for developing embryos and larvae before exogenous feeding (Schilling et al. 2015, Reading et al. 2012).

Vitellogenenins (Vtgs) are the primary yolk proteins in Striped bass ovaries that contribute to the appropriate egg buoyancy and critically timed nutrient delivery to embryos (Reading et al. 2011, 2014, 2018, Hiramatsu et al. 2013, 2015, Williams et al. 2014ab). Striped bass and other Acanthomorph (i.e. spiny-rayed teleosts) express three distinct forms of vitellogenin (VtgAa, VtgAb, and VtgC) (Finn and Kristofferson 2007, Reading et al. 2009). Vtgs consist of 2 identical subunits with phosphate, lipid, carbohydrate, and proteins. They are large particles consisting of approximately 20% lipid and serve as carriers of ions such as calcium, magnesium, and iron. Pre-vitellogenesis is the stage of primary growth for the ovarian follicle and the beginning of lipid storage that takes place roughly August-September. Vitellogenesis is the secondary growth stage during November-March and is separated into five steps: 1) hepatic synthesis of yolk precursors induced by estrogen, 2) delivery of vtgs to oocyte via bloodstream, 3) selective uptake of vtgs through receptor mediated endocytosis, 4) processing vtgs into yolk proteins, and 5) storage of yolk proteins in the oocyte. Gonadotropin releasing hormone (GnRH) is mediated by biorhythms, nutrition, seasonal day length and temperature, which then induces follicle stimulation hormone followed by estradiol-17B that instructs the liver to synthesize and secrete Vtgs. Hepatic secretion levels vary for different types of Vtgs: VtgAb is dominant (peak in early-mid vitellogenesis), VtgAa is intermediate (peak in mid-late), and C is minor throughout whole process. Uptake is variable between species and mediated by membrane receptors (Vtgrs) stored in yolk granules: Lrp13 for VtgAa and Lr8 for VtgAb. VtgC has no oocyte receptor and is stored in the lipid droplet as an incomplete type of yolk protein (Reading et al. 2011, 2014, Reading et al. 2017, 2011).

Marine species have a secondary yolk proteolysis that occurs during ovary maturation just prior to ovulation. During this time, yolk proteins derived from Vtgs are cleaved into small peptides and free amino acids (FAAs) that dissociate and act as osmotic effectors to drive an influx of water (Reading et al. 2019, 2018). When VtgAa is broken down into FAAs they form an essential osmotic gradient to promote oocyte hydration, which is related to buoyancy in the form of lift for pelagic eggs. VtgAb and VtgC largely survive this degradation and are consumed by later stage embryos and larvae (Williams et al. 2014). A previous study on cod eggs showed the most important cause for increased hydration of eggs was their higher content of FAAs and inorganic ions. Differences in the protein content of cod eggs from marine and brackish water treatments were observed relative to the FAA content during final maturation (Thorsen et al. 1996).

Egg characteristics are inevitably linked to many other fundamental adaptive traits, for example, length of larval development, size at metamorphosis, and length of facultative feeding period (Moran et al. 2009). For the first 7 to 14 days after hatching, Striped bass larvae survive on the energy derived from their stored yolk sac. The proportional ratio of VtgAa:VtgAb:VtgC in the pre-ovulatory oocytes of fishes will determine the buoyancy of the ovulated eggs (Schilling et al. 2015).

Previous studies suggest preliminary data that the egg yolk composition in White Perch (*M. americana*) is influenced by water salinity (Schilling et al. 2015a). White Perch reared indoors in recirculating aquaculture systems (< 5 ppt salinity) had a 8:16:1 vitellogenin ratio in pre-ovulatory oocytes, whereas in perch reared outdoors in a flow through system supplied with estuary creek water (7-15 ppt salinity) the proportional vitellogenin ratio was 5:6:1. It is unclear

if the oocytes and eggs of Striped bass similarly adapt to changes in water salinity, however if they are unable to do so then this may relate to poor recruitment in some systems.

Our goal was to increase understanding into the influences of genetic and environmental factors on the development and portrayal of physical characteristics of Striped bass eggs and validate our observations through experimental analysis. We studied whether fertilized egg characteristics (e.g., buoyancy) of Striped bass was a fixed genetic effect or a plastic physiological response due to environmental conditions of staging females prior to upstream migration and spawning. To accomplish this goal, we employed observational and experimental methods to evaluate the influences of genetic origin and estuary salinities on the physical characteristics (i.e., specific gravity, size of chorion, yolk, and oil globule) and proteomic composition of eggs from wild-caught and domestically propagated Striped bass. Our objectives included; 1) determining genetic origin of females and physical characteristics of fertilized eggs from wild-caught Striped bass collected from a diversity of staging habitats and salinity conditions; 2) evaluating the proteomic composition of eggs prior to spawning from wild-caught Striped bass to ascertain the underlying biological mechanisms responsible for fertilized-egg characteristics; and 3) validating observational results by experimentally evaluating the influence of salinity during staging on the physical characteristics of fertilized eggs through the use of a domesticated Striped bass population of similar genetic stock held at differing salinities during simulated staging durations. We hypothesized that fertilized egg characteristics of Striped bass may be variable due to environmental conditions during staging but may be limited by genetic constraints among populations due to isolation. Our research provides information necessary to understand early life history and adaptation of Striped bass that is essential to manage and

conserve populations that are exploited and subject to altered environmental conditions (e.g., saltwater intrusion).

METHODS

Study Area

North Carolina Striped bass populations comprise the third largest along the coast with the Albemarle Sound as a source of key tributaries. Striped bass is a euryhaline fish that is typically considered anadromous, meaning it migrates from marine environments to freshwater rivers each spring to spawn. The coastal populations of North Carolina have natal estuaries that vary in salinity which potentially require different life history adaptations to be successful. Some Striped bass, however, complete their entire life cycle without marine migrations, such as, populations north of Cape Hatteras, populations upstream of the Santee-Cooper reservoir (South Carolina), and populations in the John H. Kerr reservoir (Virginia-North Carolina) (ASMFC, 2019).

In North Carolina, Striped bass in the Tar, Neuse, and Cape Fear Rivers are known as the Central Southern Management Area (CSMA). Inland waters are under the jurisdiction of the Wildlife Resource Commission (NCWRC) while coastal waters are monitored by the Division of Marine Fisheries (NCDMF). Striped bass caught recreationally in the Atlantic Ocean are limited to 1 fish per person per day with a 28-inch minimum length requirement and a year-round season. The recreational fishing season in the Albemarle Sound is October 1 through April 30 with 2 Striped bass per person per day using hook and line fishing only, and an 18-inch minimum. Roanoke River regulations are similar, but the season is open March 1 through April 30, and only 1 Striped bass exceeding 27 inches may be taken per day. Commercial fishing has a quota of 68,750 lbs of Striped bass annually. Commercial and recreational fishing for Striped bass is currently closed in the CSMA (NCDENR 2019).

Striped bass are considered a stock of concern in the CSMA by the North Carolina Division of Marine Fisheries (NCDMF) due low abundance, truncated size and age distributions,

and overall lack of adequate data for stock assessment (ASMFC 2019). Parentage based genotyping from all rivers has demonstrated further concern among CMSA rivers with a predominance of hatchery-origin fish in three of the four populations. Specifically, over 80% of Striped bass sampled in in Tar, Neuse, and Cape Fear Rivers were hatchery progeny, while no hatchery contribution has been observed in the Roanoke River (O'Donnell et al. 2015, 2016, 2017). One early management practice put in place to preserve declining North Carolina populations was to stock Roanoke River Striped bass into the other CSMA Rivers. Historically, the Roanoke River has had one of the most productive populations and continues to this day to have a strong naturally reproducing population. However, the Roanoke River environmental conditions vary greatly from the other coastal rivers, therefore, fish adapted to the Roanoke River may have trouble reproducing successfully in other rivers without drastic changes to their biology. These stocking efforts were partially successful, however, Striped bass populations in the CSMA are still supported primarily by annual stocking. These rivers are essentially a put, grow, and take fishery. Management has been amended such that broodstock used for stocking are now captured in the natal estuaries of specific river systems (ASMFC 2019).

The waters sampled for Striped bass and their salinities are:

1. High Salinity System (5-25 ppt, parts per thousand): Cape Fear River (Cape Fear River Estuary)
2. Intermediate Salinity Systems (0.5-5.5 ppt): Neuse /Tar River (Pamlico Sound)
3. Low Salinity System (0-0.5 ppt): Roanoke River (Albemarle Sound)
4. Zero Salinity System: Santee-Cooper Reservoir (SC)

Salt water has a greater density (1.025 g/ml at 4°C) than fresh water (~1.0 g/ml at 4°C) due to dissolved solutes, which contributes to the stratification of the water column in coastal areas, in particular estuaries.

Working in conjunction with NWRC and NCDMF personnel during annual spawning season (April-May 2018-2019) egg samples were obtained from female Striped bass. A replicate set of samples were obtained in Year 2 (2019), with the addition of evaluating Striped bass from the Dan River in North Carolina and the Santee-Cooper Reservoir at the Jack Bayless Hatchery (SC). The Dan River (freshwater system) sample size was smaller (spawns from 2 females) and was statistically not considered in the analysis but was examined for trends. Female Striped bass were analyzed for general population data (total length, weight, and fin clips for genetic year class analysis) as well as unfertilized and fertilized egg characteristics. The egg characteristics evaluated are: 1) size of chorion (outer diameter), 2) size of egg yolk (volume), 3) size of oil globule (diameter), and 4) buoyancy (specific gravity). Procedures were consistent across both years of sampling.

Pulse-DC boat electrofishing was used at all sites to obtain samples from the minimum of 10 gravid females throughout the season. Two dip-netters located on the front of the boat collected Striped bass of all sizes. Fish were handled quickly and carefully to reduce stress, but without the use of MS-222 sedation. All fish were weighed (g) and measured for total length (mm). Water temperature and salinity were tested using an YSI and recorded upon arrival at each sample location.

The larger females selected by the NC WRC to serve as broodstock were then transported to one of the two main NC hatcheries: 1) Watha State hatchery (Watha, NC) which stocks Striped bass from the Roanoke and Cape Fear Rivers, and 2) Edenton National hatchery which

stocks Striped bass from the Tar and Neuse Rivers (Edenton, NC). Santee-Cooper freshwater control fish were available for sampling at the Jack D. Bayless hatchery (St. Stephen, SC).

Egg buoyancy and size measurements

Fish were injected with human chorionic gonadotropin (hCG; Chorulon) to induce ovulation upon arrival at the hatchery. The fish were tank spawned according to methods outlined in Harrell et al. 1997. Spawning tanks each containing 1 female and 3 males were checked for the presence of eggs throughout the night (midnight and 4 am). Light was shined into the egg collector to detect the presence of eggs. As eggs appeared in the tanks, they were observed for two hours until the eggs became water hardened after fertilization. A sample of viable eggs was then collected via gravity pipet for evaluation. Eggs from female Striped bass spawns from each river and the freshwater reservoir were sampled for size and density analysis at two developmental stages (Hardy 1978): 1) stage 1 (<10 h post-fertilization) defined as fertilized eggs prior to formation of the germ ring; and 2) stage 4 (>30 h post-fertilization) the advanced embryonic phase, characterized by tail release from the embryonic bundle.

From a subsample of several hundred eggs at both stages, half were placed in a petri dish containing ambient hatchery water, and the other half were placed in a petri dish containing USEPA (1987) standardized freshwater control made with 48 g of sodium bicarbonate, 30 g of calcium sulfate, 30 g of magnesium sulfate, and 2 g of potassium chloride in 1 L of double-distilled, de- ionized water. After a 5-min stabilization period, randomly selected eggs were recorded regarding the specific female, river origin, and photo id. Photographs of 20 eggs per female and treatment spawned at both developmental stages (40 total per spawn) were taken using a dissecting scope at 20x magnification. Size measurements of the outer chorion, yolk, and

oil globule were repeated for all photographed eggs. Size measurements were calculated using ImageJ software calibrated to the same standard measurements using pixel number to represent length in mm. Figure 1A and 1B, respectively, show a stage 1 fertilized Striped bass egg and a stage 4 embryo from Watha State fish hatchery.

