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THE EFFECTS OF SALINITY ON ZOOTECHNICAL PERFORMANCE OF
SPOTTED SEATROUT (*CYNOSCION NEBULOSUS*) IN RECIRCULATING
AQUACULTURE SYSTEMS

by

Eric Michael Gigli

A Thesis
Submitted to the Graduate School,
the College of Arts and Sciences
and the School of Ocean Science and Engineering
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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ABSTRACT

The spotted seatrout (*Cynoscion nebulosus*) is a euryhaline finfish candidate for marine aquaculture in the northern Gulf of Mexico. Aquaculture of this species currently uses salinities of 25 psu or greater making production challenging in areas where high salinity water is not available. This study aimed to document the effects of salinity on zootechnical performance of spotted seatrout embryos, early larvae, and juveniles in recirculating aquaculture systems to assist with the development of protocols for low salinity culture.

Embryos obtained from a volitional spawn at 29 psu were incubated at three salinities (12.5, 18.75, or 25 psu) until unfed larvae expired. The neutral buoyancy salinity of embryos was 25 psu in all groups. Hatch rate was reduced in the 12.5 psu group where embryos tended to fall out of suspension, but larval survival post-hatch did not differ between treatments. Vitelline reserves were utilized more slowly in the lower salinity treatment groups than in the high salinity treatment.

Juveniles from two age groups (42 and 210 days old) were cultured in parallel for 267 days at four salinities (10, 15, 20, 25 psu). The 15 psu group was lost due to a parasitic infection early in the experiment. Survival in the other groups was low (average ~12%) but did not differ between treatments. Total length was highest in the 10 psu group. These results suggest that embryos and newly hatched larvae tolerate low salinity culture after hatch, and that grow out of juveniles at low salinity would be beneficial.

ACKNOWLEDGMENTS

DEDICATION

To Sarah and Harper, thank you for the sacrifices you made during my academic career. Without the love and support from the two of you, none of this would have been possible. To my parents and brothers, thank you for the support of my childhood dreams to pursue a career working with marine finfish. To Nora and Bill, thank you for your support and assistance every day, which made this possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

HPS	Hours Post-Spawn
DPH	Days Post-Hatch
DPT	Days Post-Treatment
DPF	Days Post-Fertilization
PSU	Practical Salinity Unit
PPM	Parts Per Million
mOsm	Milliosmole
SE	Standard Error
GSI	Gonado-Somatic Index
HSI	Hepato-Somatic Index
CF (k)	Fulton's Condition Factor
USM	The University of Southern Mississippi
GCRL	Gulf Coast Research Laboratory
TCMAC	Thad Cochran Marine Aquaculture Center
MDMR	MS Department of Marine Resources

CHAPTER I – GENERAL INTRODUCTION

1.1 General

Fish is a rich source of easily digested, high quality protein featuring high

quantities of essential amino acids, long-chain omega-3 fatty acids, vitamins (A, B, and

D), and minerals (calcium, iodine, zinc, iron, and selenium) (FAO 2019). The human

population will reach almost 10 billion by 2050 and per capita seafood consumption,

currently at 20 kg per year, also will rise (FAO 2019), thus creating a tremendous

increase in the demand for protein. All told, the demand for seafood products is expected

to grow 21% over the next 19 years (World Bank 2013). Static capture fishery production

has not met the increasing demand and cannot be expected to meet additional increases in

demand. Aquaculture, which contributed only 7% of the seafood supply in 1974, has

filled the gap between demand and wild fisheries production and grown to contribute

over 50% of the seafood supply. Further growth of demand can be filled only through

growth in aquaculture. Aquaculture also can contribute to maintaining commercially or

recreationally important fish stocks through restocking or stock enhancement, which seek

to restore depleted spawning biomass or augment the natural supply of juveniles by

releasing cultured animals (Blankenship and Leber 1995; Bell et al. 2008; Lorenzen et al.

2010).

Currently the United States imports 91% of the seafood it consumes,

approximately 50% of which is farmed. The corresponding trade deficit amounted to $14

billion in 2016 (NOAA 2019). Aquaculture production in the United States has focused

largely on freshwater species such as catfish and rainbow trout. The southeast region of

the United States, and Mississippi in particular, has led the development of hybrid blue-

channel catfish culture into a substantial industry producing over 30 million pounds of catfish per year since the 1990's (Hanson and Sites 2009). However, almost half (46%) of the per capita consumption of the top ten seafood products in the United States is of marine finfish (17% salmon, 29% other species), to which U.S. aquaculture contributes almost nothing (FAO 2018).

Technological limitations, land use issues, and other economic factors currently make it unrealistic to expect aquaculture to replace every popular seafood species in the United States. However, several factors including political instability in exporting countries, recent tests identifying tainted import products, and the possibility of bio-terrorism raise serious questions about the long-term safety and reliability of a seafood supply based overseas (Institute of Medicine 2006, Berkeley Wellness 2012, Love et al. 2011). Thus, the U.S. would benefit from the development of a domestic marine aquaculture industry. Coastal states like Mississippi are well suited to lead the development of a sector that will diversify and strengthen the economy, stimulate growth of research capabilities, and promote revitalization of the fisheries industry. Efforts to develop aquaculture on the Gulf coast of Mississippi so far have included research programs evaluating the feasibility of aquaculture for selected marine and anadromous species of regional importance such as striped bass, marine shrimp, blue crab, eastern oyster, spotted seatrout, and red snapper.

Options for expanding production include traditional extensive pond culture systems and more technologically complex net pen and recirculating systems. The potential for pond culture to produce marine species along the Mississippi Gulf coast is limited because of the scarcity of coastal land and the variable salinity of coastal waters.

2

Suitable sites for net pens in Mississippi coastal waters are limited by the requirement for depth and adequate currents to provide sufficient water exchange and waste flushing. Deployment in offshore locations would address the issue of local benthic and water column fouling, but other issues such as potential escapement, predation by marine life and associated mortality remain. Further, increased costs of engineering required to withstand the offshore environment and the expenses of operating at appropriate distances from the coast may outweigh the benefits of net pen culture (Langan 2009). Aside from these technical considerations, the current regulatory environment is complex and provides no clear path to a permit for offshore net pen aquaculture. Recirculating aquaculture systems (RAS) evolved as filtration technology advanced and concerns over the environmental impacts of other methods became widespread. RAS utilize several components to filter and recycle water in a closed system with limited discharge. This approach has been used successfully to produce several marine species in Mississippi at a research scale. Its spread to the commercial sector for expanded production is limited essentially by the high infrastructure and operational costs.

The variable salinity of ambient coastal water in Mississippi prevents the direct use of local seawater sources for most aquaculture. Artificial salt may be used for aquaculture, but associated costs can be prohibitive especially for extensive production systems. Recirculating aquaculture circumvents this problem to a certain extent because it maximizes the fish biomass produced per unit water volume through high stocking density and re-use of water by recirculation, but salt costs remain consequential. Strategies that can minimize the requirement for artificial salt such as low salinity culture could improve the applicability of RAS and the feasibility of commercial aquaculture in

coastal Mississippi. Therefore, the tolerance for low salinity and performance in these conditions must be investigated for those species with potential for low salinity culture.

1.2 Spotted Seatrout

The spotted seatrout (*Cynoscion nebulosus*) is a euryhaline, estuarine species residing in coastal waters of the US from Texas to Virginia. Common habitats include but are not limited to brackish marshes, saltwater marshes, mangroves, oyster reefs, mud flats, beaches, sea grass beds, and channels (Overstreet 1983, USM - GCRL 2019). The spotted seatrout is the most popular recreational fish in the Gulf of Mexico (NOAA 2015). Accessibility, propensity for taking a variety of baits and lures, and mild flavor result in recreational harvests of nearly one million pounds of spotted seatrout per year in Mississippi (Leaf et al. 2016); Commercial catches amount to only a small fraction of the landings. Management of the fishery focuses on maintaining spawning biomass through creel, season, and size restrictions. The spawning potential ratio (SPR), the number of eggs that could be produced by an average recruit in a fished stock divided by the number of eggs that could be produced by an average recruit in an unfished stock, is a measure of the impact of fishing on the potential productivity of a stock (Goodyear 1990). Since 2009, the Mississippi population has maintained a SPR of only 9.3% compared to the recommended minimum of about 18%. This assessment triggered an increase in the minimum size limit from 13 to 15 inches (Total Length) in 2017.

Stock enhancement is a potential approach to increase the recruitment in a fishery through the release of cultured juveniles. The Thad Cochran Marine Aquaculture Center at the University of Southern Mississippi in partnership with the Mississippi Department of Marine Resources formed the SPEC (Seatrout Population Enhancement Cooperative)

in 2004 to investigate the feasibility of spotted seatrout stock enhancement. The goals of the SPEC include the development of RAS culture methods, broodstock maintenance, volitional spawning in captivity, and intensive grow out of progeny to produce juveniles for release. The SPEC also seeks to develop protocols for tagging, releasing, and monitoring of released individuals to determine their contribution to the wild population.

Spotted seatrout broodstock spawn volitionally when subjected to a photothermal cycle derived from the protocol described by McCarty et al. (1987). Fertilized embryos are cultured through the larval stage in recirculating systems and the resulting 1-inch fingerlings are either released or further cultured in recirculating tanks or ponds to 4-inch juveniles that are tagged with coded-wire tags and released. Broodstock and larvae are cultured in artificial salt water (Crystal Sea Marinemix) that costs approximately $0.10-0.11 gal^{-1}. Broodstock and larval rearing production uses a minimum of 1.08×10^5 gallons of new artificial water annually assuming year-round production. A 50% reduction in the amount of artificial salt water required would reduce the commodity costs of production by an estimated 5% (GCRL-TCMAC communications). Therefore, the tolerance of spotted seatrout to low salinity in culture requires investigation.

1.3 Salinity and Osmoregulation

Salinity is a measure of the quantity of salts dissolved in water. Typically, salinity is measured as the number of parts of salt per 1,000 parts of water (parts per thousand-ppt or practical salinity unit-psu). Vertebrate animals maintain a salt content in their blood of approximately 9 psu (Wurts 1998). When fish live in water that differs from 9 psu, they need to osmoregulate through active adjustments to the ionic balance of their internal fluids. In fresh water where the salinity is less than 1 psu, animals gain water from the

environment through osmosis and must eliminate the excess water and conserve ions. Blood pressure forces water and ions into the kidney tubules where ions are returned to the blood and water is excreted. In full strength salt water where the salinity is greater than 30 psu, animals drink to replace the water lost to the environment. Blood pressure forces water and ions into the kidney tubules where water and important solutes are returned to the blood. Excess ions and waste products are concentrated and excreted in urine. Ions are also excreted by passive diffusion through the mitochondria-rich chloride cells of the gills.

Species inhabiting offshore habitats characterized by high (>30 psu) and relatively stable salinity are typically stenohaline which means they tolerate only a narrow range of salinity. Those that inhabit inshore estuarine waters experience variation in salinity that results from fluctuations in fresh water influx. These species are typically euryhaline meaning that they tolerate a wider range of salinity. The effects of salinity on fitness, growth, and well-being of an organism are therefore species specific.

Osmoregulation capacity also can vary among life stages depending on the conditions encountered by a species during successive ecophases of the life cycle. Early life stages of marine fishes such as embryos and newly hatched larvae usually have limited osmoregulation capacity (Varsamos et al. 2005). As the gills, kidneys, and urinary bladder develop, the larvae progressively acquire the greater osmoregulatory capability typical of juveniles and adults. Therefore, it is possible that the range of salinity tolerance for a species changes during ontogeny as larvae metamorphose into juveniles equipped with the osmoregulation structures of the adult.