Stage 1 embryos have yolks that are circular when rendered in a 2D image, whereas stage 4 embryos have yolks that are elliptical. In order to account for the oviform shape of stage 4 embryos, volume was estimated as the measure of analysis rather than using a single diameter for the stage 1 embryos. Stage 1 volume was calculated with the equation for volume of a sphere ($V=4/3*\pi r3$) whereas stage 4 volume was calculated using the equation for volume of an ellipsoid ($V=4/3*\pi rabc$, where a=longest radius, and both b= and c=shorter radius). The measurement procedure is shown in Figure 1.

Egg density was determined by placing 13-15 groups of randomly selected eggs (10 per group) into salinity solutions ranging from 0 ppt to ~15ppt increasing by individual ppt and standardized to 17°C (Bergey et al. 2003). This range of salinity solutions is representative of the differing estuarine conditions that serve as pre-spawning and larval nursery habitats. Specific gravity (a measure of density) of salinity solutions was verified using a refractometer before use (1.001 g/cm^3-1.013 g/cm^3). Each group of 10 eggs was gently placed in a test cylinder with an eyedropper, and the number of eggs that sank, floated, or remained neutral (no longer moving down the cylinder after a few seconds) were counted and recorded. The procedure was repeated for 13 groups of 10 randomly selected eggs held in ambient or USEPA control water.

Ovary biopsies and proteomic composition analyses

Ovary biopsy samples were obtained from gravid female Striped bass during 2018 and 2019 spawning seasons with a subset of 36 submitted for proteomics analysis (Table 2). Ovary biopsies were collected in accordance with Rees and Harrell 1990 procedures. Briefly, a 3mm diameter catheter was inserted 2-3 inches into the urogenital pore at an 30-45° angle, and mouth pipette suction was applied to draw egg samples into the catheter tube. The eggs were then divided via pipet and transferred to corresponding pre-labeled Nalgene 2.0ml cryogenic vials and recorded in data sheets. Egg samples for proteomics analysis were immediately placed on dry ice.

Samples were photographed to determine ovary stage (13-15 hour Bayless or post-vitellogenic stage oocytes are desired), transported on ice to North Carolina State University, and stored at -80°C prior to proteomics analysis (Reading et al. 2013, Williams et al. 2014b).

Mass spectrometry

The proteome is the final product of genomic expression and a link to cellular activities. Dr. Reading pioneered Striped bass “Omics” as part of the U.S. National Animal Genome Project (NRSP-8) providing publicly available transcriptome, proteome, and genome resources (Reading et al. 2012, 2013). With previous work on identification of a transcriptomic fingerprint for Striped bass, the next step is to examine a possible proteomic fingerprint. Therefore, ovarian proteomics is a subject of interest for continued understanding of egg quality.

The NC State University METRIC Core Facility analyzed prepared samples from each River in partnership with Dr. David Muddiman and Dr. Taufika Williams. Semi-quantitative non-targeted proteomics and absolute quantification of vitellogenin proteins

(VtgAa:VtgAb;VtgC) of ovary biopsies were implemented using tandem mass spectrometry (MS/MS) in triplicate. Stable isotope-labeled (SIL) surrogate peptides uniquely identifying their respective protein were synthesized by New England Peptide (Gardner, MA). The Striped bass genome (Reading et al. *in preparation*, GenBank: 10722), ovary transcriptome (Reading et al. 2012), and vitellogenin sequences (Williams et al. 2014a) were used as a reference for these unique peptides corresponding to each form of Vtg, as well as to perform the protein identifications from final MS/MS data. Unique peptides for each form of Vtg selected had 'heavy' forms that were added to the mass spectrometry as stable isotope-labeled versions to match with the 'light' naturally occurring forms. Sequences corresponding to human keratins and porcine trypsin were also included in the database.

For MS/MS preparation, ovary samples were thawed and diluted with Tris- buffered saline (20 mM Tris-HCL pH 8.0, 150 mM NaCl, and 2 mM $CaCl_2$) to a final protein concentration of 1 mg/mL. Protein concentrations of the digests were obtained using a Nanodrop at A280 (Thermo Scientific) as determined by Bradford assay (Bradford 1976). A modified filter-aided sample preparation (FASP) protocol (Schilling et al. 2014) was used to prepare ovary samples from each fish. Briefly, samples were incubated for 30 min at 56°C with 15 µL of 50 mM DTT per 200 µL (200 µg) of sample to reduce disulfide bonds. Proteins were denatured by adding 200 µL of 8 M urea in 0.1 M Tris- HCl pH 8.0 and then centrifuged at 14,000 × g for 15 min at 21°C and conducted in duplicate. Each sample was on-filter alkylated by adding 64 µL of 200 mM iodoacetamide (50 mM final) prepared in 8 M urea. Samples were incubated in the dark at 37°C for 1 hr and then centrifuged at 14,000 × g for 15 min at 21°C. Each filter was washed three times with 100 µL of 2 M urea/10 mM $CaCl_2$ by centrifugation for 10 min at 14,000 × g followed by three washes with 100 µL of 0.1 M Tris pH 7.5. Modified trypsin prepared in 0.1 M

Tris pH 7.5 was added to each sample at an enzyme-to-protein ratio of 1: 5. Following eight hours of digestion at 37°C, trypsinization was quenched with 50 µL of 0.001% zwittergent 3–16 (Calbiochem, La Jolla, CA)/1% formic acid and tryptic peptides were collected by centrifugation at 14,000 × g for 10 min at 21°C. A second quench/wash step was carried out to maximize tryptic peptide recovery. Samples were dried using a SpeedVac (Thermo Fisher Scientific, San Jose, CA) and stored at -20°C (Schilling et al. 2015).

Standards were subjected to direct infusion into a TSQ Quantum triple quadruple mass spectrometer (Thermo Fisher Scientific, San Jose, CA) at 50 µM to identify the most abundant charge state for each peptide precursor. The most abundant precursor of each standard was fragmented, and the 6 most abundant product ions of each precursor selected for further characterization. Collision energy optimization experiments were per- formed by optimization in the tune software, testing a range of collision energies and choosing the energy for which maximum signal intensity of each peptide was obtained.

Targets were analyzed using selected reaction monitoring mode. Instrument parameters included an argon collision gas pressure of 1.5 mTorr, a full width half maximum of 0.7 m/z for Q1 and Q3, a scan width of 0.01 m/z, a scan time of 0.050 sec/transition and a chrom filter peak width of 45 seconds. Data from targeted LC-MS/MS experiments were imported into Skyline v.2.5.0.6079 where reproducible co-elution of native and SIL peptides along with their respective transitions was used to confirm the presence of the target peptide. Data were exported into Excel, and peak area (area under the curve) ratios of native-to-SIL peptides were multiplied by the amount of each SIL peptide added during digestion to obtain absolute quantities in femtomoles (fmol) of target protein/µg of total protein (Schilling et al. 2015). The peak area value represents absolute quantification of a Vtg protein.

Another method of data analysis was based on normalized spectral counting procedures (Reading et al. 2013, Williams et al. 2014b, Schilling et al. 2014, 2015ab). Spectral count represents the total number of spectra identified for a protein to identify protein abundance. The spectral count for each protein divided by the protein length (# of amino acids) generated a spectral abundance factor (SAF) for that specific replicate. To account for trial variation, individual SAF values were normalized (NSAF) by dividing the SAF value by the sum of all SAFs for proteins in the complex. The NSAF values resulting from these analyses provide relative quantification of Vtgs. Raw MS/MS of data-dependent acquisition (DDA) and parallel reaction monitoring (PRM) files were downloaded with Proteome Discoverer (Thermo Scientific, San Jose, CA). PRM quantification is considered biased as it focuses on known amounts of internal standards. DDA is considered unbiased as it examines the top 40 most intense peaks to take a full picture of the spectrum. Sample preparation was the same for both methodologies and produced comparable results.

NSAF data were analyzed by analysis of variance (ANOVA) and The Tukey–Kramer Honestly Significant Difference (HSD) was used as a post hoc test for comparisons of Vtg-ratio by river using 0.05 as the nominal level of significance.

Machine learning analyses of ovary proteomics

Machine learning uses data 'training sets' and 'testing sets' to create algorithms for classification prediction using pattern recognition. Machine learning of "shotgun" protein discovery MS/MS data was used to identify proteins most predictive of differences between ovary biopsy samples collected from fish in the different rivers. Once the machine learning algorithms perform the pattern recognition, Information Theory is used to assign weights to each

variable (i.e., protein) in the dataset and those with the highest weights are then considered to be the most important factors in the pattern recognition predictions; those with lower weights are excluded from the model to reduce data dimensionality. The goal of these methodologies was an explorative analysis of all proteins identified with high confidence by the mass spectrometer. Using the program WEKA, the data were analyzed with three different machine learning classifiers: SMO (SVM), J48 decision tree, and Random forest decision tree. SVMs are non-probabilistic binary linear classifiers that predict patterns in which data points are modeled in space and separated into groups by the decision boundary maximum margin hyperplane. Decision trees build classification models in a tree structure using entropy calculations.

The two methods used for cross-validation (CV) were percent split and stratified fold-out (fold). The default percent split had 66% of instances used for training and 34% of instances for testing. The default for CV folds is 10. Three numerical summary outputs were used to compare model performances: percent correct classification, Kappa statistic, and area under the receiver operating characteristic curve (AUROC). The InformationrGainAttributeEval method (Information Theory) was used to reduce data dimensionality by eliminating less important (lower ranked) variables from the dataset.

The attributes with an information gain value of 0 were removed from the dataset first, then attributes with less than 10% contribution (infogain <0.1) were removed from the dataset next. The top 26 attributes were then used in negative control for learning. It is possible that the machine-learning model was able to correctly identify data based on random chance. Negative control procedure involved repeated randomizing of the sample designation and rerunning the model. The average % correctly classified, Kappa statistic, and AUROC values should be similar to values predicted by random assignment.

This data has three classes; therefore, the random probability is 33.3%. Using the reduced dataset with 26 attributes and SMO classifier, 10 negative control runs were completed. The 66% split CV runs resulted in an average of 44% correctly classified instances, 0.45 Kappa statistic, and 0.26 AUROC. The 10-fold CV runs resulted in an average of 53.125% correctly classified instances, 0.379 Kappa statistic, and 0.1889 AUROC. The percent correctly classified values are close to 30%, which indicates true learning has occurred.

Genetic analyses

Genetic differences between wild female Striped bass captured from four rivers with estuaries that vary in water salinity and a freshwater reservoir were determined in partnership with the NC WRC and SC DNR (2010-2018 reports). Information from these studies provided a possible mechanism for lack of Striped bass recruitment in major river drainages in North Carolina. For example, if female Striped bass are genetically adapted to spawn in a particular river system based on the salinity of a natal estuary, then their female offspring may return there to spawn as adults and produce eggs tailored to that salinity dynamic. Stocking fish in rivers with estuaries of a different salinity than natal origin may lead to recruitment failure.

Fin clip samples were collected from all wild-caught Striped bass in various river systems throughout 2018 and 2019 spawning seasons. Fin clip samples were collected using forceps and surgical scissors. Samples were stored in centrifuge tubes with 95% ethanol. Parentage based genetic tagging (hatchery origin) was conducted in conjunction with NC WRC and Dr. Tonya Dardin at South Carolina Department of Natural Resources (SC DNR). Samples were genotyped at 12 loci to reconstruct pedigrees in collaboration with the SC DNR (Hollings Marine Laboratory). These pedigrees were used to evaluate the population structure of Striped bass

captured from the different water systems and to assess genetic relatedness that may underlie physiological adaptations (NCRWRC 2020, LeBlanc et al. 2020, Wirgin et al. 2020)

Domestic Striped bass experiment

It is unclear whether the adaptation of egg buoyancy observed in Striped bass is a fixed genetic effect or a plastic physiological response. In order to confirm the physiological adaptation of Striped bass to the differing water salinity, domestic Striped bass reared in captivity at low and high salinities were used as an experimental control group for our observations of the wild caught fish. Using genetically similar domestic fish in these captive studies allowed us to isolate the effect of environmental conditions on egg quality aside from genetic adaptation. The focus of these experiments was the influence of salinity on egg buoyancy and other characteristics described above. Although North Carolina coastal Rivers vary in many environmental parameters, using these controlled aquaculture rearing conditions at particular water salinity eliminated the potential effect of genetics and other parameters that may vary year-to-year in the wild systems.