1.4 Salinity Regimes in Spotted Seatrout Culture

Seatrout broodstock systems are currently maintained at 25 to 32.5 psu, which is consistent with the spawning ecophase for wild adults. Incubation of embryos and subsequent culture of early stages throughout the 25-day larval period are performed at 25 psu. Twenty-five-day old fingerlings are stocked into growout production tanks or ponds and maintained at 18-25 psu, which is likely higher than the salinity experienced by post-larval seatrout in the wild (GCRL unpublished results), for an additional 50-65 days. As the release approaches, salinity at the release site is monitored and the rearing salinity in the tanks is adjusted to match. Release site salinity is seasonally variable and ranges between 5 psu and 20 psu. Preliminary, unpublished results suggest that juveniles can be cultured in ponds or tanks at salinities lower than the standard 20-25 psu with little or no effect on growth rate, but a formal assessment of juvenile growout production under low salinity conditions is lacking. Information on the effect of salinity on early stages of spotted seatrout is still limited. Fitness traits including neutral buoyancy and rate of yolk utilization were shown to be affected by salinity (Ponwith and Neill 1995, Bortone 2002, Banks et al. 1991) and the response of these traits to changes in salinity varied among populations. Thus, the responses of Mississippi seatrout to changes in salinity during early development must be characterized.

1.5 Objectives

The main goal of this study was to determine the effects of salinity on the zootechnical performance of spotted seatrout embryos, early larvae, and juveniles in recirculating aquaculture systems to assist in the evaluation of the feasibility of low salinity culture protocols. The specific objectives were to: 1) measure the effects of

salinity on the growth, vitelline reserve utilization, and survival of spotted seatrout embryos and larvae; and (2) evaluate the effects of salinity on the growth, survival, and carcass traits of spotted seatrout juveniles. These objectives were addressed in two experiments that were conducted in conditions matching those applied in large-scale production to ensure the findings are applicable to the practical needs of commercial or stock enhancement programs.

CHAPTER II – THE EFFECTS OF INCUBATION SALINITY ON EGG QUALITY

TRAITS AND EARLY DEVELOPMENT OF SPOTTED SEATROUT (*CYNOSCION*

NEBULOSUS)

2.1 Introduction

The development of land-based aquaculture for marine species in coastal

Mississippi is limited by the low and variable salinity of ambient coastal water. However,

the feasibility of commercial aquaculture could be improved by focusing on organisms

that can be cultured at low salinity. The spotted seatrout (*Cynoscion nebulosus*) is the

most popular recreational finfish species in the Gulf of Mexico and its culture is of

interest to resource management agencies. Juveniles and adults of the species frequent

habitats featuring a broad range of salinity from nearly fresh water upper estuaries to high

salinity barrier islands, but available data suggest that spawning occurs primarily at high

salinity in the wild (GSMFC 2006). The current protocol for spawning and larviculture in

the hatchery therefore involves high salinity (> 25 psu). This study aimed to document

the effects of salinity on spotted seatrout embryos and early larvae and evaluate the

feasibility of low-salinity culture at these stages.

Salinity can affect the fitness of embryos and early larvae in multiple ways. A

first trait of importance to embryonic and early larval fitness is buoyancy. In the wild,

eggs rely on buoyancy to avoid mortality in the benthos and access currents near the

surface that will carry them and maintain them near the planktonic food sources they will

need as they develop. In aquaculture, incubation of embryos relies on maintaining their

position in the water column. Negatively buoyant embryos sink to the bottom of

incubators where they experience degraded conditions that quickly become anoxic and

lead to rapid mortality. Similarly, newly hatched larvae have limited capacity to actively swim up during the first few days of life and, therefore rely on buoyancy to maintain themselves in the water column. Negatively buoyant larvae at that stage also tend to sink to the bottom of larval tanks where they have reduced access to live feeds and water quality is degraded leading to heavy mortality during the first few days of culture.

The internal salinity of embryos and the oil globule both contribute to the density and buoyancy in the water column. If the egg's total density is greater than the surrounding water, it will be negatively buoyant and sink. If the egg's density is less than the surrounding water, it will be positively buoyant and float. The density and osmolarity of the egg are determined primarily during the hydration stages of oogenesis when the oocytes intake low-osmolality fluids (Craik and Harvey 1987). Density and osmolarity of fish eggs are primarily influenced by the prevailing salinity experienced by the female in several species (Banks et al. 1991, Bortone 2002). After eggs are released, adjustment to density (and osmolarity) will take place in the perivitelline fluids through interaction with the surrounding water and as a result of metabolic activity. However, this adjustment capacity is limited and determines the range of salinity compatible with the maintenance of embryo buoyancy. The oil globule contributes to buoyancy with low density material, and this contribution decreases over time as pre-feeding larvae consume vitelline reserves. The active adjustment of this component of buoyancy by the larva is limited. The membrane of the egg, however, is permeable to water which permits adjustment of the internal salinity and the egg's buoyancy within the water column. Water will travel from an area of low osmolarity to an area of greater osmolarity until equilibrium is

reached or the membrane expands to its maximum internal capacity and lysis occurs (Freeman et al. 2011).

The fitness of embryos and larvae also can be impacted by the osmotic stress induced by too broad or too sudden changes in salinity. Moderate osmotic stress may not result in immediate mortality. Rather, it may impact overall fitness by requiring premature use of vitelline reserves to cope with the salinity change, thereby preventing the newly hatched larva from surviving until exogenous feeding can begin. If the larva survives to exogenous feeding, it would likely require more frequent feeding with reduced feed conversion efficiency.

The parental acclimation salinity is also an important determinant of the response of newly hatched larvae to different salinity regimes. Kucera et al. (2002 B) compared the rate of yolk utilization in spotted seatrout offspring from parental stocks maintained at 20 psu to that in offspring from parents maintained at 40 psu. When larvae were cultured at 20 psu, yolk utilization was slower for offspring from the 20 psu parental group than for those of the 40 psu parental group indicating that the optimal use of yolk reserves was achieved when larvae were maintained at a salinity matching that experienced by the parental stock. Similarly, Swanson (1996) concluded that milkfish larvae cultured at a normal salinity (30 psu) for the species exhibited the most efficient yolk utilization.

This study aimed to evaluate the salinity tolerance of spotted seatrout embryos and early larvae produced from broodstock held at the TCMAC. The experiment compared the hatch rate, embryonic survival to hatch, neutral buoyancy, rate of resorption of vitelline reserves, mortality rates over time, and growth rate of embryos and unfed larvae exposed to different salinity regimes.

11

2.2 Materials and Methods

Embryos from a natural spawn produced by broodstock maintained at 29 psu were directly transferred to 100-L incubators at 25, 18.75, and 12.5 psu. The three treatments were replicated three times. The embryo diameter and the amount of vitelline reserves (based on an estimate of the size of the yolk sac and oil globule) were measured 12 hours after the beginning of the experiment in all 9 groups. The 9 incubators were then sampled every 45 minutes to measure buoyancy and characterize the neutral buoyancy adjustment (Kucera et al. 2002 A). The hatch rate was then monitored every 30 minutes beginning 8 hours post-spawn until the hatching was determined to be complete. Because it is difficult to assess hatch time accurately in the 100-L incubators, the monitoring of hatch rate over time also was performed using samples of embryos in microplates. Following hatch, the 9 groups were monitored every 12 hours for larval density (taken as an indicator of larval survival), larval length, and size of remaining vitelline reserve. The timing of the completion of mouth development also was recorded.

2.2.1 Broodstock Management

Wild-caught local seatrout adults are maintained in spawning tanks containing approximately 20 adults (1:1 sex ratio) each connected to an independent recirculating system featuring photothermal control. Gamete maturation is induced by exposing fish to a 150-day cycle as described in McCarty et al. (1987). Spawning conditions (26.5°C with a 13-hr photophase) are typically maintained for 183 days. Salinity is maintained between 25 and 35 psu throughout the cycle. Stocks are fed a diet composed of frozen shrimp and cigar minnows during the spawning season and cigar minnows and squid during the rest of the year. The feed ration is 5% of the tank biomass three times per week, but this target

may be adjusted based on fish consumption to accommodate decreased activity during the winter cycle. During the spawning season, a vitamin supplement (Sea Tabs BTFS) is fed weekly to compensate for potential deficiencies in the frozen diet. Water quality is maintained through a combination of sand or bubble bead (trademark) particle filters, fluidized bed biological filters to control ammonia (0-3ppm), air stones (O^2 maintained > 80% saturation), protein skimmers and ozone generators. The pH is maintained at approximately 8 by addition of sodium carbonate as needed. Activated carbon is used as necessary to remove drugs or chemicals required to treat occasional disease outbreaks. Each recirculating system is equipped with a side egg-collector that diverts the surface water of the tank into a basin to collect and concentrate the fertilized embryos. Broodfish are replaced at an average rate of 25% per year to increase genetic diversity among released fish.

2.2.2 Incubation Management

Embryos from one spawning event were collected from the spawning tank at 0015 hours (24-hour time) on July 19, 2017 using a fine-mesh net and immediately placed in a 20-L cambro provided moderate aeration to facilitate suspension and thorough mixing. Five sub samples (1mL each) were removed from the cambro and placed separately onto counting wheels where they were examined using a dissecting microscope. The fertilization rate and development stage were assessed, and the number of eggs was estimated by counting every embryo within sub-samples and extrapolating to the total volume (20L) in the cambro (Bonn et al. 1976). Additional embryos entered the collector after the initial collection. These embryos were collected at 0100 and added to the cambro. Fertility and fecundity of the spawn were reassessed as previously described.

The selected spawn contained 6.39×10^5 fertilized eggs (fertilization rate of 93%) and was used to stock the nine 100-L incubation tanks at a density of 0.59 embryos mL^{-1}. Embryos were randomly assigned to one of nine incubators (three salinity treatments - 12.5, 18.75, and 25 psu x three replicate incubators per treatment). Stocking was performed by thoroughly mixing the spawn and filling incubators progressively in a rotational scheme to ensure incubators received a similar sample of the spawn. In such manner, each incubator received 700mL three times and 122mL once to disperse the 20L volume of stock. Embryos were not acclimated to treatments. Rather, they were instantly immersed and thoroughly aerated to maintain suspension during the anticipated buoyancy adjustment phase.

Prior to hatch, aeration in the incubation tanks was adjusted to induce movement of the eggs at approximately 15cm sec^{-1}. Larvae become increasingly vulnerable to damage associated with current after hatching. Therefore, aeration was lowered at hatch to reduce current velocity to 2.5cm sec^{-1}. Incubators were operated in static conditions (i.e., with no water exchange or filtration). Water temperature, salinity, ammonia, nitrite and nitrate levels were monitored every 12 hours. Water temperature was maintained by controlling ambient room temperature (26°C) and measured using an infrared thermometer gun. Salinity was measured in each incubation tank using a light refractometer, and ammonia, nitrite, and nitrate levels were monitored with Hach test strips (Hach – Loveland, CO).

2.2.3 Sampling and Measurements

Embryos from each incubator were sampled to assess neutral buoyancy salinity prior to treatment, changes of neutral buoyancy salinity over time following transfer to

incubators, stabilized neutral buoyancy salinity, embryo diameter and oil globule reserves (12 hours after transfer to incubators), hatch rate, and hatch time. Larvae from each incubator were sampled at hatch and every 12 hours post-hatch to assess density, survival rate, larval notochord length, and oil globule and yolk sac dimensions (visible 2D surface area). The time of completion of mouth development also was recorded.

2.2.3.1 Neutral Buoyancy.

Initial neutral buoyancy salinity, rate of change, and adjusted/stabilized neutral buoyancy salinity were measured as follows. Ten 50-mL samples were taken from each incubator by plunging a plastic tube (150cm length, 1cm diameter) vertically to the bottom of the incubator to sample the entire water column. Fifty-mL samples were expected to contain on average 30 embryos based on the initial stocking density. Each of the 10 samples obtained from a given incubator were transferred to one of 10 treatment beakers pre-filled with artificial seawater prepared at 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, or 32.5 psu. The 50-mL samples were sieved using a 50-μm screen to remove residual water from their original incubator prior to transferring eggs to a treatment beaker. The sampled embryos were then rinsed out of the sieve into the treatment beaker using water at the salinity of the recipient beaker (Gray and Colura 1988). Embryos were left in the salinity treatment beakers for 10 minutes prior to recording buoyancy. The location of embryos in each beaker was then scored as bottom, intermediate, or top. The neutral buoyancy salinity was determined as the lowest salinity value at which embryos were suspended in the water column. If only observed as top and bottom with no intermediate values, the lowest salinity at which the eggs were buoyant (top) was recorded. This process was repeated every 45 minutes until buoyancy was stable over

15

three consecutive measurements for each treatment at this point the stabilized neutral buoyancy salinity was recorded.

2.2.3.2 Hatch time and rate.

Incubators were observed every 30 minutes for the presence of hatched larvae. If hatched larvae were observed, a 100-200mL sample was taken from each tank using 2 to 4 pulls of the plastic tube described above as needed to yield a sample size of approximately 100 larvae. The sample was concentrated in a sieve and placed on a counting wheel. The number of hatched larvae and unhatched embryos or unfertilized eggs was counted using a dissecting microscope and used to estimate the hatch rate at the sampling point. Sampling was repeated every 30 minutes until hatch percentage stabilized. Upon stabilization of the hatch percentage, aeration in the incubator was reduced as described previously. The first time point at which the hatch rate reached at least 95% of the final stabilized value was recorded as the hatch time.

Hatch time also was determined in microplates. Nine plates, each containing 6 wells, were randomly assigned to three salinity treatments in triplicate (12.5, 18.75, and 25 psu) when the spawn was stocked in incubators. Wells were filled at a working volume of 6 mL and embryos were stocked at a density matching the density in incubators (3-4 per well) and left in static conditions with no water exchange or filtration. Salinity was measured when embryos were stocked into the plate and at the completion of the study using a light refractometer. Water temperature was maintained at 26 ℃ via control of the ambient air temperature and monitored using an infrared thermometer gun every 12 hours throughout the study. Well plates were monitored visually every 30 minutes from the time of stocking to determine the number of hatched larvae, un-hatched

16

embryos and unfertilized eggs. Counting was repeated until the hatch percentage was stable during three consecutive counts and the first time point at which the hatch rate reached its final value was recorded as the hatch time.

2.2.3.3 Survival.

The density of larvae in each incubator was estimated at the time buoyancy stabilized, at hatch, and every 12 hours after hatch by counting the number of embryos and larvae in a 200-ml sample obtained from four pulls of the 50-ml sampling tube described above. The survival rate was estimated as the ratio between the estimate of density at stocking and the estimate of density at a given time point.

2.2.3.4 Embryo and Larval Measurements.

Thirty embryos from each incubator were retained once buoyancy in the incubator had stabilized but before the embryos began hatching. The samples were examined using a dissecting microscope and photographed using a digital camera. ProgRes Capture 2.5 digital image software (Jenoptik, Germany) was used to capture images; i-Solution Lite (IMT Inc.) was used to perform measurements. Images were saved as Jpegs with known pixel dimensions and magnification. Images were acquired using the same procedure for anesthetized larvae sampled every 12 hours from the time full hatch was recorded until the yolk and oil globule reserves were completely exhausted. In cases where the density sampling procedure yielded less than 30 larvae, additional 50-ml pulls were obtained as needed to collect a minimum of 30 larvae. Images were measured using the calibrated software i-Solution Lite. The length of larvae was measured from the outermost extension of the mandible, along the notochord to the caudal tip of the notochord using the "spline tool" that creates a bicubic spline function on the given points yielding a

distance drawn automatically during line creation with the measurement value as length. The notochord was expected to bend upward when undergoing flexion which may affect measured length if treatment groups undergo slower development. The "spline tool" was used for measurement instead of a straight-line tool to reduce any bias with respect to growth over time during the flexion timeframes of each group. Embryo diameter, yolk, and oil globule volumes were estimated through measurement of the visible two-dimensional surface area of the respective structures using "best-fit by n points tool" that connects all the points selected with measurement value as the average radius.

2.2.4 Data Analysis

Larval length, surface area of the oil globule, surface area of the yolk sac, initial neutral buoyancy salinity, amount of time for neutral buoyancy salinity to stabilize, stabilized neutral buoyancy salinity, and hatch time data were compared using a two-factor hierarchical ANOVA where the replicate incubator was treated as a random factor nested in the fixed factor salinity treatment. Homogeneity of variance was checked through the Bartlett's test; normality was tested using the Shapiro-Wilks test. The Tukey test was used to determine differences between treatment means. When data were not normally distributed or variances were heterogeneous among treatments, data were subjected to natural log, square root, or arcsine square root transformations to improve conformance to these assumptions. Attempted transformations did not resolve departures from normality or homoscedasticity and the results of the traditional, parametric ANOVA were confirmed through the Welch-James statistic with Approximate Degrees of Freedom as implemented in welchADF r package (Villacorta 2017).

18

The embryonic mortality rate was calculated as fertilization rate minus hatch rate. The hatch rate was standardized to the fertilization rate (i.e., standardized hatch rate = hatch rate/fertilization rate) to distinguish the rate of success during embryonic development from fertilization rate. Similarly, post-hatch survival was standardized to the hatch rate to distinguish mortality post-hatch from embryonic mortality. Data expressed in percentages (standardized hatch rates) were arcsine square root transformed. The percentage of surviving larvae (compared among time points) and all other percentage data were tested using a mixed effects model ANOVA accounting for the fixed effects of time crossed with treatment. Analyses were completed using R and R-Studio programs (R Studio Team 2019) (R Core Team 2019).

2.3 Results

The initial neutral buoyancy salinity was 32.5 psu. The time to stabilize buoyancy differed significantly among treatments. It did not differ significantly between the 12.5 and 18.75 psu groups (less than 45 minutes), but the 25 psu group took significantly longer (two hours) than the other two treatments (Figure 2.1). The stabilized neutral buoyancy salinity (25 psu) did not differ significantly among treatment groups (Figure 2.1). Total diameter of embryos measured 12 hours post-spawn was significantly smaller in the 25 psu group (Figures 2.2). Oil globule surface area measured 12 hours post-spawn did not differ significantly among treatment groups (Figure 2.3).

19

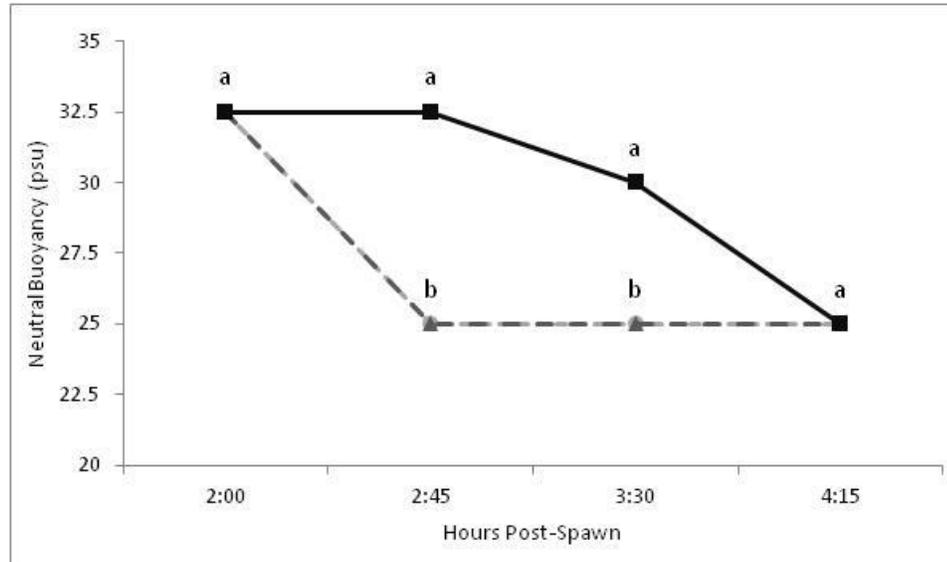Figure 2.1 Embryo neutral buoyancy.

Neutral Buoyancy salinity measured at different times post-spawn for *Cynoscion nebulosus* embryos incubated at three salinities

(- - 12.5 psu , - -18.75 psu , and — 25 psu).
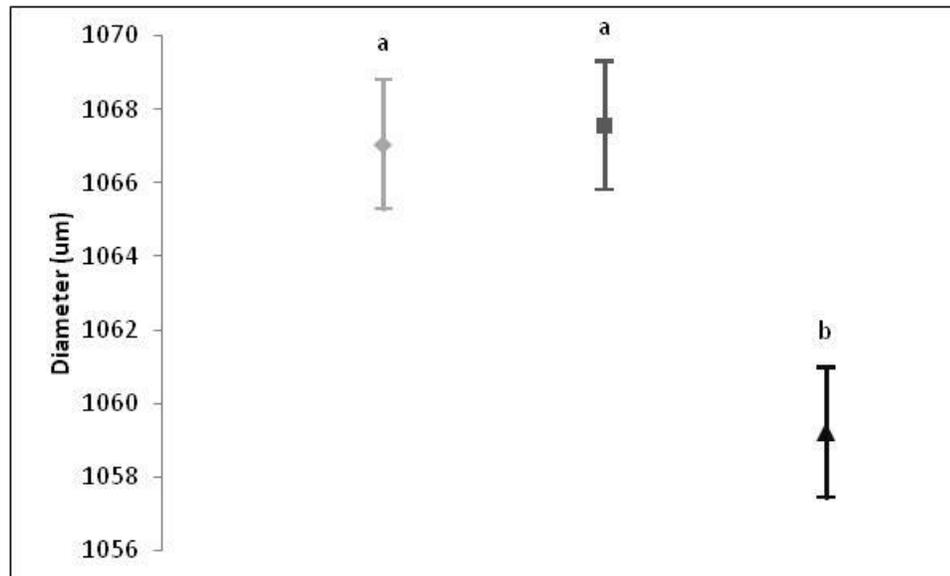

Figure 2.2 Embryo diameter.

Mean diameter ± SE measured at 12 hours post-spawn for *Cynoscion nebulosus* embryos incubated at three salinities

(♦ 12.5 psu , ■ 18.75 psu , ▲ 25 psu).
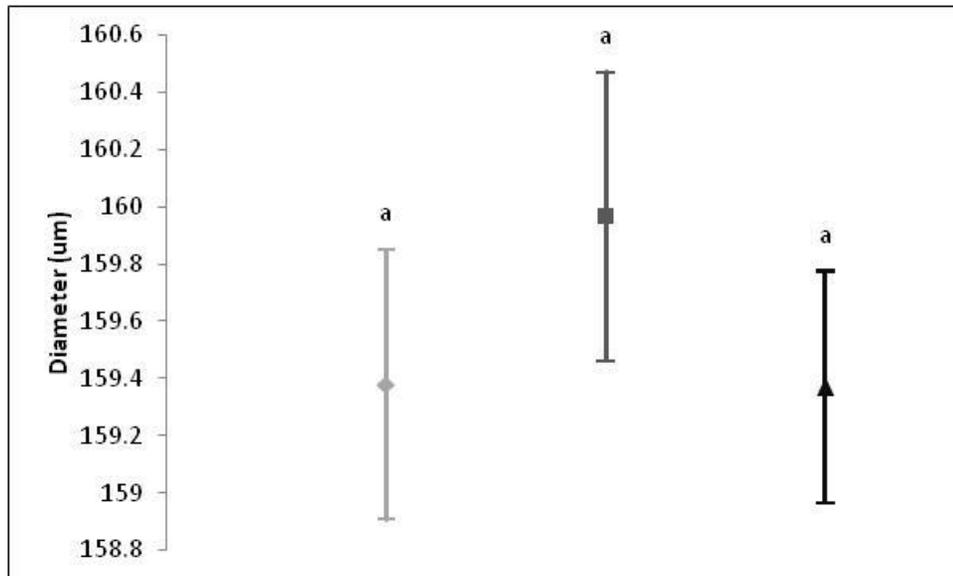
Figure 2.3 Embryo oil globule diameter.