Domesticated Striped bass were reared at the North Carolina State University Pamlico Aquaculture Field Laboratory (NC State PAFL) as part of the National Program for Genetic Improvement and Selective Breeding for the Hybrid Striped bass Industry for 8 generations. These Striped bass broodstock were developed from 400 crosses of 6 distinct stocks (Canadian, Hudson River, Roanoke River, Chesapeake Bay, Santee-Cooper Reservoir, Florida Gulf of Mexico) that were collapsed into a homogenous domestic stock. The Striped bass breeding program is spread across 4 year-classes, and typically consists of crosses of 150-200 females with 100-200 males (Reading et al. 2017, 2011)

Sexually mature, age-4 domestic female Striped bass were reared in outdoor flow through pools (24 ft diameter; 10,153 gallons; flow rate 20 gallons per minute) under a natural photothermal cycle at the NC State PAFL. Female fish were fed Bass Brood (13.0 mm Bass Brood, 45% crude protein and 15% crude fat, Zeigler Bros.) during the period of vitellogenic oocyte growth (early October through spawning in April-May). PAFL is equipped with pumps that draw surface brackish water from South Creek and well water from fresh and salt aquifers (Castle-Hayne aquifer) to maintain desired water salinities.

Females were randomly sorted into two groups (N = 15 fish each) that were reared at higher (20 ppt) or lower (0-1 ppt) water salinities during vitellogenisis beginning in August 2018 and ending in April/May 2019 when the fish spawn. This simulated Cape Fear River conditions (water salinity 8-12 ppt) and Roanoke River conditions (water salinity < 0.5 ppt) (Tables 3,4). Water temperature data was collected from each tank with a HOBO Pendant Temperature Data Logger (Onset Computer Corporation) and salinity was measured daily using an YSI 63 multi-meter. Fish (N > 3 from each water treatment) were sedated with MS-222 and weight (kg) and total length (mm) of the fish were recorded.

Female fish were then warmed to spawning temperature (approximately 18°C at 0.5-1°C per day) and photoperiod increased to 12:12 to induce final ovarian maturation. Females from each group were initially spawned using batch procedures first described in 2016 to spawn fish en masse as the success rate is much better overall and the number of harvested eggs is greater than compared to the small individual female tank spawns (Reading et al. 2015, 2018). Additionally, the methods do not rely on the use of hCG to induce ovulation and thus are more relevant to “natural spawning” circumstances. Fifteen females from each group along with 15 males were placed in separate 32,176 L replicate tanks equipped with dual 200 L upwelling egg

collectors and allowed to volitionally spawn undisturbed. We have determined the minimum number of fish required for each group-spawning unit to be 10 of each gender (Presented by Reading et al. 2015, Reading et al. 2018).

After a period of three days, the fish were sorted, and any spawned females were identified and removed. Unspent female fish were anesthetized with MS-222 and subjected to ovary biopsy (Rees and Harrell 1990). Females identified as stages 13 hour to 10 hour (or better) were injected with 330 IU/kg hCG; Chorulon. One female and 3 spermiating male Striped bass were placed into 635-gallon tanks (6 ft diameter) and allowed to volitionally spawn undisturbed; 10 such spawning trials were conducted for each salinity group. This approach provides for natural spawning data as well as replicated individual tank spawning trials.

Eggs from each spawn were harvested from upwelling incubators and enumerated (settled egg volumes were measured for each of the spawns and the total number of eggs/mL recorded and used to estimate the total fecundity of each female fish). Fertilized eggs were scored for viability based on symmetry and according to normal progression of development (Rees and Harrell, 1990). These parameters were used to determine spawn fertility at 4 hours post-fertilization as most Striped bass embryos experience mortality prior to the blastula stage of development (Chapman et al. 2014). Percent hatch and fry survival (3 days post-hatch) were recorded. Egg and oil globule diameters were recorded (10 eggs per female spawned). Fertilized and water hardened eggs were tested for buoyancy as described in earlier methods.

Statistical analyses

Statistical methods used throughout this project focused on ANOVA, Tukey HSD, and Kruskall-Wallis tests with a $p<0.05$. Omnibus ANOVA test was used to identify outliers and

determine if the Kruskall-Wallis was a more appropriate test. Kruskall-Wallis is a rank-based test that accounts for outliers rather than removing them from the dataset for analysis. Post-hoc Tukey HSD tests were used to compare the means of every treatment to the means of every other treatment for pair-wise analyses. Figures were labeled with letters to represent significant differences such that the same letter designation supports the equal means hypothesis while different letters represent statistically significant differences in the datasets. All analyses were conducted on the program R (R Core Team 2013).

RESULTS

Egg buoyancy and size measurements

A total of 22 and 39 female Striped bass spawns from 2018 and 2019 were sampled from broodstock collected in each NC coastal river and one freshwater reservoir in SC as shown in Table 1.

Differences in egg buoyancy were seen between rivers (ANOVA, $\alpha = 0.05$, $p < 0.001$), but no differences between sampling years (ANOVA, $\alpha = 0.05$, $p = 0.656$) (Figure 2). Santee-Cooper (freshwater control), Dan (low salinity), Roanoke (low salinity), and Cape Fear (high salinity) eggs were similar eggs the most buoyant. Eggs from the Tar and Neuse Rivers had intermediate buoyancy eggs. There were few significant differences in buoyancy between stage 1 (grey boxes) and stage 4 (white boxes) developmental stages (Figure 2).

Differences in egg oil globule diameter were observed between rivers (ANOVA, $\alpha = 0.05$, $p < 0.001$; Figure 3). Differences in egg oil globule were also seen between years for stage 1 (ANOVA, $\alpha = 0.05$, $p < 0.001$), but were similar between years for stage 4 embryos (ANOVA, $\alpha = 0.05$, $p = 0.898$; Figure 3). Lower salinity systems tended to demonstrate larger oil globule diameters than higher salinity systems. There were also significant differences in size between eggs at stages 1 and 4. The Roanoke and Cape Fear River oil globule sizes decreased during development, Tar and Neuse Rivers remained similar, and the Santee-Cooper Reservoir increased in relative size throughout development. The Cape Fear River eggs showed high oil globule size variability.

The outer egg chorion diameter data combined from both years (Year 1 and Year 2) of wild caught fish is shown in Figure 4. The results showed significant differences in outer egg diameter between rivers (ANOVA, $\alpha = 0.05$, $p < 0.001$), but no significant differences between years (ANOVA, $\alpha = 0.05$, $p=0.05339$). There was high variability, with few rivers having any

statistical overlap. However, trends observed indicated that the Roanoke River and Santee-Cooper Reservoir (freshwater systems) diameter sizes were larger and similar to the Cape Fear River (high salinity system). The Tar and Neuse Rivers (intermediate salinity systems) were similar and smaller than other systems.

The yolk volume measurements proved to be slightly less straightforward and showed high individual variability. Differences were observed between rivers (2018, 2019) for stage 1 yolk volume (Kruskal-Wallis, $p < 0.001$) (Figure 5). There were no significant differences between the Cape Fear and Roanoke Rivers (Bonferonni, $p = 0.1385$). There were no significant differences between the Neuse and Tar Rivers (Bonferonni, $p = 0.0839$). The trend showed that the Cape Fear and Roanoke River yolks were slightly larger. There were no significant differences between the Dan and Tar Rivers, as well as the Dan and Neuse Rivers (Bonferonni, $p = 1$), however, the sample size for the Dan River was extremely small (2 spawns) and not considered essential to this aspect of the project.

Significant differences were observed between rivers (2018, 2019) for stage 4 yolk volume (Kruskal-Wallis, $p < 0.001$). There were no significant differences between the Santee-Cooper and Cape Fear, and Santee-Cooper and Neuse Rivers (Bonferonni, $p = 1$). Yolk measurements were highly variable with many outliers throughout both years of sampling.

Vitellogenin ratios

36 ovary biopsy samples from 2018 wild caught Striped bass were submitted to the METRIC core facility at NC State University for semi-quantitative proteomics analysis (Table 2).

Based upon results of NSAFs (relative quantification) there were significant differences between VtgAa concentrations from the Cape Fear, Neuse and Roanoke Rivers (Kruskal-Wallis, $p < 0.001$). Cape Fear had significantly less VtgAa than the Roanoke River (Bonferroni, $p < 0.001$). The Cape Fear River high salinity system was not significantly different from the Neuse River intermediate salinity system (Bonferroni, $p > 0.05$). The Roanoke River freshwater system was not significantly different from the Neuse River Bonferroni, $p > 0.05$). This showed a trend of decreasing VtgAa yolk content with increasing environmental salinity (Figure 9).

There were significant differences between VtgAb concentrations from the Cape Fear, Neuse and Roanoke Rivers (Kruskal-Wallis, $p < 0.05$). Cape Fear had significantly more VtgAb than the Roanoke River (Bonferroni, $p < 0.05$). The Cape Fear River high salinity system was not significantly different from the Neuse River intermediate salinity system (Bonferroni, $p > 0.05$). The Roanoke River freshwater system was not significantly different from the Neuse River (Bonferroni, $p > 0.05$). This showed a trend of increasing VtgAb yolk content with increasing environmental salinity (Figure 9).

There were no significant differences between VtgC concentrations from the Cape Fear, Neuse and Roanoke Rivers (ANOVA, $p > 0.05$). There were no trends observed for differences in VtgC content (Figure 9).

Results from area under the curve (absolute quantification) analyses showed the Roanoke River had a 16:57:1 proportion of VtgAa:Ab:C. The average proportion for the Neuse River was 17.3:61:1. The average proportion for the Cape Fear River was 16.3:64:1. A method comparison of area under the curve and NSAF quantification showed highly similar detection rates.

Machine learning analyses of ovary proteomics

The goal of machine learning analyses was to identify patterns within the entire protein dataset. We aimed to find proteins specific to each river system which could provide potential differences related to internal mechanisms in the ovary. There were 134 river samples suitable for analysis based on the original 36 submitted and their replicates. Machine learning software WEKA was trained and asked to identify what river the sample originated from based on the composition of proteins using three algorithms (SMO, J48, Random Forest).

Rank analysis using information gain was performed on the full dataset in order to reduce the size of the dataset and focus on those particular proteins which provided the most information. The information gain values determined those protein attributes that were removed from subsequent models. Beginning with all 2424 protein attributes that were identified by the mass spectrometer with high confidence present in the dataset, the three different classifier methods were run. The best performing model used SMO and fold cross validation, with 91% correctly classified instances, 0.83 kappa statistic, and 0.80 AUROC (Table 5). Following the analyses of the full dataset, attributes with an information gain value of 0 were removed from the dataset resulting in a new dataset with 533 top ranked attributes. Analyses were run on the reduced dataset and rank analysis was run again. Attributes with an information gain of 0 were removed again resulting in a new dataset with 297 attributes. Attributes with an information gain value less than 0.1 were removed next resulting in a dataset with 173 attributes. Further removal of attributes with information gain less than 0.2 resulted in a dataset of 26 attributes. The optimal model resulted in 173 top ranked protein attributes of interest: 1 attribute with an information gain value of 0.34, 2 with an information gain ~0.3, 8 with >0.2, 162 with >0.1. The best

performing model on this dataset used SMO and fold cross validation with 96% correctly classified instances, 0.93 kappa statistic, and 0.96 AUROC (Table 5).

The top 5 ranked protein attributes indicative of river origin for Cape Fear (high salinity) or Roanoke (low salinity) were: nqo1 (7 of 10) protein AED:0.06 eAED:0.06 QI:53, rps2 protein AED:0.03 eAED:0.03 QI:7, rps19 (2 of 2) protein AED:0.04 eAED:0.04 QI:0, rpl3 protein AED:0.18 eAED:0.18 QI:0, NACA protein AED:0.01 eAED:0.01 QI:151. These are primarily ribosomal proteins involved in a variety of processes. Most notably, the NACA (nascent polypeptide associated complex subunit alpha) attribute is related to protein transport and DNA-binding.