Mean oil globule diameter ± SE measured at 12 hours post-spawn for *Cynoscion nebulosus* incubated at three salinities

(♦ 12.5 psu , ■ 18.75 psu , ▲ 25 psu).

The hatch rates measured in incubators differed significantly among groups. The 12.5 psu group had a lower hatch rate than either the 18.75 or 25 psu groups, which did not differ from one another (P = 1.573e^{-08}) (Table 2.1). The embryonic mortality in incubators was calculated from the density estimation at hatch after correcting to account for the fertilization rate of 93%. It was 31% at 18.75 and 25psu versus 58% at 12.5psu. The hatch rate measured in plate was lower (59%) and did not differ significantly among groups (Table 2.1). The time at hatch (both in incubators and in plates) did not differ significantly among groups, with all groups hatching at ~14.5 hps. Survival for the remainder of the study was standardized to the hatch rate. Survival of hatched larvae did not change significantly over time in any of the incubators until the 120-132 hours post hatch time period where most of the larvae expired in all groups (Figure 2.4).

Table 2.1 Hatch Rates of *Cynoscion nebulosus* embryos incubated at three salinities

Group	12.5 psu	18.75 psu	25 psu
Well Plates (6 mL)	41.82%	36.59%	37.14%
Incubators (100L)	52.09% (b)	81.75% (a)	78.22% (a)

Hatch rate of *Cynoscion nebulosus* embryos incubated at three salinities (12.5 psu , 18.75 psu , and 25 psu) in well plate or tank incubation trials for100-L incubators. Homogeneous groups from Tukey's a posteriori classification are labeled with the same letter.
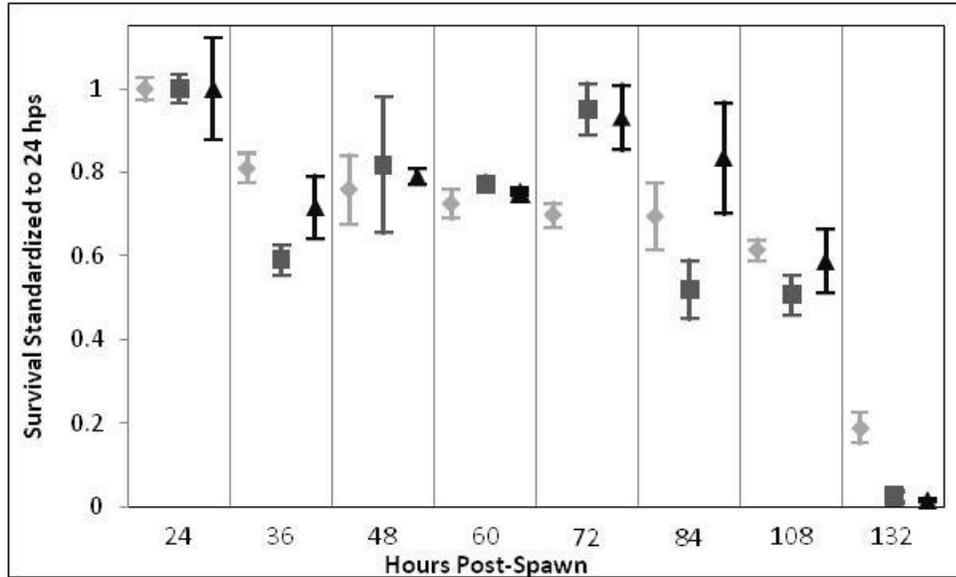
Figure 2.4 Standardized survival of larvae at different times post-spawn.

Mean Survival ± SE at different times post-spawn for *Cynoscion nebulosus* larvae incubated at three salinities
(♦ 12.5 psu , ■ 18.75 psu , ▲ 25 psu). Survival rate values are standardized to the hatch rate estimated for a given group.

Larval length did not change significantly over time (Figure 2.5). Larvae were significantly longer in the 12.5 psu group from the first measurement post hatch. The average difference was 8.7% during the monitoring period. Larvae in the 18.75 psu treatment were intermediate in length between the 12.5 and 25 psu groups.
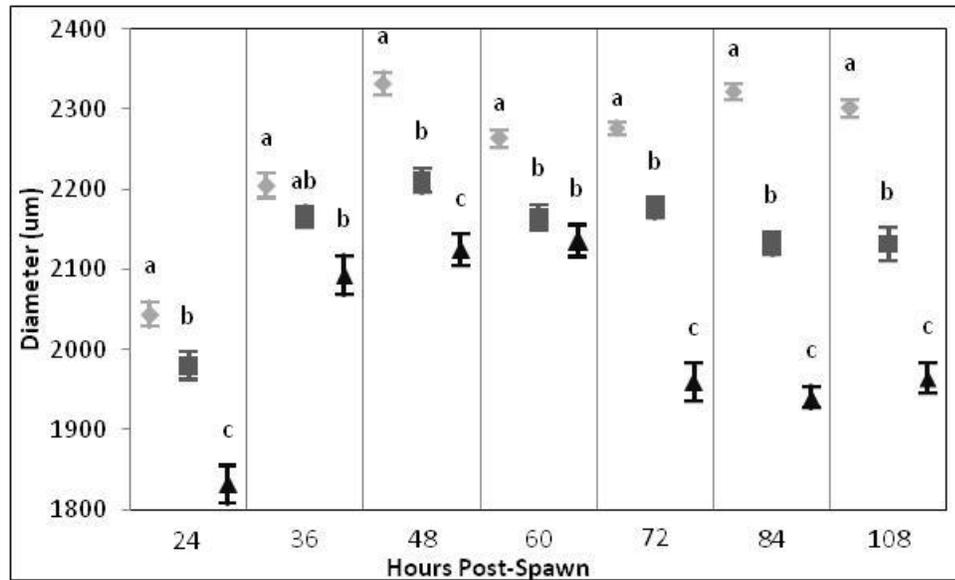
Figure 2.5 Total length of larvae at different times post-spawn.

The size of the yolk sac did not differ significantly among treatments at the first measurement point (Figure 2.6) but subsequently there was a significant effect of both salinity and time on this trait. All treatments showed a reduction over time with minimal vitelline reserves left at 48 hps. The 12.5 psu group had the largest yolk sac remaining at 24 hps (13.8% larger than the 25 psu control) and the 18.75 had an intermediate value but was not significantly different from the 25 psu group at 48 hps.

Figure 2.6 Yolk sac diameter of larvae at different times post-spawn

Mean yolk sac diameter ± SE measured at different times post-hatch for unfed *Cynoscion nebulosus* larvae incubated at three salinities (♦ 12.5 psu , ■ 18.75 psu , ▲ 25 psu). Homogeneous groups from Tukey's a posteriori classification are labeled with the same letter.

The initial size of the oil globule did not differ among treatments (Figure 2.7) but subsequently there was a significant effect of salinity and time on this trait. The oil globule did not differ among treatments at 24 hps. The oil globule was larger in the 12.5 psu group from 36 hps to 48 hps. At 60 hps, all 3 groups had equivalent oil globule size. At 72 hps, the oil globule did not differ among treatments. From 84 hps to depletion, the 12.5 had larger oil globule than controls but not the 18.75 psu group. Altogether these results show that vitelline reserves and oil globule were used at a slower pace in the 12.5 and 18.75 psu groups.
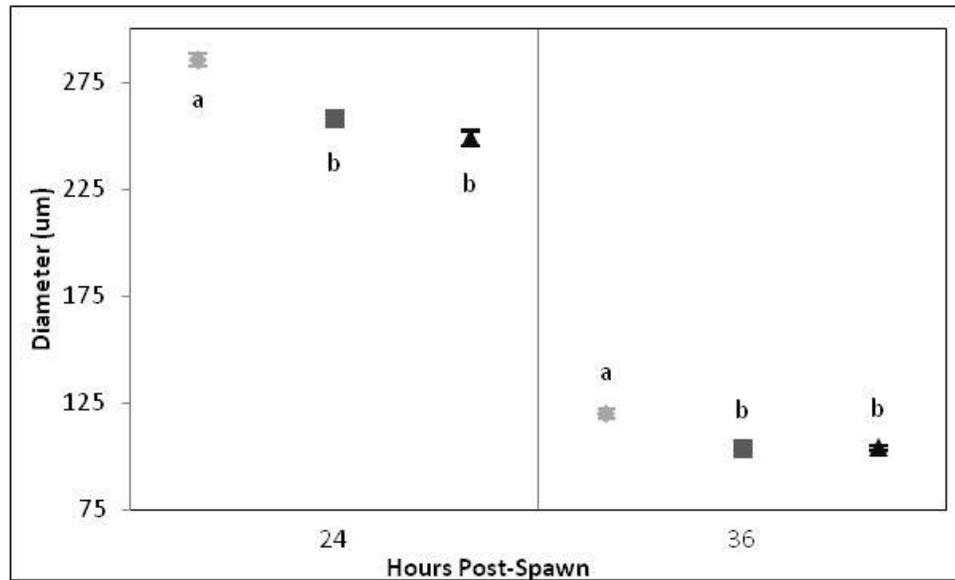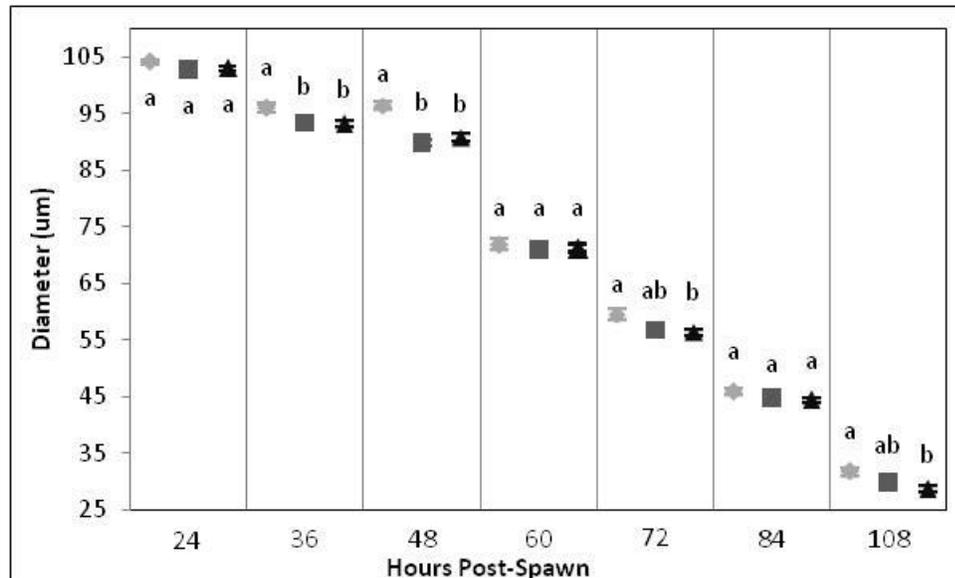
Figure 2.7 Oil globule diameter of larvae at different times post-spawn

Mean oil globule diameter ± SE at different times post-hatch for unfed *Cynoscion nebulosus* larvae incubated at three salinities
(♦ 12.5 psu , ■ 18.75 psu , ▲ 25 psu). Homogeneous groups from Tukey's a posteriori classification are labeled with the same letter.

Larval mouth formation occurred between 48 and 60 hps in all groups.