Machine learning analyses of 2424 Striped bass ovarian proteins resulted in several successful models. The optimal models used SMO or Random forest classifiers (Table 5). Negative control indicated true learning occurred. Specific proteins indicative of River origin were identified.

Genetic analyses

All wild-caught Striped bass resulting from 2018 spawning season sampled from the Cape Fear, Tar, Neuse, and Roanoke Rivers were genotyped in partnership with the NCWRC and SC DNR Hollings Marine Laboratory. Results indicated that out of a total of 607 wild-caught Striped bass 427 were identified as hatchery origin. All Striped bass collected for this study from the low salinity Roanoke River were of wild origin (NCWRC 2018).

Fish sampled for this study from the high salinity Cape Fear River were included among the 92.4% of fish originating from hatcheries, with 25.3% of that contribution coming from the 2013-cultured year class and the remaining contributions represented by 2010 and 2014 year

classes (NCWRC 2018). Among the 16 females sub-sampled for this study 100% were of hatchery origin.

The total NCWRC collection of Neuse River samples resulted in 77.6% hatchery contribution, which was similar to previous years (2014, 2015, 2016). Striped bass sampled that were designated as "wild" match size classes of previous cultured year classes but may provide minimal evidence for some natural recruitment. The strongest contribution came from the 2015-cultured year class (NCWRC 2018). The 12 female-only sample sub-set for this project resulted in 9 hatchery fish and 3 designated as wild and represented ages 3 through 8.

The complete set of 2018 Tar River samples resulted in 40.5% hatchery contribution to the system, which decreased from 2015 (93.3%) and 2016 (87.7%) (NCWRC 2018). The female-only subset for this project represented 7 females with 100% hatchery origin.

Domestic Striped bass experiment

The average weight and total length for females in each group were comparable: 4kg each for both, and 642 mm and 647 mm for high salinity and low salinity groups, respectively. High salinity group spawning run resulted in 1 female spawn, while the individual tank spawns resulted in 7 female spawns. Low salinity group spawn resulted in 0 spawns, while individual tank spawns resulted in 9 female spawns. The high salinity group had a total of 61.5% of 13 females spawn versus 81.8% of 11 females spawned in the low salinity group. Average fertility was similar for high and low salinity treatments, 6.1% and 6.4% respectively. Average percent of eggs producing fry was similar for high and low salinity treatments, 4.1% and 4.7% respectively. The average egg volume produced per spawn for high salinity treatment was 1687.5 ml compared to the average egg volume for the low salinity group of 4172.2 ml. The total number

of fry produced from the high salt group was ~150,268 and the total number of fry produced from the low salt group was ~271,643.

The domestic Striped bass conditioned for spawning in high and low salinity water at the Pamlico Aquaculture Field Laboratory exhibited differences in egg characteristics. The egg oil globule measurements showed no significant differences between water salinity treatments (ANOVA, $\alpha = 0.05$ $p = 0.65$), but there were significant differences between embryo stages 1 and 4 (ANOVA, $\alpha = 0.05$, $p < 0.001$) (Figure 6). The egg outer diameter from domestic Striped bass held in either high salinity or freshwater conditions showed no significant differences between embryo stages 1 and 4 (ANOVA, $\alpha = 0.05$, $p = 0.1428$), but there were significant differences between salinity treatments (ANOVA, $\alpha = 0.05$, $p < 0.001$) (Figure 7). Egg buoyancy measurements (g/cm3) from domestic Striped bass held in either high salinity or freshwater conditions showed no significant differences between stages or treatments (ANOVA, $\alpha = 0.05$, $p = 0.25$, $p = 0.46$) (Figure 8). There appeared to be a trend, however for the eggs spawned from the fish conditioned in high salinity to have a greater density.

DISCUSSION

This research was focused on three objectives; 1) determining genetic origin of females and physical characteristics of fertilized eggs from wild-caught Striped bass collected from a diversity of staging habitats and salinity conditions; 2) evaluating the proteomic composition of eggs prior to spawning from wild-caught Striped bass to ascertain the underlying biological mechanisms responsible for fertilized-egg characteristics; and 3) validating observational results by experimentally evaluating the influence of salinity during staging on the physical characteristics of fertilized eggs through the use of a domesticated Striped bass population of similar genetic stock held at differing salinities during simulated staging durations.

The results largely supported the pattern that fertilized Striped bass eggs from lower salinity and freshwater systems had larger and more buoyant compared to eggs from higher salinity systems with the exception of the Cape Fear River. Egg size and buoyancy fell in line with that to be predicted from environmental salinity for the Roanoke, Tar and Neuse Rivers. The eggs appear to be correctly adapted for their specific river systems and may support natural recruitment in the Roanoke River. This observation suggests that failure of recruitment in the Tar and Neuse Rivers may be due to other causes as improper egg yolk formation was not noted here.

Figure 4 shows that fertilized Striped bass eggs from lower salinity systems are larger than those from intermediate salinity systems. It is apparent that there is a trend in larger egg size characteristics of the Striped bass sampled from the lower salinity systems such as the Roanoke River (0-0.5 ppt) and Santee Cooper reservoir (0 ppt) as compared to rivers with intermediate salinity (0.5-5 ppt) such as the Tar and Neuse Rivers. The same trend is observed with egg buoyancy; fertilized eggs from the lower salinity systems are more buoyant than those from the intermediate salinity environments (Figure 2).

Very few statistical differences between years for all egg measurements indicate that the egg characteristics are generally consistent within river year to year among the wild captured fish. Links between physical characteristics and buoyancy suggest larger outer diameter occurs in more buoyant eggs. Diameter changes could be due to hydration effects related to egg yolk processing and absorption during ontogeny.

A previous study by Bergey et al. 2003, showed that the fertilized eggs of Striped bass females captured from higher physical energy watersheds (inland, lower salinity) have been shown to be denser than the eggs of females captured from lower energy (coastal, higher salinity) watersheds. Buoyancy results from this project did not support those of this previous study by Bergey et al. perhaps due to slight differences in methodologies. In the present study care was taken to only evaluate the buoyancy of viable fertilized eggs after the water hardening process was complete. As this methodology was not completely detailed in Bergey et al. these authors may have inadvertently included non-viable eggs in measures of buoyancy. In the present study, buoyancy of eggs was evaluated by physically sorting and separating the eggs into water-hardened, non-water hardened, and mixed groups. The average buoyancy of these groups differed. Non-water hardened eggs will always sink to the bottom of the tube regardless of water salinity, and mixed eggs may produce inconsistent results, such as those reported in Bergey et al. for the Roanoke River. The results presented here in Figures 2 and 4 indicate that the Roanoke River, considered a high-energy watershed, produced lighter and larger Striped bass eggs.

The Cape Fear River Striped bass had significantly larger eggs (ANOVA, $p < 0.05$, Figure 4) compared to the eggs of Striped bass from the intermediate salinity rivers (Neuse, Tar). For example, the average size of Cape Fear River Striped bass eggs (stage 1 and stage 4) was 2.42+0.26 mm standard deviation in 2018 (Year 1) and 2.51+0.25 mm standard deviation in

2019 (Year 2) compared to 2.01+0.08 mm from combined Tar and Neuse. The Cape Fear River Striped bass eggs were slightly smaller than the Roanoke River, but comparable in size to the no salinity Santee-Cooper system. The Cape Fear River Striped bass also had more buoyant eggs similar to the Striped bass in the Roanoke and Santee-Cooper despite their differences in environmental salinity (Figure 2). We would expect that the buoyancy would follow trends of increasing density along with increasing salinity conditions, which was observed for all river systems except the Cape Fear River.

One explanation for these results concerns the specific gravity of the fertilized eggs. Specific gravity refers to the ratio of the density of a liquid to a solid. This follows that the eggs will be ovulated and tailored to specific water salinity so that they float appropriately in the water column (Figure 2). Freshwater is less dense than saltwater which is supported by the observation that the control group (Santee-Cooper Reservoir) fish reared completely in freshwater had the eggs with the lowest specific gravity, Tar and Neuse River fish had eggs that were more dense and had higher specific gravity than Roanoke River fish, as the Albemarle Sound has lower salinity compared to the Pamlico Sound where the females stage their eggs. However, the fertilized eggs from the Cape Fear River had a lower specific gravity than expected based on the salinity of the Cape Fear River system. The specific gravity of eggs from the Cape Fear River fish were predicted to have a specific gravity of about 1.009 g/cm^3, which is based on the salinity of the mouth of the Cape Fear River (10-12 ppt) and also possible incursion into the freshwater system from the Atlantic Ocean. The Striped bass eggs from the Cape Fear River had an average specific gravity of 1.0045 g/cm^3, which would have neutral buoyancy in 4-6 ppt salinity similar to the intermediate Tar and Neuse rivers. The Cape Fear River Striped bass had significantly larger eggs (ANOVA, $p < 0.05$) compared to the eggs of Striped bass from the Neuse, Tar, Dan,

and Santee-Cooper systems. (Figure 4). One explanation is that the Cape Fear River Striped bass have undergone some physiological compensation (response) to spawning the eggs under increased salinity such that the Cape Fear River fertilized eggs appear more similar to wild type eggs found in lower salinity systems such as the Roanoke River.

One potential explanation for this observation is that the eggs of the Cape Fear River Striped bass form their yolks in preparation for buoyancy adaptation at the higher estuarine salinity but undergo hydration effects that increase the outer diameter. The yolk protein composition accounts for the densest part of the egg, and if formed correctly would imply that the incorrect buoyancy of Cape Fear eggs may be due to other factors. The differences between VtgAa concentrations from the Cape Fear, Neuse and Roanoke Rivers showed a trend of decreasing VtgAa yolk content with increasing environmental salinity. This falls in line with expectations for freshwater systems as increasing amounts of VtgAa broken down into FAAs provide the egg with more lift in the form of hydration.

Differences between VtgAb concentrations from the Cape Fear, Neuse and Roanoke Rivers showed a trend of increasing VtgAb yolk content with increasing environmental salinity. This would be consistent with predictions based on the prevailing salinity of these river systems and the evaluated egg buoyancy characteristics, with the exception of the Cape Fear River fish. There were no trends observed for differences in VtgC content. These results confirmed differences in vitellogenin proportions of fish of the same species acquired from waters of different salinities and confirms the previous study by Schilling et al. 2015 examining a closely related species of White Perch (*Morone americana)*.

The Cape Fear River eggs are of specific interest because their buoyancy and size fell in line with that of Freshwater system fish which was unexpected. However, their vitellogenin egg

yolk systems did fall in line with what was expected based on previous literature. Cape Fear River fish could have buoyancy issues because although they have the correct ratios of Vtgs they are not processing it correctly (aka cleavage). The amount of VtgAa cleaved may be altered due to environmental hydration. It appears that they can control the rate of hydrolysis, but in the last couple hours of development may not be processing it correctly. Very little is known about this process at this time and should be considered as a future direction for study.

Since Lock and Dam 1 on the Cape Fear River essentially traps the Striped bass at this location during the spawning run, it may be that the females complete ovarian maturation at that location after a very short spawning run (swimming distance). The Striped bass do not appear to use the rock arch ramp as a passage upstream as not many broodfish are collected upstream of Lock and Dan 1 (Jeff Evans, personal communication). The Striped bass eggs may be designed to float down river until they reach the salt wedge of the estuary. As such, the eggs may take up additional "metabolic" water during oocyte hydration and/or water hardening in preparation for eventually flowing into a relatively high salinity estuary located a short distance (32 miles) from the only available spawning area (Lock 1). This hydration would explain the significantly larger egg size and the increase in egg buoyancy, as the uptake of water would make the eggs swell and additionally reduce their densities. Such a compensatory mechanism would be required for the eggs to survive dehydration in the estuary, as they otherwise cannot osmoregulate at this time, however possibly at the cost of providing adequate buoyancy as observed. Additional information is necessary to validate this hypothesis.