2.4 Discussion

In this study, the development and fitness of spotted seatrout embryos and newly hatched larvae following transfer to low salinity were investigated. Fertilized eggs were transferred directly from the egg collector of a spawning tank at 29 psu to incubators at 25, 18.75 or 12.5 psu. The 25 psu salinity treatment corresponds to routine incubation and larval culture conditions (Blaylock et al. 2005) and thus is viewed as a control. The 25 and 18.75 psu had similar hatch rates, but incubation at 12.5 psu led to a 35% reduction of hatch rate indicating that embryonic mortality occurred in this group as a result of the treatment. The higher embryonic mortality in the 12.5 psu group could be related to embryo buoyancy in this treatment. The neutral buoyancy of embryos in all groups was 25 psu. Therefore, the 18.75 and 12.5 psu groups were expected to display negative

buoyancy and sink in their incubator treatment if incubators had been maintained in static conditions similar to those in the beakers used to measure buoyancy. Sinking embryos accumulate at the bottom of tanks where they experience anoxic conditions and overall degraded water quality. These conditions would be expected to induce stress and mortality. Incubators were not maintained in static conditions during the experiment. Water was gently circulated using aeration and was renewed at a rate consistent with the Standard Operating Procedure for spotted seatrout larviculture. Water circulation successfully maintained embryos suspended in the water column in the 25 and 18.75 psu groups, but a substantial fraction of embryos in the 12.5 group sank to the bottom of incubators and likely accounted for the higher mortality rate in this group. The lower hatch rate at 12.5 psu also could be due to the osmotic stress experienced by embryos during the transfer. Teleost embryos have a limited osmoregulatory capacity (Varsamos et al. 2005), but during the development to hatch, they are protected from the external environment by the chorion. The chorion is an impermeable barrier that restricts diffusion of molecules in and out of the embryo. The presence of the chorion is thus expected to help the embryo maintain a constant internal environment (including osmotic pressure) even when external salinity changes. Accordingly, the active adjustment and stress induced by the transfer to low salinity before hatch in our experiment may have been minimal. Data from experiments varying salinity during the incubation period in other species indeed indicate that embryos tend to tolerate a wide range of salinity, including values as low as 5 psu (e.g. Berlinsky et al. 2004, Zhang et al. 2010, Perez-Robles et al. 2016). However, Lee and Menu (1981) reported that the tolerance of embryos to salinity change in another estuarine dependent fish, the grey mullet *Mugil cephalus*, varies during

the embryonic development; Embryos transferred before gastrulation were sensitive to osmotic stress and showed reduced viability. Embryos were transferred to treatment salinity within two hours post-fertilization (i.e., before they had reached gastrulation) in our experiment. Therefore, it is possible that the direct transfer operated at that sensitive stage of early development induced some stress. The magnitude of the difference between the original incubation salinity (29 psu) and the treatment salinity was highest for the 12.5 group, which would suggest that a greater amount of stress was experienced by embryos in that group following transfer and could contribute to the higher embryonic mortality.

The salinity in the 12.5 psu group was expected to be close to neutral internal osmolarity (Varsamos et al. 2005). Accordingly, embryos in that group may have experienced less osmotic stress once they overcame the effects of the transfer and adapted to the new salinity conditions. The latter hypothesis is supported by the observations conducted on larvae after hatch. Survival of hatched embryos was close to 100% until the 108 hps sampling point, after which all groups experienced rapid mortality likely reflecting exhaustion of vitelline reserves and lack of energy sources because larvae were not fed in this experiment (Bardon and Saillant 2017). Indeed, the yolk reserves were nearly exhausted at 48 hps and the oil globule was almost consumed by the 108 hps time point when mortality began. There was no difference among treatments in survival during that period indicating that the embryonic mortality in the 12.5 psu group did not translate in continued stress and mortality post hatch. Newly hatched larvae of marine fishes have limited osmoregulatory capacity (Varsamos et al. 2005), but studies challenging larvae to varying salinities in other teleosts indicate that

27

the capability of larvae to tolerate relatively broad ranges of salinities is species

dependent. Berlinsky et al. (2004) observed that newly hatched larvae of *Centropristis*

striata, a strict marine species, experienced lower survival at salinity lower than 20 psu

indicating limited euryhalinity. However, a study in a coastal brackish water species,

Takifugu flavidus, revealed tolerance to salinities as low as 5 psu, which is consistent

with conditions in their coastal habitats and comparable to the values tested in the present

experiment (Zhang et al. 2010). The high survival in the low salinity treatments tested in

this study on spotted seatrout seems consistent with the range of estuarine conditions

spotted seatrout larvae are expected to experience during early life stages in their natural

environment.

The lower salinity treatments, particularly the 12.5 psu treatment, were closer to

osmotic equilibrium and were therefore expected to require less energy to maintain

internal homeostasis. The slower depletion of vitelline reserves and oil globule in the

lower salinity group (intermediate at 18.75 psu, slowest at 12.5 psu) is consistent with

this hypothesis with the 12.5 psu group expected to require less energy for

osmoregulation (because external salinity was close to internal osmolarity) while the

energy needs of the 18.75 psu group would be intermediate. The slower depletion of

yolk reserves at low salinity would be expected, a priori, to lead to longer survival of

unfed larvae and consequently a potentially longer critical period where larvae transition

to exogenous feeding. The critical period is defined as the end of the endogenous feeding

phase when larvae have sufficiently developed to initiate exogenous feeding and still

have energy reserves left from the residual yolk that they can use for maintenance and

prey capture until energy from the ingested nutrients become available for metabolism

(Kamler et al. 2008, McCasker et al. 2014). Larvae that have exhausted all their reserves prematurely do not have energy left to capture prey and transition to energy and nutrients from exogenous food and are, therefore, expected to die. A slower depletion of yolk reserves is, therefore, expected to be associated with a longer critical period and higher survival rate through the initiation of first feeding. Survival did not appear to be longer at low salinity in this experiment despite a slower depletion of vitelline reserves in these groups. However, it is possible that larvae did survive longer in the 12.5 and 18.75 psu groups but that the differences in survival duration between groups were too small to be revealed by the comparison of survival rates at 12-hr time intervals used in this study. Monitoring the fitness of larvae at low salinity during the feed transitional period and the subsequent larval culture is warranted to fully understand the feasibility of culture at low salinity.

Larvae did not grow significantly in length during the monitoring period. During the pre-feeding period, larvae undergo major ontogeny changes including the development of digestive organs, eyes, and mouth and usually do not grow much in length (Govoni et al. 1986). Larvae were slightly longer in the low salinity groups and this was observed from the first measurement point 24 hours post-spawn. Fridman et al. (2011) reported that larvae hatched from embryos incubated at 0 psu were longer than those hatched from embryos incubated at higher salinities in *Oreochromis aureus* and Brown et al. (2011) found that *Fundulus grandis* larvae hatched from embryos incubated at 20 psu were longer than those from embryos incubated in 10 psu salinity water. In the latter study, the authors discussed a delayed hatch leading to longer larvae at 20 psu and/or mortality of shorter larvae during incubation at that salinity. Embryo diameter was

29

larger at low salinity in this experiment suggesting that the size advantage for the lower

salinity groups may have been acquired at least in part before hatch. The relative size of

the three groups did not change during the experiment, which is also consistent with the

hypothesis that the size advantage was acquired during the embryonic development or

shortly thereafter (before the first measurement point on larvae). It is possible that the

higher embryonic mortality observed at low salinity, in particular in the 12.5 psu group,

selected for larger eggs and consequently longer hatched larvae. This hypothesis could be

evaluated by monitoring the size of dead and live embryos during a similar challenge.

2.5 Conclusion

This study indicates that a reduction in hatch rate is to be expected when embryos

produced from a broodstock spawned at high salinity are incubated at low salinity (as in

the 12.5 psu treatment), while a moderate reduction in salinity to 18.75 has no significant

effect on hatch. This embryonic mortality is likely due to the negative buoyancy at 12.5

psu and may be resolved with increased circulation in tanks, although the level of water

movement, if induced by aeration, may end up negatively affecting the survival of

embryos and newly hatched larvae (Sakakura et al. 2007). Protocols for incubation at low

salinity (e.g., 12.5 psu) also could account for the embryonic mortality by increasing the

stocking density to achieve the desired quantity of larvae needed for production.

Alternatively, transfer to low salinity could be delayed until hatch and/or be performed

more progressively during incubation to avoid embryonic mortality. The tolerance of

newly hatched larvae or embryos to the osmotic stress incurred by such protocols remains

to be evaluated. The lack of mortality post-hatch, even in groups incubated at 12.5 psu,

until the first feeding stage is encouraging and suggests that hatched larvae incubated at

low salinity may remain viable and initiate feeding. The success at first feeding and subsequent development of larvae incubated at low salinity therefore needs to be documented to determine the feasibility of larval culture at low salinity. Finally, acclimation and spawning of broodstock at low salinity may improve the fitness of embryos and larvae at low incubation salinity as shown by previous studies conducted using seatrout broodstock from other bay systems (Kucera et al. 2002 A). Evaluation of maturation and spawning of Mississippi seatrout broodstock at low salinity as well as the salinity tolerance of embryos produced by these broodstocks is warranted.

CHAPTER III – THE EFFECTS OF SALINITY ON GROWTH AND SURVIVAL OF

SPOTTED SEATROUT (*CYNOSCION NEBULOSUS*) REARED IN

RECIRCULATING AQUACULTURE SYSTEMS

3.1 Introduction

The spotted seatrout (*Cynoscion nebulosus*) is the most recreationally targeted

fish in the northern Gulf of Mexico. The species is cultured for stock enhancement in

several states including Texas, Mississippi, and Florida. There also is some interest in

commercial production in the region. Aquaculture in recirculating aquaculture systems

(RAS) or in closed ponds presents several potential advantages over alternative culture

methods such as cage culture including reduced environmental impacts. Currently,

culture of seatrout for stock enhancement in Mississippi is performed at a salinity of 25

psu. Culture at low salinity would be beneficial in inland areas where saltwater is not

easily accessible and for production in low-salinity coastal ponds.

The spotted seatrout, as most other sciaenids, is an estuarine dependent fish that

frequents brackish habitats characterized by low and fluctuating salinity. The species is

considered euryhaline meaning that it can tolerate a broad range of salinity during its

estuarine ecophase which extends from the late larval stage to adult. Salinity at or near

that of the internal environment has been shown to be beneficial in other euryhaline

fishes with improved zootechnical performance (survival, growth and food conversion

efficiency) and water clarity in tanks (e.g., Saillant et al. 2003). Pilot studies of spotted

seatrout at the GCRL suggested that the species can be cultured at low salinity during the

grow out period, but no formal assessment of the performance of the species at different

salinities has been conducted to date.

This study aimed to document the effects of rearing salinity on the zootechnical performance of juvenile and sub-adult potted seatrout including survival, growth, and carcass quality during grow-out in RAS to assist in the evaluation of protocols involving low salinity for the species.

3.2 Materials and Methods

The experiment was conducted using two groups of spotted seatrout at different stages of development. One group was 42 days old (i.e., 42 days post-hatch, dph) at stocking while the other group was 210 dph corresponding to the beginning and second half of grow out. Fish were stocked into four systems featuring four 1-m^3 tanks each. Each of the systems was run at a different salinity (10, 15, 20, or 25ppt). Two tanks in each system were stocked with 42 dph fish and the other two tanks in each system were stocked with 210 dph fish. All tanks were treated identically throughout the experiment except for the salinity treatments. Groups were monitored for survival and growth traits every 75 days until fish reached 365 dph at which point the experiment was terminated and all remaining fish were harvested for carcass quality trait measurements.

3.2.1 Experimental Animals

Fish used in the experiment were produced from a spontaneous spawn of a captive group of spotted seatrout broodstock. Broodstock management followed methods described in Chapter II. The two groups of fish (42 dph and 210 dph) were from the same broodstock population.

Embryos produced during spontaneous spawning events were cultured in intensive recirculating systems as described in Blaylock et al. (2005). Culture is performed at a constant salinity and temperature of 25 psu and 27 °C, respectively, under

a 24 hours photophase (200 lux). The initial density is 15 larvae L^{-1}, and larvae are fed a sequence of enriched rotifers and Artemia nauplii and transitioned to prepared feeds from 9-22 dph. Oxygen is checked daily and maintained above 80% saturation. Ammonia and nitrite levels are checked daily and kept below 2.5 ppm. At approximately 23-26 dph, fingerlings were harvested, counted, and transferred to nursery or growout systems.

3.2.2 Growout Management

The first group of fish was harvested from larval rearing and relocated to 10-m^3 growout tanks for 157 days and then transferred to the experimental 1-m^3 nursery tanks on April 15. The second group of juveniles was harvested from larval rearing and relocated to the experimental 1-m^3 nursery tanks on April 11. Both groups were maintained at 25 psu, 26 °C, and a 24 hr photophase until the beginning of the experiment.

On April 29, 2016, the first and second groups of juveniles (first group aged 42dph and second group aged 210 dph) were considered acclimated into experimental systems. There were four recirculating systems and each system was comprised of four 1-m^3 tanks. Two culture tanks in each system were stocked with 42-dph fingerlings at 280 individuals tank^{-1} while the other two were stocked with 210-dph juveniles at 210 individuals tank^{-1}. Each system of four tanks was assigned one of 4 salinity treatments (10, 15, 20, or 25 psu). Salinity was gradually brought to the treatment salinity over a period of 14 days for all treatments (i.e., at the rate of 0.36 - 1.07 psu per day depending on treatments).

Fish were fed a commercial pellet (Skretting, 57% protein and 15% lipid) at a target 5% body weight daily by automated belt feeder. Daily rations were adjusted to

satiation based on observations of food intake. The photophase was maintained at 24 hr with light intensity at the surface of the tanks averaging 100 lux. The temperature, salinity, pH and dissolved oxygen were monitored daily prior to each morning feeding using a YSI556. Temperature was maintained at 26 ±1°C, salinity at 10, 15, 20, or 25 psu depending on treatment, pH at 8.1 (±0.3), and dissolved oxygen between 4 and 10mg L^{-1}. The pH was maintained by addition of sodium carbonate as needed. Alkalinity, ammonia, nitrite and nitrate concentrations were measured daily for the first month and every other day thereafter using Hach test strips (Hach – Loveland, CO). These parameters were maintained between 180-240 ppm CaCO$_3$ for Alkalinity, 0-1 ppm for ammonia, 0-1 ppm for nitrite, and $< \sim$100 ppm for nitrates. Water was changed if water quality parameters departed from the above ranges. Water lost in each system was replaced with well water adjusted to the appropriate salinity.

3.2.3 Sampling and Measurements

Mortalities were recorded daily throughout the study. Every three months, all individuals in each tank were lightly anesthetized in 50-150 ppm MS-222 (dependent on reaction to dosage), weighed to the nearest g, measured for total length to the nearest mm, and returned to their original tanks. The 210-dph age class was harvested when the average length of fish reached 28-30 cm, an appropriate size for the collection of carcass quality data. The 42-dph age class was terminated at the same time. For the final measurement, fish were killed with an overdose of MS-222. A suite of carcass quality traits was measured on 30 specimens from each culture tank. The measurements included total weight (whole fish), fillet weight (two fillets combined), gonad weight, sex

(M/F/Unknown), liver weight, and combined weight of other organs (heart, swim bladder, and digestive tract).

3.2.4 Data Analysis

The two groups (42-dph and 210-dph stocking age) were analyzed separately. The fillet weight, gonad weight, liver weight and combined weight of other organs were standardized to body weight for analysis to allow comparison among individuals and treatments. Fulton's condition coefficient was calculated from weight and length data as $100,000 \text{ x } (W/L^3)$ where weight is in grams and length is in millimeters.

A hierarchical ANOVA accounting for the fixed effect of treatment and replicate tank nested within treatment was used to test the effect of salinity treatments on growth and carcass quality parameters. For the last measurement date (267 days after the beginning of the experiment), sex was included as a fixed factor along with the sex x salinity interaction treated as fixed. The assumptions of homogeneity of variance and normality were checked through Bartlett's Test and Shapiro-Wilks test, respectively. The overall survival rate (calculated as the number of fish alive at the time of a given measurement /initial number of fish stocked in the tank) was compared between sampling dates using a one factor ANOVA after arcsine square root transformation. The Tukey test was used to determine differences between treatment means. When assumptions of normality and homoscedasticity were rejected, transformations were attempted (natural log, square root, or arcsine square root) to improve fit of the transformed datasets to these assumptions. When transformation of data was ineffective, the WelchADF package for robust hypothesis testing in unbalanced multivariate mixed models with heteroscedastic and non-normal data (Villacorta 2017) was used to confirm the results of parametric

ANOVA. Analyses were completed using R and R-Studio programs (R Studio Team

2019) (R Core Team 2019). A p-value threshold of 0.05 was used in all tests. Survival

rates were compared among treatments using one factor ANOVA.

3.3 Results

Ninety six percent of the fish from the 15 psu treatment were lost between day 61

and 75 due to infection with a parasitic mite. This treatment was therefore excluded from

further analysis. There were no significant differences in survival among the other

treatments over the course of the study (Figures 3.1 and 3.2).

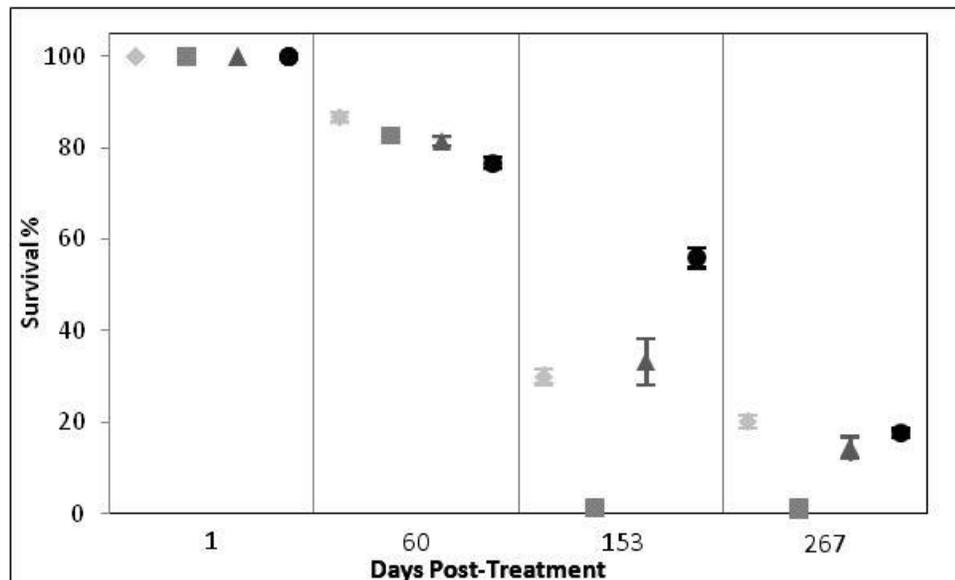Figure 3.1 Survival rate during the experiment (210-dph age class).

Survival rates ±SE of juvenile *Cynoscion nebulosus* groups exposed to four salinity treatments

(◆ 10 psu, ■ 15 psu, ▲ 20 psu, ● 25 psu) beginning at 42 days post fertilization.
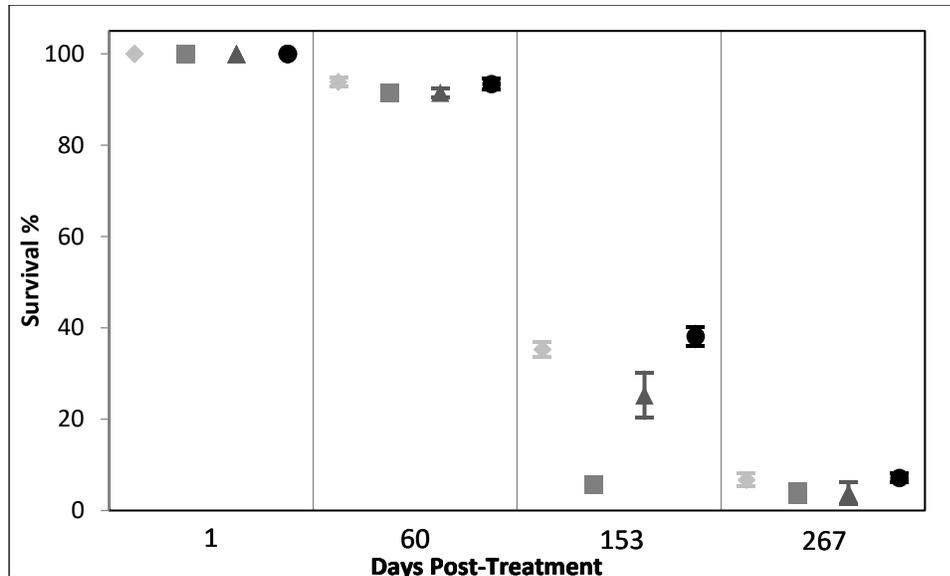
Figure 3.2 Survival rate during the experiment (42-dph age class).

Survival rates ±SE of juvenile *Cynoscion nebulosus* groups exposed to four salinity treatments

(♦ 10 psu, ■ 15 psu, ▲ 20 psu, ● 25 psu) beginning at 210 dpf.

Large fish suffered heavy mortality during the last phase of the experiment in all groups. Mortality was due to injuries incurred hitting the side of the tanks when fish reacted to movement around tanks or other forms of stress. Larger fish were preferentially impacted because they hit the sides of the tanks harder.

Total length differed significantly among groups at all three measurement points (Figures 3.3 and 3.4). In the 42-dph age class at day 60, the 15 psu group was the longest and the 10 psu group the shortest while the 20 and 25 psu groups could not be distinguished from the 10 psu or 15 psu groups (Figure 3.3). In the 210-dph age class at day 60, the mean length in the 25 psu group was shorter than in the other 3 salinity groups (Figure 3.4). At day 153, fish in the 10 psu group were longer than those in the 20 and 25 psu groups in both the 210-dph and 42-dph age classes. At day 267, the 20 psu group fish were shorter than the 10 psu group fish. The 25 psu group did not differ

38

significantly from either the 10 or 20 groups for the 42-dph age class (Figure 3.3). For the

210-dph age class, the 10 psu group was the largest and the 25 psu group the shortest

while the 20 psu group was intermediate and could not be distinguished from either the

10 or 25 psu groups (Figure 3.4).
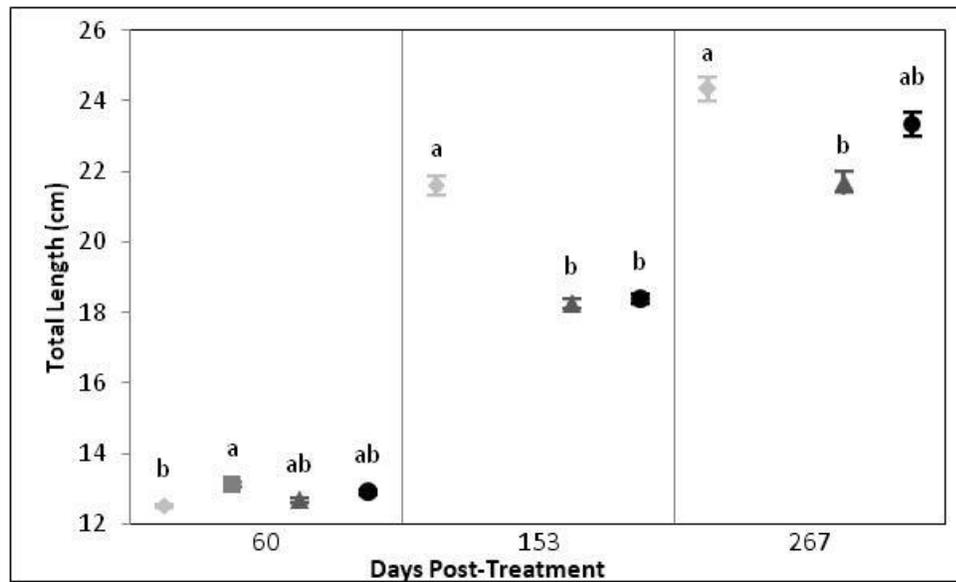
Figure 3.3 Total length at different measurement dates (42-dph age class)