Also, understanding flow rate of the river also would be useful. For example, if eggs are spawned at Lock and Dam 1, how long does it take them to flow downstream and eventually reach the Atlantic Ocean? If flow rate were such that the eggs would reach the mouth of the river

within 48 hours or less, then the eggs would be exposed to those salinities. Conversely, if they reach the mouth of the river within 72 hours, then the recently hatched fry would similarly be exposed to those same salinities, as they lack strong swimming capabilities until they are at least 3 or more days post-hatch. Since the Lock and Dam 1 is located only 32 miles upriver from Wilmington, NC, this would indicate that eggs carried in a flow rate of even 1.3 miles per hour (1.9 foot per second) would allow them to reach the mouth of the river before hatching. Thus, habitat modification and regulation of flow on the Cape Fear River may be a problem with recruitment, however the additional data collected on this project needs to be analyzed before such contention should be supported.

One potential management strategy that may be considered is to physically move gravid, captured female and male broodfish upstream of Lock 1. This could be performed alongside the annual Striped bass stock assessment on the Cape Fear River during the spring and when they are collected for spawning to produce fingerlings for supplemental stocking. Instead of releasing the fish back into the water below Lock 1, they could be transported and released into the water upstream of Lock 1 after length and NC DMF and NC WRC collect weight data. This may be advantageous and allow the fish to spawn in a more favorable location and allow the eggs to hatch in transit to a more favorable nursery.

Tar and Neuse River Striped bass eggs, along with Roanoke River, appear normal and follow the original trend of salinity impacts on egg buoyancy and size (Figures 2, 3,4,5). Fish from these rivers produced eggs with specific gravity of 1.006 g/cm^3, which are designed to be neutrally buoyant in 8-9 ppt water. This is closely comparable to the Pamlico Sound estuary salinity (0.5-5 ppt) and therefore the eggs appear to be fairly suitable for this spawning area and nursery. It does not appear that any reproductive dysfunction is related to egg characteristics that

have already been assessed in the Tar and Neuse River Striped bass that may be related to recruitment failure, unlike the Cape Fear River. In this regard, Striped bass recruitment failure in the Tar and Neuse Rivers may be related spawning stock biomass and natural mortality rates. Egg characteristics of the Tar and Neuse Striped bass are relatively suitable for the system but there are other life stages that are vulnerable to recruitment failure. The Tar River is subject to erratic spring flows and habitat modification. High juvenile Striped bass mortality has been reported in the Neuse River (Bradley et al. 2018), which may impact year class recruitment strength along with overfishing and reduction of spawning biomass since about 2007 (Rachels and Ricks 2018). Bradley et al. (2018) suggest that if larger Striped bass are disproportionately targeted in the fishery, then the negative effects of exploitation may impact spawning success. Recently, a moratorium of 2 years was passed on both recreational and commercial fishing for Striped bass in the Tar-Pam system with the goal of increasing spawning stock biomass for future production. Thus, the failure of recruitment in these rivers may be due to overfishing, whereas the Cape Fear River population may have recruitment failure due to insufficient spawning territory or adequate nursery habitat as identified here through egg characteristics.

The results from objective 1 genetic analysis did not support our original hypothesis that genetic differences of Striped bass among rivers in North Carolina of lower and higher estuarine salinities may result in recruitment variation due to broodstock origin. The majority of Striped bass identified from the Tar, Neuse, and Cape Fear Rivers were hatchery origin (NCWRC 2018) and mean admixture coefficients of Striped bass collected at 15 sites to 6 genetic clusters, calculated using 1,256 SNP loci showed few differences in North Carolina Coastal populations (LeBlanc et al. in review 2020). Although these rivers may have once supported distinct genetic populations, recent evidence suggests there is little differentiation. Specifically, the Cape Fear

River and Roanoke River Striped bass are genetically indistinguishable (LeBlanc et al. in review 2020). The observed differences in egg sizes and buoyancy thus suggest adaptation to environmental conditions regarding egg quality or other parameters rather than genetic adaptations to the Rivers. This result was further supported by experiments using genetically similar domestic Striped bass that similarly exhibited differences in egg characteristics based on the salinity of the rearing conditions.

Another study recently published (Wirgin et al. 2020) shows genetic divergence of Roanoke River fish from that of the Santee-Cooper Reservoir and other Atlantic Striped bass populations. Generally, these findings are consistent with LeBlanc et al. and Wirgin et al. that indicate mid-Atlantic populations of Striped bass including the Roanoke River are highly migratory and exhibit much lower levels of lower genetic divergence. For example, individuals may be captured as far north as the Bay of Fundy, Canada. This migratory behavior would allow for gene flow of Roanoke River fish into other northern Striped bass populations and this has been shown in both studies (Wirgin et al. 2020, LeBlanc et al. in review 2020). Therefore, the influence of genetic origin on differences observed in fertilized egg characteristics is minimal. Although the exact causes of the differences were undetermined, it is the hypothesis of objective 2 that vitellogenin-derived egg yolk content plays a key role.

Machine learning analysis of the complete proteome identified from Striped bass ovary biopsies revealed that there are other proteins indicative of river origin besides Vtgs. The differences between the Cape Fear and Roanoke Rivers were primarily a collection of ribosomal protein coding genes with varying functions. The results from machine learning provides a framework for future studies regarding the complex internal mechanisms of yolk formation to consider besides the most prevalent and well-studied Vtgs. None of these proteins indicate an

obvious effect of water pollution, for example, on the proteomes of fish from either the Cape Fear or Roanoke River. Ribosomal proteins are generally involved in protein translation and therefore have a wide variety of functions, which makes it not possible to pinpoint an exact proximal cause of this difference observed here. Further research will be required to fully understand this.

The third objective was to experimentally evaluate the observed results from wild-caught Striped bass. We anticipated that fertilized egg characteristics from domestic Striped bass would show differences between water treatments similar to that observed in the wild. Results largely supported this hypothesis; thus, the overall conclusions are that Striped bass are able to adapt to different environmental salinities with no genetic influence.

General spawning data such as average fertility, egg volume, and fry produced supported that the fish spawned in low salinity conditions performed slightly better than those in the high salinity group, however this was not statistically significant. Both groups had similar numbers of female spawns and these females were all comparable in body size and condition, however, differences in egg characteristics were observed.

Results observed for oil globule of fish reared in high salinity versus freshwater indicated two points: Firstly, the egg lipids were not different between treatment groups, suggesting that any differences in spawned egg buoyancy may not be regulated by lipid deposition as is suggested for the buoyant Chesapeake Bay Striped bass eggs. Second, the egg lipid content appears to change as the embryo develops from stage 1 to 4 and this is to be expected. The observed increase in oil globule size is perhaps an artifact of the absorption of the protein yolk (i.e., vitellogenin yolk proteins) and subsequent expansion of the lipid droplet. This likely does not reflect an actual increase in lipid content and may be due to the nature of the lipid as stored.

The vitellogenin yolk proteins are typically utilized early during embryonic development, compared to the oil globule, which is consumed later by hatched larvae (Schilling et al. 2015, Williams et al. 2014a, b).

Results from the egg outer diameter of domestic Striped bass held in either high salinity or freshwater conditions suggests that the fish conditioned in high salinity produced significantly smaller eggs with less water content (i.e., denser) in accordance to our initial hypothesis. Further, it supports the notion that the fish can adapt or respond to environmental salinity, as the Striped bass used in this aspect of the study were genetically similar. Results from the egg buoyancy of domestic Striped bass held in either high salinity or freshwater conditions were in agreement with the outer egg diameter observation. Thus, Striped bass conditioned for spawning in high salinity water produced eggs that were significantly smaller (and hence have less water content) and have a trend to be more dense, which would better match the specific gravity of the water in which they are being spawned (e.g., they are designed for neutral buoyancy).

Domestic Striped bass experiments indicated that egg characteristics, such as, size and buoyancy were influenced by environmental salinity. Genetic similarities between wild-caught Striped bass from varying systems also supports that differences in egg characteristics is more likely a physiological response to environmental conditions as opposed to genetic adaptations. Overall, the combination of wild-caught and domestic Striped bass egg characteristics provides a wide-ranging picture of key components affecting egg quality and potential natural recruitment.

The goal of this study was to understand the influence of genetics and environment on egg characteristics and whether specific adaptations are suited to different salinities. Eggs are the most vulnerable life stage contributing to recruitment and this study was the first step to understanding observed recruitment failure of Striped bass stocks in some NC rivers. We have

shown that egg characteristics are influenced by environmental salinity and that the vitellogenin egg yolk system is adaptable. This is the first demonstration of plasticity in primary yolk protein ratios of fish. Our observed results were further confirmed by an experimental evaluation of Striped bass. This examination of early life development can provide valuable information to management agencies for the continued study of Striped bass.

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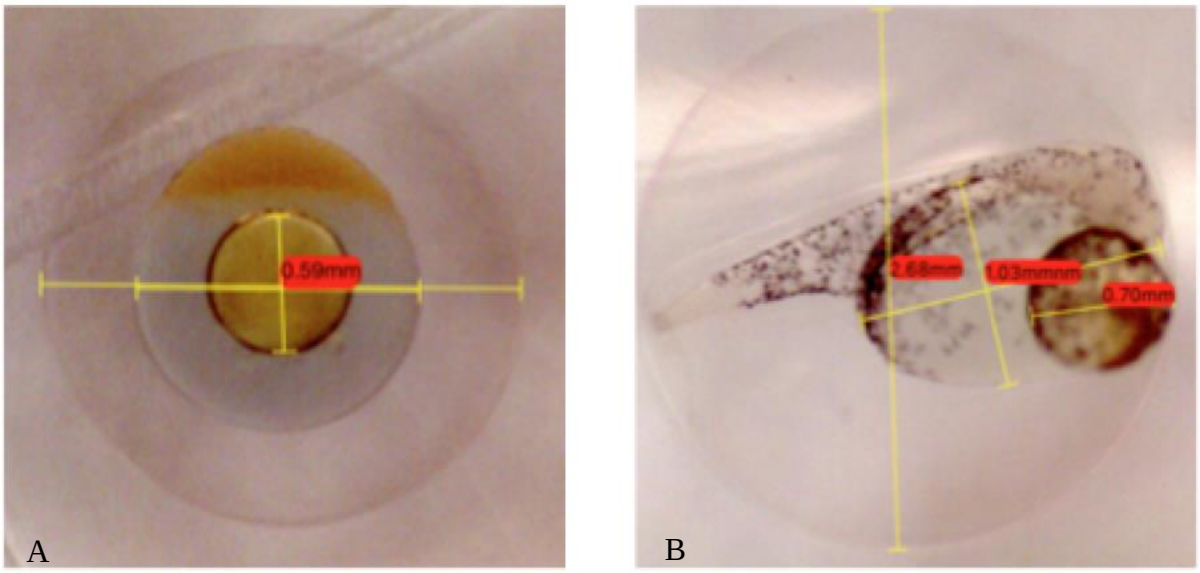

Figure 1. Representative images of a stage 1 fertilized Striped bass egg (*A*) and a stage 4 embryo (*B*) from Watha State fish hatchery, which were measured and tested for buoyancy. Yellow lines indicate measurement procedure using ImageJ software.

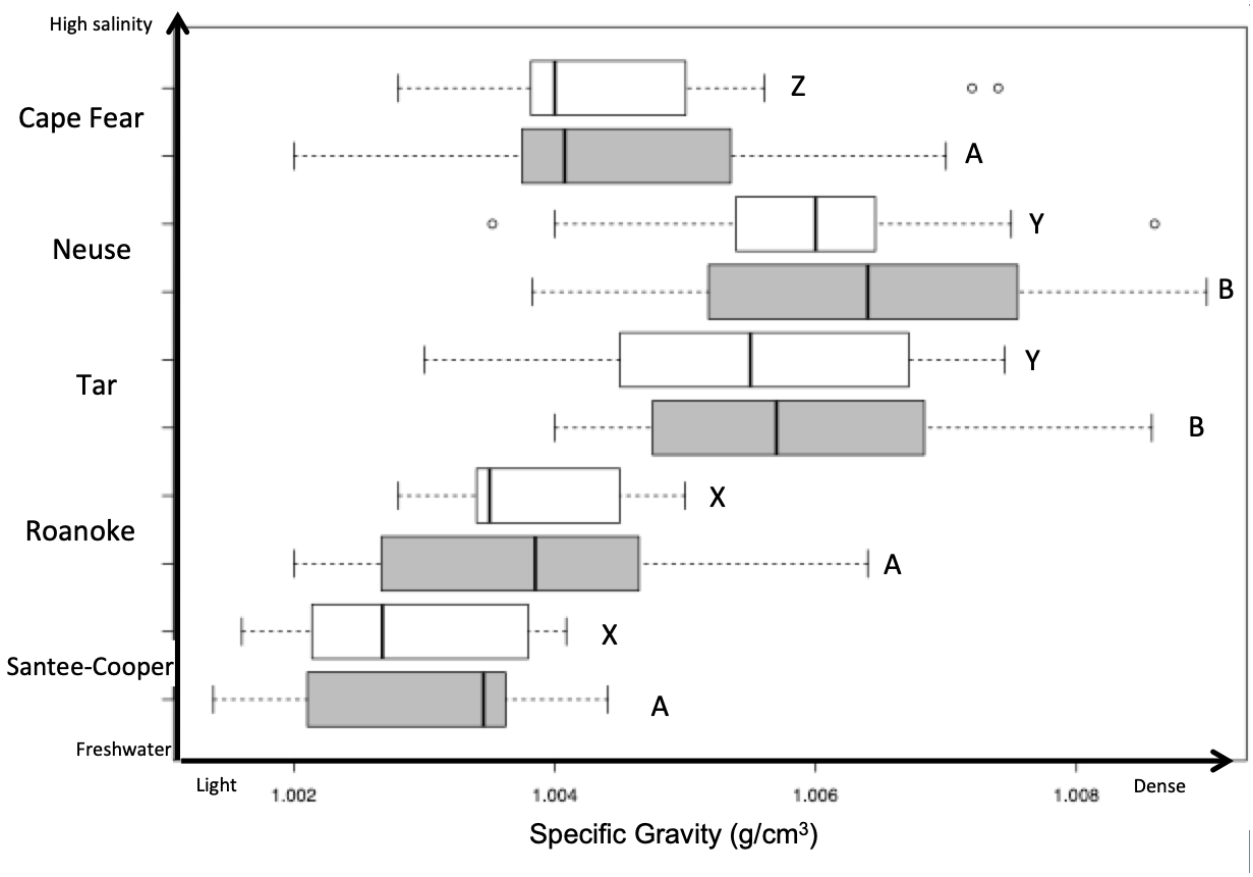

Figure 2. Buoyancy (g/cm3) boxplot of stage-1 (gray boxes) and stage-4 (white boxes) fertilized eggs from wild-caught broodstock sampled for hatchery propagation in 2018 and 2019. Rivers are ordered according by salinity conditions of estuaries. Letters indicate significant differences (Tukey HSD, α=0.05).

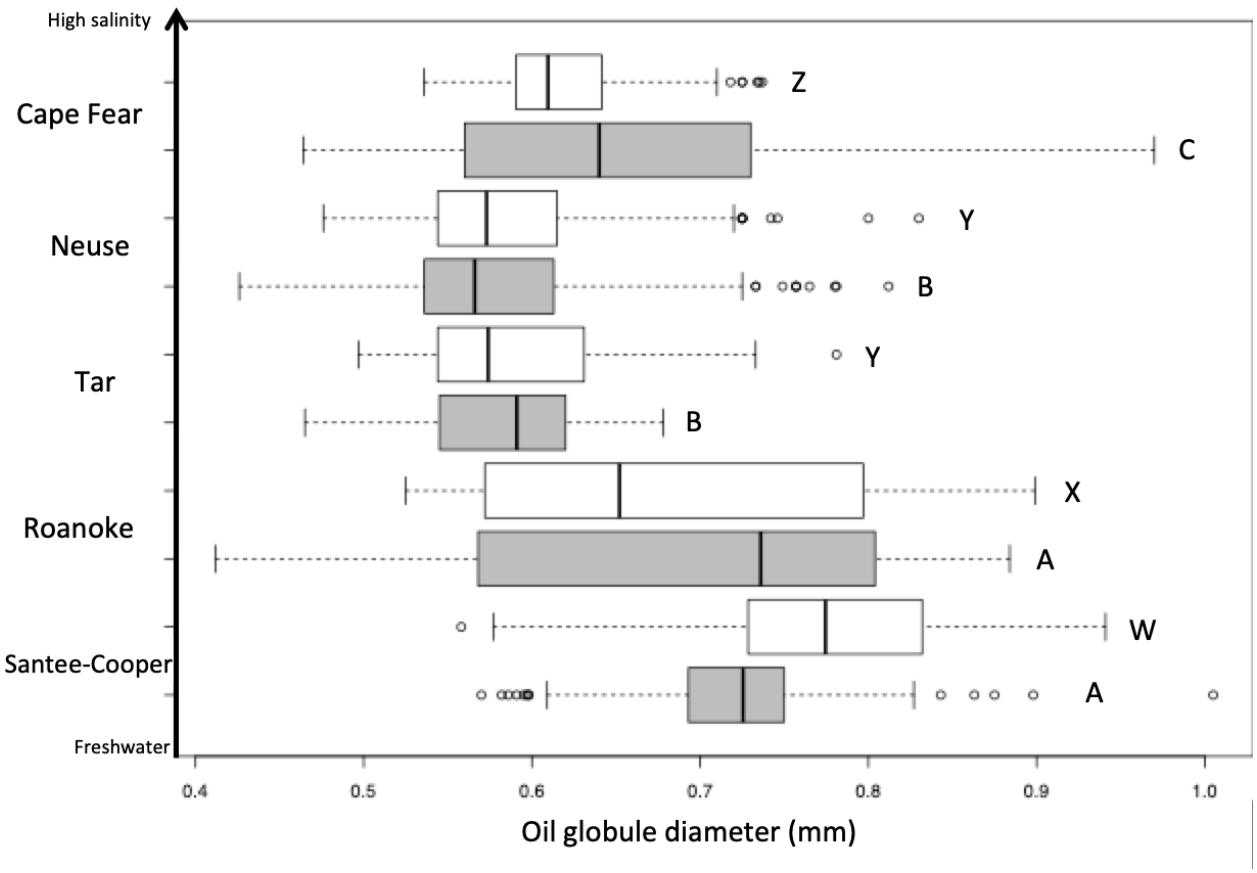

Figure 3. Egg oil globule diameter (mm) measurements from stage 1 (gray boxes) and 4 (white boxes) fertilized eggs from 2018 and 2019 hatchery broodstock sampling. Tukey pair-wise comparisons are labeled above the boxplots with ABCD indicating stage 1 significant differences and WXYZ indicating stage 4 significant differences (Tukey HSD, α=0.05).

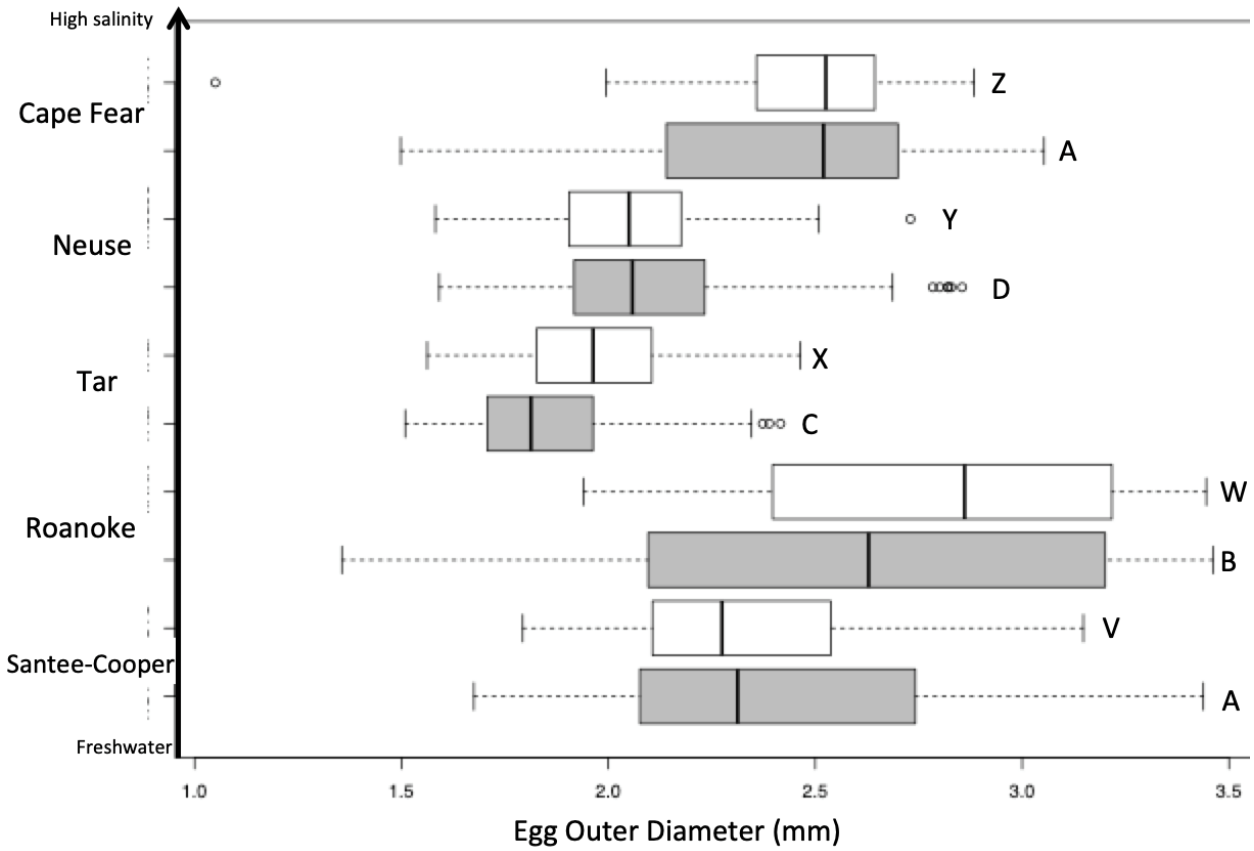

Figure 4. Egg outer diameter (mm) measurements from stage 1 (grey boxes) and 4 (white boxes) fertilized eggs from 2018/2019 hatchery broodstock sampling. Tukey pair-wise comparisons are labeled above the boxplots with ABCDE indicating stage 1 significant differences and WXYZ indicating stage 4 significant differences (Tukey HSD, α=0.05).