Mean length ± SE of juvenile *Cynoscion nebulosus* groups subjected to 4 salinity treatments (♦ 10 psu, ■ 15 psu, ▲ 20 psu, ● 25 psu)
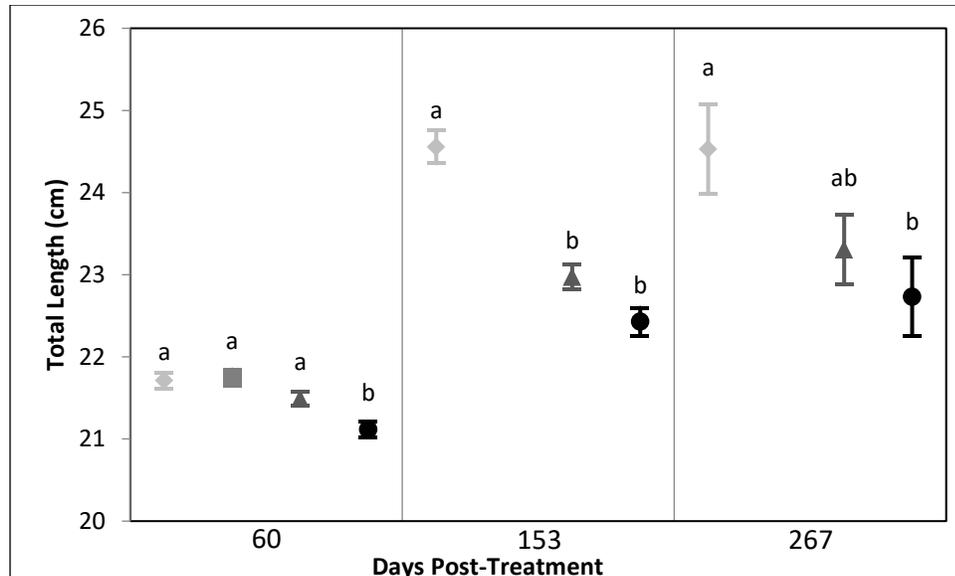
beginning at 42 dph.

Figure 3.4 Total length at different measurement dates (210-dph age class)

Mean length ± SE of juvenile *Cynoscion nebulosus* groups subjected to 4 salinity treatment (◆ 10 psu, ■ 15 psu, ▲ 20 psu, ● 25 psu) beginning at 210 dph.

The 10 psu group had a higher condition factor than the 20 and 25 psu groups at day 60 in small fish, while the 15 psu groups could not be distinguished from any of the other groups (Figure 3.5). In large fish, the 15 psu groups had a higher coefficient than the 25 psu groups while the 10 and 20 psu groups were intermediate (Figure 3.6). At day 153, the 10 psu group had a higher condition factor than the 20 psu which was higher than the 25 psu group in both small fish and large fish groups. At day 267, the three treatments were homogeneous in small fish or the 10 and 25 psu groups had a higher condition factor than the 20 psu group (large fish).

Figure 3.5 Condition factor of 42-dph age class fish at different measurement dates

Mean Condition Factor ± SE of juvenile *Cynoscion nebulosus* exposed 4 to salinity treatment

(◆ 10 psu, ■ 15 psu, ▲ 20 psu, ● 25 psu) beginning at 42 dph.

Figure 3.6 Condition factor of 210-dph age class fish at different measurement dates

Mean Condition Factor ± SE of juvenile *Cynoscion nebulosus* groups subjected to 4 salinity treatment

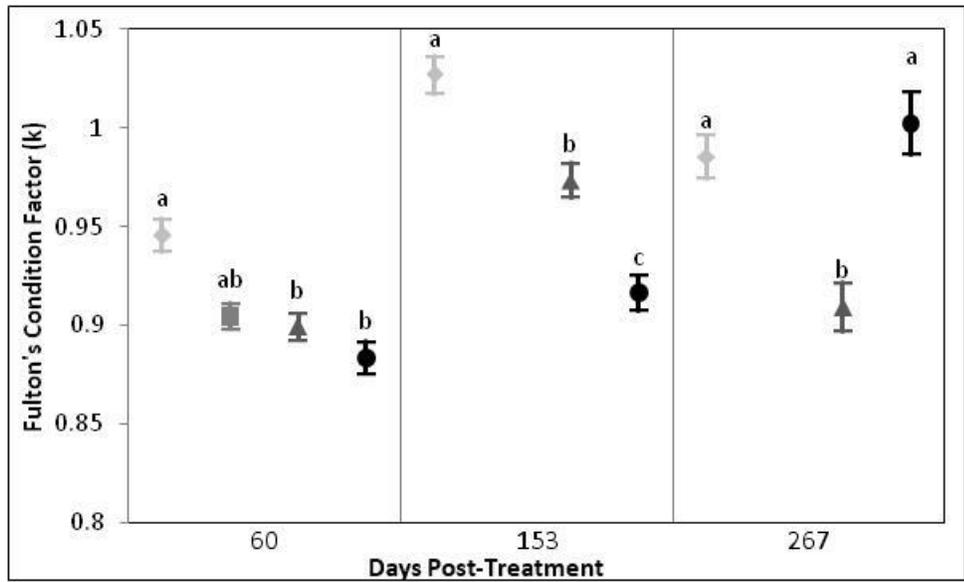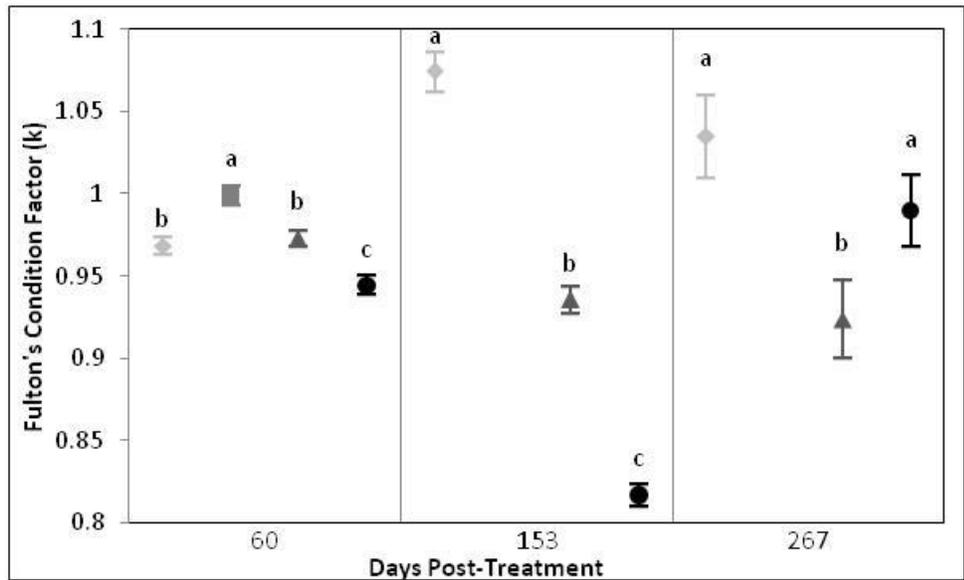(◆ 10 psu, ■ 15 psu, ▲ 20 psu, ● 25 psu) beginning at 210 dph.

The analysis of carcass traits focuses on females because only one male could be processed in the 10 psu group and the other two treatments are very close in salinity. The ratio of liver weight to body weight (hepato-somatic index) was larger in the 10 and 25 psu group than in the 20 psu treatment. The ratio of gonad weight to body weight (gonado-somatic index, GSI) differed significantly among the groups with the highest GSI found in the 10 and 25 psu group while the lowest GSI was recorded in the 20 psu group. The other carcass traits did not differ among treatment groups (Table 3.1).

Table 3.1 Mean ±SE of carcass quality traits in *Cynoscion nebulosus* groups reared at different salinities

	10 psu		20 psu		25 psu	
Variable	Mean	±SE	Mean	±SE	Mean	±SE
Fillet	0.3427	0.0073	0.3147	0.0097	0.3300	0.0092
Carcass	0.5867	0.0070	0.6314	0.0099	0.6201	0.0092
Liver	0.0137	0.0016	0.0116	0.0007	0.0165	0.0014
Gonad	0.0040	0.0004	0.0022	0.0004	0.0035	0.0006
Other Viscera	0.0528	0.0034	0.0401	0.0015	0.0298	0.0013

Mean ±SE of the ratio of each carcass component weight to body weight measured in females cultured at 3 salinities for 267 days.

A high positive correlation was identified between body weight and all the carcass component wet weight (r > .57 data not shown). Table 3.2 shows the correlation between carcass components standardized to body weight. The correlation between the ratio of fillet weight to body weight (fillet yield) and body weight was positive and intermediate while the carcass yield showed a negative and intermediate correlation to body weight. The other carcass component ratios were uncorrelated to body weight.

Table 3.2 Standardized carcass traits correlation matrix

X	Body weight	Fillet	Carcass	Liver	Gonad	Viscera
Body weight	1					
Fillet	0.571121	1				
Carcass	-0.57139	-0.9326	1			
Liver	-0.05845	0.086692	-0.15121	1		
Gonad	-0.01991	-0.02602	-0.10354	0.11393	1	
Viscera	0.120385	-0.05917	-0.26736	-0.20177	0.15685	1

Correlation matrix of standardized carcass quality traits (ratios of body compartment wet weight to body weight).

The Body weight, total length and gonadosomatic index were greater in females than males. The small fish group had 21% more females than the older fish group but allocation of females to salinity tanks was random and uncorrelated to salinity treatments.

3.4 Discussion

The experiment was impacted by a parasitic mite infestation in the system containing the 15 psu treatment group during the early phases of the trial. The infection led to near total mortality of fish in that system preventing further evaluation of the 15 psu treatment. The four recirculating systems hosting individual salinity treatments were independent and the other three treatments (10, 20 and 25 psu) showed only minor impacts of the outbreak. The trial therefore continued with the remaining three treatment groups. While overall survival throughout the experiment was low, mortality rates did not differ significantly between the three salinity treatment groups, indicating that the tested range of salinity is compatible with survival of spotted seatrout juveniles. This result is consistent with the range of salinities encountered by juvenile spotted seatrout in

the wild (Brown-Peterson et al. 2002, GSMFC 2006) and with the results of preliminary growth trials evaluating similar low salinity treatments in our laboratory (GCRL unpublished results).

Fish grew larger at low salinity (10 psu group) in both the 42-dph and 210-dph age class groups during the experimental period. Faster growth at low salinity was reported for other euryhaline estuarine dependent fish such as the red drum (Forsberg et al. 1996, Tomasso and Kempton 2000) and the European seabass (Saillant et al. 2003). Improved growth at low salinity in these species and the spotted seatrout is consistent with the low salinity habitat frequented by juveniles during their estuarine growth ecophase. The life history of juvenile spotted seatrout is still not well understood due to difficulties sampling and monitoring the species before specimens recruit to the fishery (GSMFC 2006), but available data suggest that juveniles commonly use habitats with salinity levels similar to the low salinity treatment (10 psu) tested in this study. The 10 psu treatment in this experiment also is closest to the fish's internal osmolarity. It was, therefore, expected that seatrout in the 10 psu group would require less energy to maintain osmotic balance than fish in the 20 or 25 psu groups. The reduced metabolic energy needed for osmoregulation in this group was, therefore, expected to translate into a lower food conversion rate and ultimately faster growth considering that food was not limiting during the trial. Higher conversion rate at low iso-osmotic salinity was indeed reported in other species that use low salinity habitats and grow faster in these conditions such as the seabass (Saillant et al. 2003), the gilthead seabream (Bodinier et al. 2010, Laiz-Carrión 2005), and the turbot (Imsland et al. 2001). Hormonal processes involved in growth also may have been influenced by culture salinity. Growth hormone and prolactin

44

are both involved in adaptation to saltwater and could have been modulated by the salinity treatments applied. The effects of salinity on growth hormone appears to be species specific. In anadromous salmonids, this hormone is assumed to be involved in saltwater adaptation, but in some euryhaline sparids, its secretion was found to be stimulated in iso-osmotic conditions (Deane and Woo 2009). Considering the life history of spotted seatrout and the faster growth at low salinity observed in this study, it is possible that growth hormone secretion is stimulated in iso-osmotic conditions in this species. Monitoring of growth hormone levels at different salinity would, therefore, be interesting for future studies. Growth hormone secretion also is strongly influenced by temperature. Studies in other euryhaline species such as turbot (Imsland et al. 2001) indicated occurrence of salinity x temperature interactions where secretion of growth hormone at high temperature was stimulated at higher salinity, while higher levels of this hormone were found at low salinity when temperature was low. It would be interesting to investigate temperature x salinity interactions in the spotted seatrout in the future, in particular to assess whether there exists an optimal combination of temperature and salinity to maximize growth efficiency at low salinity.