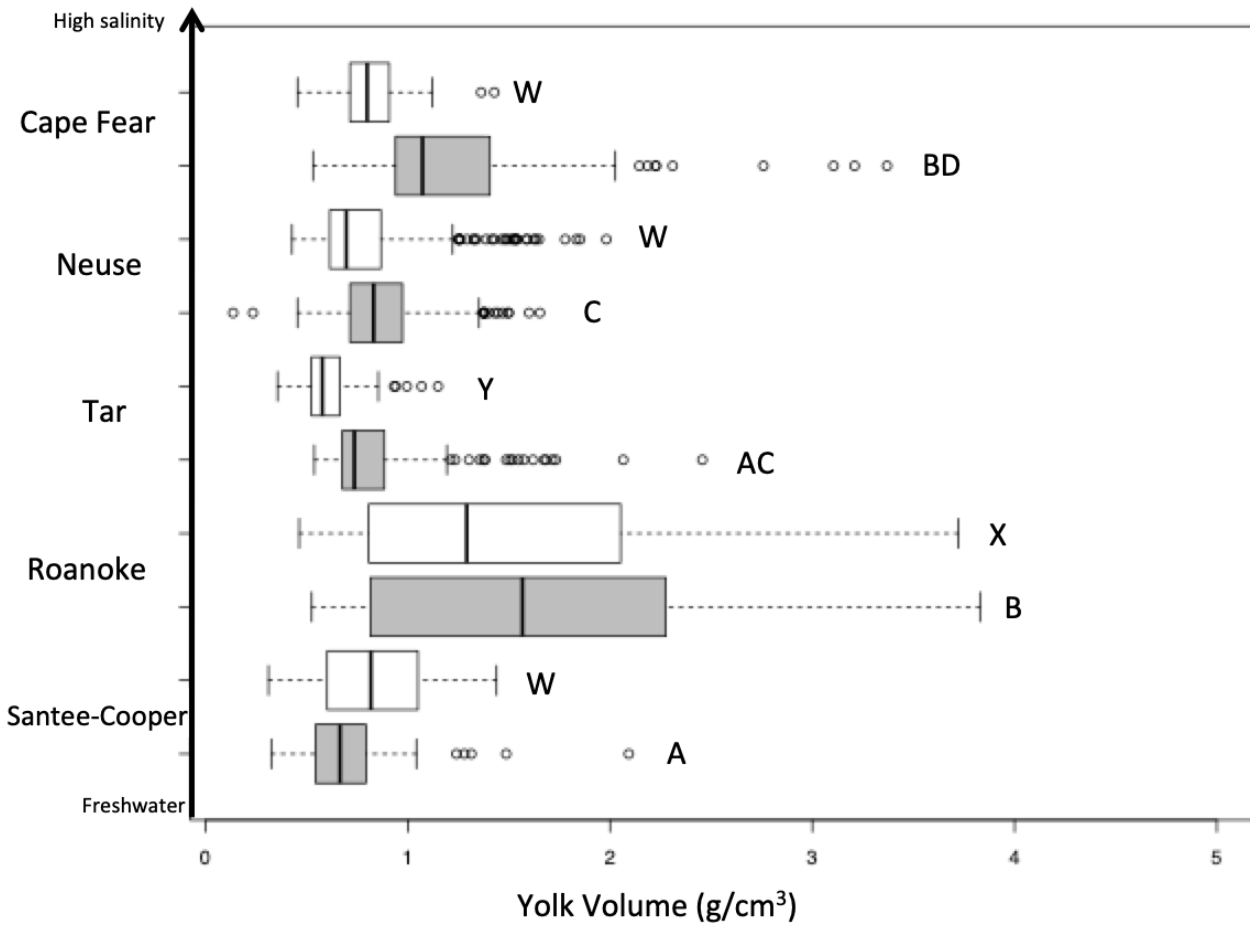

Figure 5. Egg yolk volume (g/cm^3) measurements from stage 1 (gray boxes) and 4 (white boxes) fertilized eggs from 2018 and 2019 hatchery broodstock sampling. Tukey pair-wise comparisons are labeled above the boxplots with ABCD indicating stage 1 significant differences and WXYZ indicating stage 4 significant differences (Tukey HSD, α=0.05).

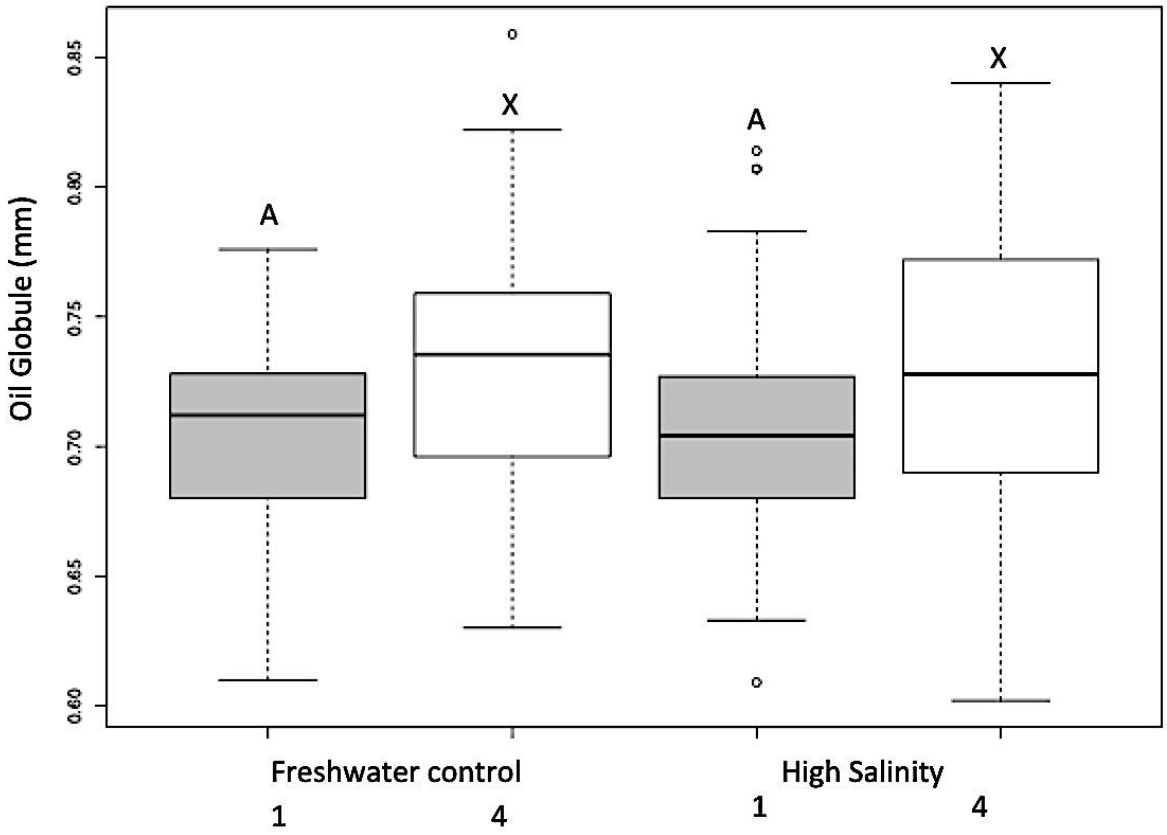

Figure 6. Egg oil globule diameter (mm) measurements from stage 1 (gray) and 4 (white) fertilized eggs from domestic Striped bass held in either high salinity or freshwater. AB represents significance for stage 1 eggs, XY represents significance for stage 1 eggs (Tukey HSD, α=0.05).

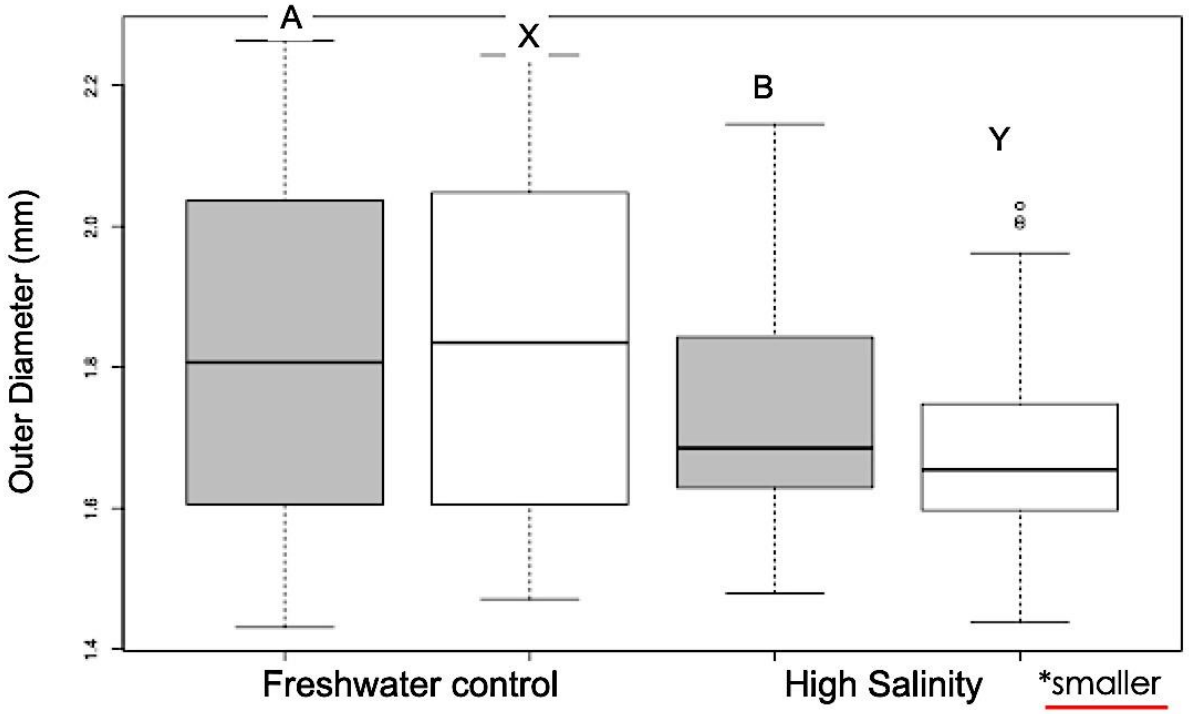

Figure 7. Egg outer diameter (mm) measurements from stage 1 (gray) and 4 (white) fertilized eggs from domestic Striped bass held in either high salinity or freshwater. AB represents significance for stage 1 eggs, XY represents significance for stage 1 eggs (Tukey HSD, α=0.05).

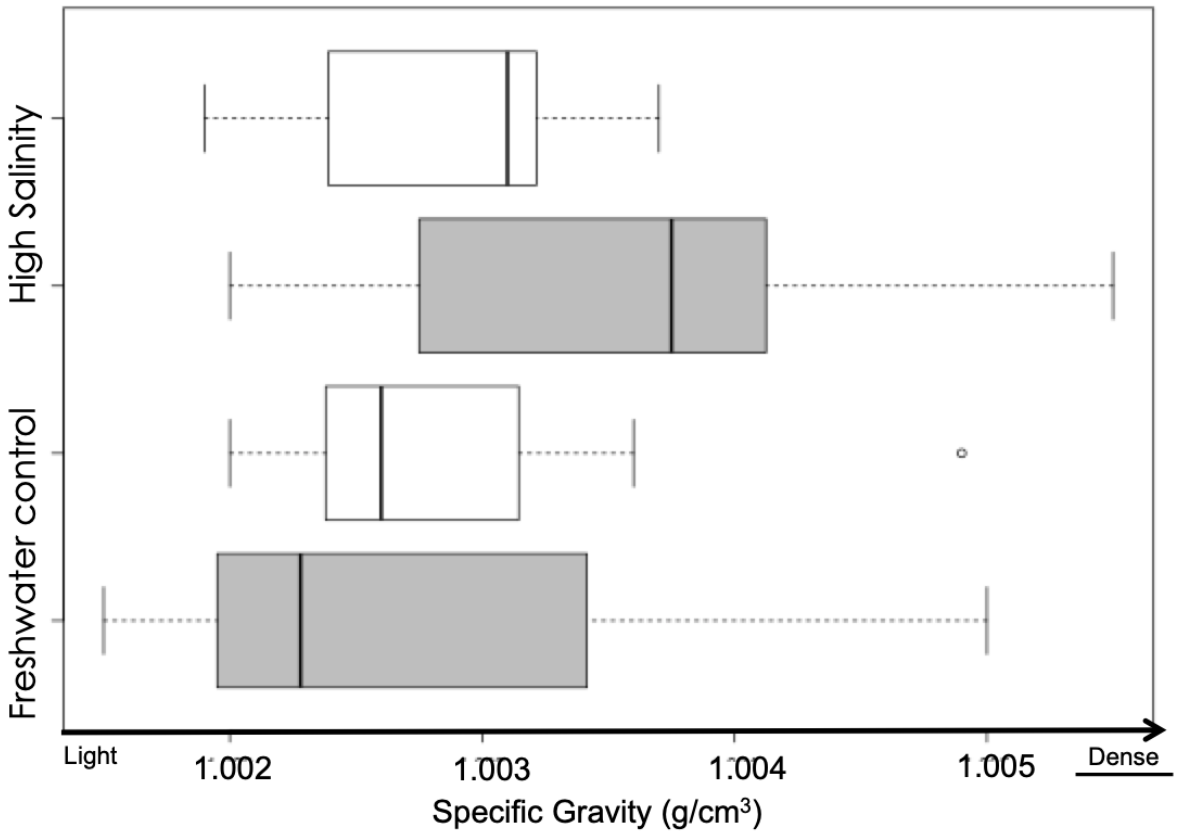

Figure 8. Egg buoyancy (g/cm3) measurements from stage 1 (gray) and 4 (white) fertilized eggs from domestic Striped bass held in either high salinity or freshwater. No significant differences observed (ANOVA, Tukey HSD, α=0.05).

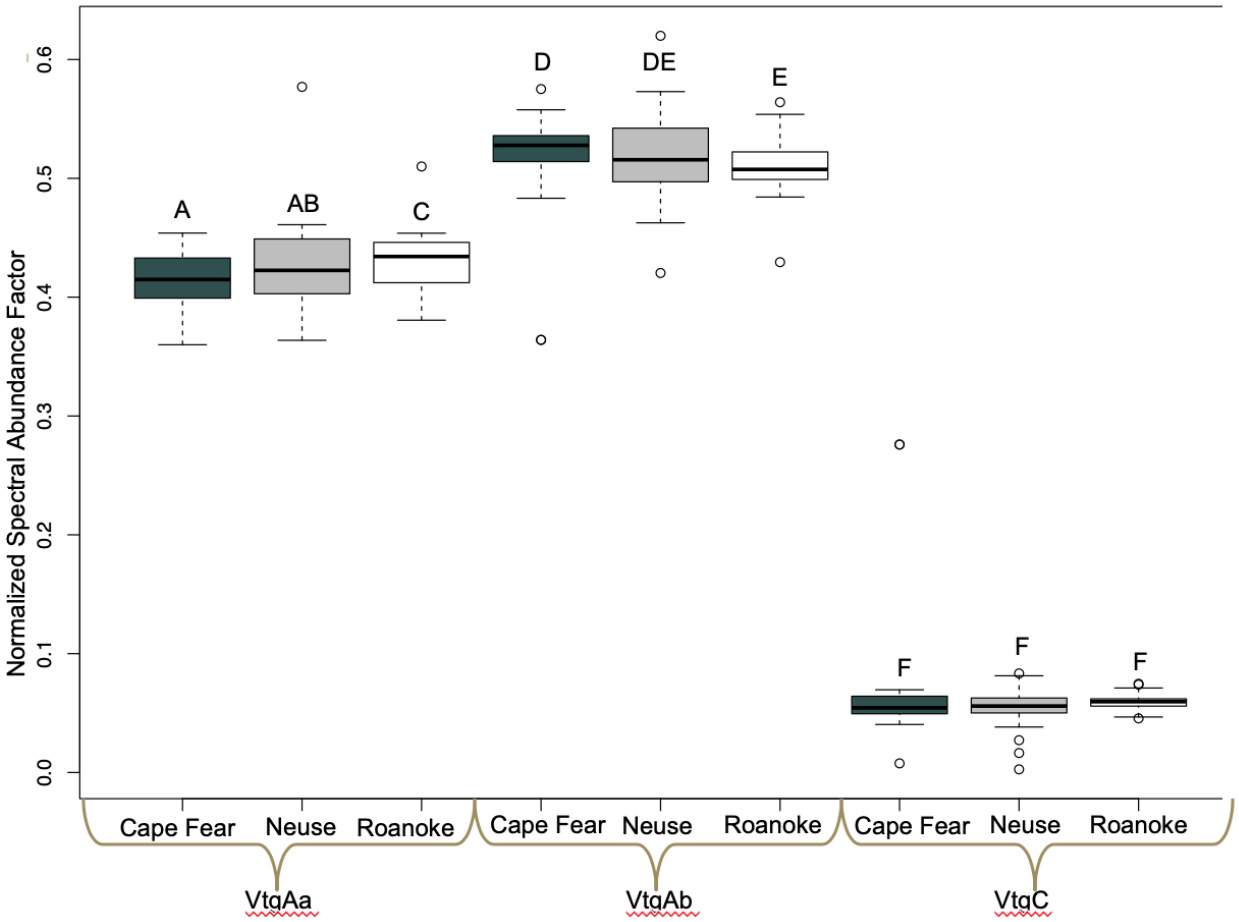

Figure 9. Vitellogenin NSAF values compared by River origin. Capital letters (A-F) above boxplots represent significant differences (Tukey HSD, α=0.05).

Table 1. Numbers of Striped bass spawns sampled at Edenton, Watha and Bayless fish hatcheries in 2018/2019. The fertilized eggs and embryos were evaluated for buoyancy, diameter, yolk, and globule size and represented fish from the Neuse, Tar, Cape Fear, Dan, Santee-Cooper and Roanoke Rivers.

River	Spawns Sampled	Hatchery	Year
Neuse	9	Edenton	2018
Tar	5	Edenton	2018
Cape Fear	4	Watha	2018
Roanoke	4	Watha	2018
Neuse	13	Edenton	2019
Tar	7	Edenton	2019
Santee-Cooper	10	Bayless	2019
Cape Fear	4	Watha	2019
Dan	2	Watha	2019
Roanoke	3	Watha	2019

Table 2. Striped bass ovary biopsy samples from Cape Fear, Neuse, Tar, and Roanoke River systems and their associated estuaries selected for submission to proteomics analyses (Year 2). Samples from the Santee-Cooper System in South Carolina will be collected in 2019 with SC Department of Natural Resources staff.

1. High Salinity System: Cape Fear River (NC, Cape Fear River Estuary)
    Cape Fear River Samples for Proteomics: 6
    Cape Fear Estuary Samples for Proteomics: 6
2. Intermediate Salinity Systems: Neuse River, Tar River (NC, Pamlico Sound)
    Neuse River Samples for Proteomics: 7
    Tar River Samples for Proteomics: 0
    Pamlico/Neuse Estuary for Proteomics: 5
    Pamlico/Tar Estuary for Proteomics: 0
3. Low Salinity System: Roanoke River (NC, Albemarle Sound)
    Roanoke River Samples for Proteomics: 8
    Roanoke/Albemarle Estuary for Proteomics: 4
4. Zero Salinity System: Santee-Cooper Reservoir (SC)
    Santee-Cooper System Samples Collected: 0
    Will be sampled in 2019 (March)

TOTAL SAMPLES SELECTED: 36 fish

Table 3. Salinities during conditioning and spawning of domestic Striped bass to mimic high salinity river systems (Cape Fear River) in North Carolina during Year 2. A total of 8 females were spawned of the 15 that were conditioned.

Day	Note	Time	Date	Temperature (degrees C)	Salinity (ppt)
0	*Start*	AM	5/2/2019	11.2	10.8
0	Target Temp 12	PM	5/2/2019	12.8	11.9
1	Target Temp 12	AM	5/3/2019	12.6	10.6
1	Target Temp 13	PM	5/3/2019	12.6	8.6
2	Target Temp 13	AM	5/4/2019	13.9	9.6
2	Target Temp 13	PM	5/4/2019	13.9	10.8
3	Target Temp 14	AM	5/5/2019	15.3	10.5
3	Target Temp 14	PM	5/5/2019	15.8	11.5
4	Target Temp 15	AM	5/6/2019	15.8	11.7
4	Target Temp 15	PM	5/6/2019	16.2	11.3
5	Target Temp 16	AM	5/7/2019	16.5	11.5
5	Target Temp 16	PM	5/7/2019	16.3	11.0
6	Target Temp 17	AM	5/8/2019	17.5	9.7
6	Target Temp 17	PM	5/8/2019	20.1	9.3
7	Target Temp 18	AM	5/9/2019	20.2	8.1
7	Target Temp 18	PM	5/9/2019	20.3	8.0
8	**1 Female spawned**	AM	5/10/2019	19.5	2.4
8		PM	5/10/2019	19.7	1.3
9		AM	5/11/2019	19.5	0.7
9		PM	5/11/2019	19.7	0.6
10	*Sort fish*	AM	5/12/2019	19.7	0.5
10	*Inject hCG*	PM	5/12/2019	19.9	0.5
11	**7 Females spawned**	AM	5/13/2019	19.6	0.5
11		PM	5/13/2019	19.8	0.5
12	*TAKE DOWN*	AM	5/14/2019	18.6	0.5
12	**END**	**PM**	**5/14/2019**	**END**	**END**

Table 4. Salinities during conditioning and spawning of domestic Striped bass to mimic low salinity river systems (Roanoke River) in North Carolina during Year 2. A total of 9 females were spawned of the 15 that were conditioned.

Day	Note	Time	Date	Temperature (degrees C)	Salinity (ppt)
0	*Start*	PM	5/9/2019	11.7	0.5
0	Target Temp 12	AM	5/10/2019	11.6	0.5
1	Target Temp 12	PM	5/10/2019	11.6	0.5
1	Target Temp 13	AM	5/11/2019	11.7	0.5
2	Target Temp 13	PM	5/11/2019	11.8	0.5
2	Target Temp 13	AM	5/12/2019	12.3	0.5
3	Target Temp 14	PM	5/12/2019	12.1	0.5
3	Target Temp 14	AM	5/13/2019	13.3	0.5
4	Target Temp 15	PM	5/13/2019	13.3	0.5
4	Target Temp 15	AM	5/14/2019	14.5	0.5
5	Target Temp 16	PM	5/14/2019	14.5	0.5
5	Target Temp 16	AM	5/15/2019	15.5	0.5
6	Target Temp 17	PM	5/15/2019	16.0	0.5
6	Target Temp 17	AM	5/16/2019	16.9	0.5
7	Target Temp 18	PM	5/16/2019	17.5	0.5
7	Target Temp 18	AM	5/17/2019	18.5	0.5
8		PM	5/17/2019	18.9	0.5
8		AM	5/18/2019	19.6	0.5
9		PM	5/18/2019	19.5	0.5
9	**0 Female spawned**	AM	5/19/2019	19.8	0.5
10	*Sort fish*	PM	5/19/2019	19.6	0.5
10	*Inject hCG*	AM	5/20/2019	19.5	0.5
11	**3 Females spawned**	PM	5/20/2019	19.4	0.5
11	**4 Females spawned**	AM	5/21/2019	no data	0.5
12	**2 Females spawned**	PM	5/21/2019	19.6	0.5
12	*TAKE DOWN*	AM	5/22/2019	no data	
13	**END**	**PM**	**5/22/2019**	**END**	**END**

Table 5. Machine learning analyses of ovary proteomics using three classifier methods (SMO, J48, and Random Forest), two cross validation methods (% split, and fold), and three measures of model performance (% correctly classified, Kappa statistic, AUROC). Datasets were reduced using backwards selection.

Dataset size (# attributes)	Classifier	Cross-Validation	% correctly classified	Kappa Statistic	AUROC
2424	SMO	fold	91	0.83	0.80
	SMO	% split	75	0.49	0.75
	J48	FOLD	76	0.54	0.64
	J48	% split	72	0.41	0.73
	RandomForest	FOLD	82	0.61	0.92
	RandomForest	% split	81	0.59	0.89
533	SMO	FOLD	96	0.92	0.95
	SMO	% split	86	0.71	0.87
	J48	FOLD	80	0.60	0.79
	J48	% split	68	0.35	0.69
	RandomForest	FOLD	87	0.73	0.97
	RandomForest	% split	86	0.69	0.96
297	SMO	FOLD	94	0.83	0.94
	SMO	% split	89	0.79	0.91
	J48	FOLD	80	0.60	0.83
	J48	% split	80	0.60	0.83
	RandomForest	FOLD	91	0.83	0.97
	RandomForest	% split	86	0.68	0.95
173	SMO	FOLD	96	0.93	0.96
	SMO	% split	93	0.85	0.94
	J48	FOLD	79	0.58	0.80
	J48	% split	79	0.58	0.80
	RandomForest	FOLD	93	0.85	0.98
	RandomForest	% split	93	0.85	0.97
26	SMO	FOLD	89	0.78	0.90
	SMO	% split	75	0.53	0.79
	J48	FOLD	87	0.74	0.88
	J48	% split	69	0.41	0.75
	RandomForest	FOLD	88	0.76	0.95
	RandomForest	% split	89	0.77	0.96