Salinity also could have influenced the growth parameters by affecting sexual maturation. Fish averaged almost 10 inches in total length in all groups at the end of the experiment and would have reached the size at which sexual maturation begins in spotted seatrout (Brown-Peterson et al. 2002). Fish investment in gametogenesis when they become reproductively active leads to reduced growth rates and higher conversion rates as energy and nutrients are diverted towards the production of gametes instead of somatic growth (Davidson et al. 2014). Spotted seatrout can start maturing gametes at one year of

age in the wild, but are assumed to delay investment in gametogenesis and completion of the reproductive cycle to their second year of life in most cases (GSMFC 2006). However, sexual maturation can occur at a younger age in captive conditions where growth rates are typically faster. Indeed, late vitellogenic females and spermiating males have been observed in one-year old captive stocks at GCRL (unpublished results). The photophase was kept constant at 24 hours during this study which would be expected to inhibit sexual maturation (Bromage et al. 1994), but culture at warm temperature (and constant photoperiod) has proved sufficient to induce gamete maturation of cultured seatrout in recirculating systems (GCRL unpublished results). In this study, most fish were not advanced in gametogenesis and were scored 2 or less on the sexual maturity scale of spotted seatrout indicating reduced investment in gametogenesis (GSMFC 2006). Gonadosomatic and hepatosomatic indices were low in all groups, consistent with the results of gonad maturity staging. However, differences among salinity groups for GSI and HSI were significant even if they were minimal in magnitude. Salinity may impact sexual maturation if fish are grown to larger sizes than in this experiment and reach more advanced stages of sexual maturity. In the wild, the final phases of gamete maturation in spotted seatrout are occurring in higher salinity waters where they spawn (e.g., offshore near the barrier islands). Therefore, high salinity could trigger late phases of gamete maturation and consequently slow somatic growth. Low salinity was found to inhibit ovulation in another estuarine dependent fish, the black bream *Acanthopagrus* butcheri (Haddy and Pankhurst 2000) and could contribute to control maturation in cultured stocks. Further monitoring of gamete maturation on the maturity scale and growth of

46

spotted seatrout as fish reach larger sizes and engage in gametogenesis more actively would be useful to assess the effects of salinity on growth to larger market sizes.

The full interpretation of results on growth parameter in the experiment is challenging because of the heavy mortality experienced by all groups during the trial. The larger fish suffered heavier mortality especially during the later phases of the experiment effectively truncating the size distribution in all groups. This may have affected the size distributions differentially in the different salinity groups if some treatments grew faster and contained larger fish as suggested by the very slow plateau growth in the large fish group. Therefore, the effects of salinity on growth and the other traits measured in this study would need to be confirmed in an experiment with higher survival rates and growth rates more typical of intensive production of seatrout.

The effect of salinity on spotted seatrout growth was also assessed using Fulton's Condition Factor (CF, Heincke 1908). As the experiment progressed, the condition of fish showed a trend at the second measurement point (day 153) of increasing condition at lower salinity. This trend was not maintained at the end of the trial where the 10 and 25 psu treatments did not differ significantly in condition coefficient. The latter result may be due in part to the poor growth performance at the end of the trial, especially in the large fish group where almost no growth was recorded. As discussed above for growth in length, mortality may have affected fish non-randomly with respect to condition and compromised the comparison of treatments, at least for the last measurement point. Condition coefficient is often used as a measured of health and fitness but may also be used as an indicator of investment in gametogenesis in particular in females. This relationship could not be studied in this work considering the very low gonado-somatic

47

indices recorded, but condition could be evaluated as an indicator of gamete maturation for larger fish in future studies.

Carcass quality traits measured in the experiment were not affected by culture salinity indicating that production in low salinity water would not be expected to induce negative effects on fillet yield or gutted yield.

There was a significantly greater percentage of females present in the 42-dph group at the end of the experiment than in the 210-dph groups. This result likely reflects the effect of mortality in the large group during the experiment. Mortality affected primarily large fish, which were expected to be females, thereby directly reducing the proportion of females. In contrast, mortality due to large fish size was less severe in the 42-dph age class and may have had less impact on the sex ratio.

3.5 Conclusion

The study was limited by the slow growth and heavy mortality recorded in all groups, particularly the 210-dph age class. Tank size was likely a major factor that led to mortality events. Larger fish injured themselves by hitting the sides of the tanks when there were even subtle movements in the culture room. Repeating this trial in larger tanks compatible with fast growth rates and high survival typical of commercial scale production of seatrout would be useful to confirm the trends observed during this experiment. The observation of a trend of faster growth at low salinity indicates that culture of spotted seatrout at the lower salinity level tested is not only possible but that it would likely be beneficial by promoting faster growth rate and improved feed conversion ratios.

CHAPTER IV – CONCLUSION

4.1 General Conclusion

The objective of this work was to assess the potential for low salinity culture in spotted seatrout. Two culture phases were investigated. Chapter II examined the effects of salinity on embryo and newly hatched larvae during the incubation period of larval culture from fertilization to initiation of feeding while Chapter III examined effects during the grow out of juveniles.

Results of the incubation experiment described in Chapter II indicate that spotted seatrout embryos and larvae can be incubated at 18.75 psu through the pre-feeding period with no notable effects on hatch rate and survival post hatch. Newly hatched larvae tolerated salinity as low as 12.5 psu with no negative impact on viability until the end of the endogenous feeding period, but significant embryonic mortality occurred attributed primarily to the negative buoyancy of eggs in that treatment. As discussed in Chapter II, the negative buoyancy and mortality at low salinity may be mitigated through improved water circulation in tanks, but this management approach may incur mortality through increased occurrences of mechanical shocks or negative effects of air bubbles on embryos (Sakakura et al. 2007, Honryo et al. 2016). This study did not reveal significant effects of salinity on survival duration post-hatch. However, a small effect of salinity on this trait might not have been detected due to the length of the monitoring interval (12 hr). An interesting finding was that the depletion of vitelline reserves was slower at low salinity. Larvae cultured at 12.5 psu may, therefore, display improved transition to exogenous feeding; although, the effectiveness of this transition at low salinity will need to be

evaluated in true larviculture conditions where buoyancy may affect feeding success as well.

The grow out challenge revealed that survival was not affected by salinity in the tested range and that culture at low salinity improved growth rate. A major issue with the implementation of the grow out challenge was the low survival and poor growth performance of fish, especially for the larger (210-dph) size-group. The experiment was conducted in 1-m^3 tanks that appeared too small to promote growth and welfare of animals once they reached larger sizes. Consequently, heavy mortality was sustained by all groups especially during the late phases of the experiment where mean size showed little change over time and plateaued as fish approached 24 cm TL. Fish were skittish and reacted with erratic swimming behaviors to any stimulus around the tanks. Injuries were sustained by hitting the sides of the tanks or jumping out of tanks during these escapement episodes which led to mortality. Larger fish appeared more susceptible to mortality due to these stress reactions, possibly because they hit the sides of tanks harder than smaller fish. The apparent lack of growth in the large fish group may be due to a combination of effects of stress impacting food intake and food conversion, but also the truncation of size distributions that resulted from the removal of the larger fish present in the tank as discussed above. The finding of improved growth at low salinity, despite these limitations, is therefore noteworthy and warrants further investigation. The effects of salinity on growth parameters should, however, be confirmed and properly quantified in larger tanks typically used for grow-out of spotted seatrout (10-ft diameter and 18-ft diameter are usually used for grow-out of this species at the GCRL-TCMAC).

This work indicates that low salinity culture at the grow out stage is possible and would be expected to be beneficial promoting faster growth and lower food conversion ratios. Growing out spotted seatrout at low salinity would lead to a major reduction in production costs considering that this phase of the culture uses the largest amount of water. Water also needs to be renewed, on average, at higher rates during grow out to remove wastes produced during large-scale intensive culture. This experiment evaluated growth and zootechnical parameters at salinity as low as 10 psu. The 10 psu treatment was expected to be close to the internal osmolarity of approximately 300 mOsm (Kültz 2015). Culture salinity matching this value is expected to achieve optimum growth and the lowest food conversion ratios due to reduced energy needs for osmoregulation. It is likely that spotted seatrout will tolerate even lower culture salinity considering the low and fluctuating salinity of their natural habitat. Further work could, therefore, explore the tolerance, growth, and food conversion ratios of spotted seatrout at salinities lower than 10 psu and determine an optimal salinity for culture accounting for all aspects of the production cost (including duration of the grow out cycle, feed conversion ratio, and salt cost). A moderate reduction of performance at a salinity lower than 10 psu may be tolerable if the savings in salt costs outweigh a moderately longer production cycle, higher mortality and/or higher food conversion ratio, for example. Further study at salinities ranging from very low values close to freshwater to a salinity matching the internal osmolarity seems, therefore, a logical next step to evaluate grow out at low salinity.

Closing the spotted seatrout life cycle at low salinity would involve culturing broodstock and larvae in these conditions. This work indicates that the early phases of

incubation may be challenging at low salinity because of the buoyancy issue discussed above, although this may be partially resolved if broodstock conditioned for maturation at low salinity spawn eggs with a lower neutral buoyancy. If this was not the case, incubation may be performed at higher salinity until hatch and lowered thereafter. Incubation is performed at high density (1 embryo mL^{-1}) and, therefore, salting up incubator systems would be a minimal cost as compared to the cost of performing other culture phases at high salinity such as the grow out as discussed above.

The culture of seatrout larvae at low salinity past the endogenous feeding phase remains to be studied. If larvae can be successfully transitioned to exogenous food at low salinity, it seems likely that they will tolerate low salinity for the remainder of larval culture. Live feed maintenance at low salinity will need to be evaluated. Both rotifers and *Artemia* spp. are reported to tolerate low salinity in the range tested in this study but impacts of low salinity culture on their nutritional value cannot be excluded. Finally, broodstock maturation and spawning at low salinity also remains to be studied. Spotted seatrout are found in low salinity waters throughout the summer spawning season although they are thought to move slightly offshore in higher salinity waters to spawn (GSMFC 2006). According to this scenario, the initial phases of gametogenesis may be achieved in low salinity water, but the completion of the final stages of gamete maturation (including ovulation and spermiation) would require higher salinity. Low salinity culture may therefore be possible for most of the year-round broodstock culture cycle, even if salinity needs to be increased temporarily to allow spawning. The broodstock maturation salinity also may impact embryo and larvae buoyancy as shown by Kucera et al. (2002 B), and manipulation of this variable may improve the survival

during incubation at low salinity as discussed above. Further study of broodstock

maturation, spawning, and egg quality at low salinity to test these hypotheses is

warranted.

APPENDIX A – IACUC

THE UNIVERSITY OF SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	17103001
PROJECT TITLE:	Development of aquaculture and stock enhancement for marine finfish at the Thad Cochran Marine Aquaculture
PROPOSED PROJECT DATES:	11/2017 – 09/2020
PROJECT TYPE:	Modification – addition of species
PRINCIPAL INVESTIGATOR(S):	Eric Saillant
DEPARTMENT:	Division of Coastal Sciences
FUNDING AGENCY/SPONSOR:	N/A
IACUC COMMITTEE ACTION:	Designated Review Approval
PROTOCOL EXPIRATON DATE:	September 30, 2020
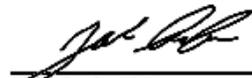
June 15, 2018

Jake Schaefer, PhD
IACUC Chair

Date

54

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