ABSTRACT

GLASS CAMPBELL, LINDSAY ANN. Direct and Indirect Effects of Hypoxia on Juvenile Fish in the Neuse River Estuary, North Carolina. (Under the direction of Dr. James A Rice).

In North Carolina and elsewhere, there is concern that excessive nutrient loading and resulting hypoxic conditions in coastal ecosystems are adversely affecting living resources, but quantifying the effects on fish can be difficult. Direct exposure to hypoxia can reduce fish growth or survival, but fish can also rapidly detect and avoid low dissolved oxygen (DO) levels. In the wild, hypoxia may reduce fish growth via direct exposure, or indirectly (e.g., costs of avoidance, reduced food availability, density-dependent effects in oxygenated refuges). I evaluated this hypothesis for juvenile spot *Leiostomus xanthurus*, a representative estuary-dependent species. By monitoring water quality and fish density across the Neuse River Estuary (NRE) during varying water quality conditions I showed that fish effectively avoided hypoxia despite rapidly changing conditions, moving away from incursions of hypoxic water and then rapidly redistributing into affected areas after these events passed. Densities of fish in nearshore oxygenated refuges increased nearly two-fold when habitat was compressed by hypoxic waters. Fish in compressed refuges also had significantly less food in their stomachs. Based on published estimates of density-dependent spot growth I estimated that habitat compression reduced average spot growth rate over summer 2007 by at least 4%. Hypoxic events during my study proved to be more spatially and temporally dynamic than anticipated. Given the fine spatial and temporal scale of oxygen dynamics in the NRE, evaluating the impacts of hypoxia on fish growth required novel, short-term growth indicators that integrate the effects of rapidly changing environmental conditions. My overall goal was to directly quantify how the frequency, duration and severity of hypoxia

events affects growth of juvenile estuary-dependent fishes in nursery habitats. To do this, I experimentally determined the sensitivity and response time of a suite of bioindicators of recent growth (RNA:DNA ratio and RNA concentration in muscle tissue, circulating plasma levels of insulin-like growth factor-I (IGF-I), IGF-I mRNA expression in the liver, Hepatosomatic Index, and Fulton's K) to changes in spot specific growth rate in laboratory experiments. Results indicated that using multiple bioindicators in a predictive growth rate model was more informative than models based on individual indicators. The best model, identified using an Information Theoretic Approach, estimated specific growth rate over the previous week based on HSI, RNA concentration, DNA concentration, Fulton's K and temperature, and accounted for 80% of the variability in specific growth rates among fish in laboratory trials. I used this model to estimate recent growth rates of spot collected from the NRE and other estuarine locations and related them to DO conditions over the week prior to collection. Estimated growth rates of spot collected after a week of Good DO conditions were almost twice those of spot collected after a week of Poor DO conditions. Using these results and DO data from the NRE in 2007-2010, I estimated that hypoxia dynamics reduced growth of spot over the summer 6-18% in these years relative to growth under constant Good DO conditions. Many studies have employed growth rate relationships with single bioindicators, but I demonstrated the value of combining multiple physiological and morphological bioindicators, along with environmental data, to produce a more robust predictive growth model that can be applied to fish in the field. This approach can be used to evaluate impacts of observed or modeled scenarios of water quality dynamics on growth of juvenile spot and serve as a template for development of predictive growth models for other species. The findings presented here could only be achieved by combining biotechnology

techniques with traditional field ecology methods. Such interdisciplinary approaches are becoming essential to address many of the increasingly complex questions and issues facing society.

Direct and Indirect Effects of Hypoxia on Juvenile Fish in the Neuse River Estuary, North Carolina

by
Lindsay Ann Glass Campbell

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Fisheries, Wildlife and Conservation Biology

Raleigh, North Carolina

2012

APPROVED BY:

_____	_____
Dr. James A. Rice Committee Chair	Dr. Russell J. Borski
_____	_____
Dr. Jeffrey A. Buckel	Dr. David B. Eggleston

DEDICATION

For my family and friends who helped me and inspired me through the many years of my journey to get to this point.

Especially for my Mom, Dad, and husband who believed in me even when I did not believe in myself and always supported me even when they didn't "get the fish stuff."

BIOGRAPHY

Lindsay was born and raised in Irving, TX where she fell in love with science and decided to make a career out of it. She earned her BS in Marine Biology from Texas A&M at Galveston in 2003, where she started off just wanting to study ocean life (possibly---gasp---marine mammals) but learned that fish were way more interesting through many internships and classes. After a short break she started working on her MS under the direction of Dr. Jay Rooker at Texas A&M studying southern flounder, finishing in 2006. She came to visit and decided on perusing her PhD at North Carolina State University after literally running into Dr. Jim Rice on an elevator at a fisheries meeting and then discussing how she cited his papers in her MS thesis. Lindsay moved to North Carolina in the summer of 2006 to begin her study of hypoxia dynamics and develop her PhD dissertation. During her time in North Carolina she met and married her husband, Bob, adopted two great cats and a dog, and made many great friends.

ACKNOWLEDGMENTS

First I would like to acknowledge my adviser, Dr. Jim Rice, for providing me the opportunity to work with him on my PhD. He provided invaluable support, guidance, and encouragement as I developed as a researcher, scientist, critical thinker, and as a person. Thank you to my committee, Drs. Russell Borski, Jeffrey Buckel, and David Eggleston, who offered comments, suggestions, and assistance in achieving my research goals. Thank you to my unofficial committee member Dr. Derek Aday who mentored me in teaching and always made time to give me feedback when asked, even though I was not technically one of his students. Thanks go to Susan Marschalk, Dawn Newkirk, and Meredith Henry who keep the Biology Department running and always help us lost graduate students navigate paper work, bookkeeping, and sometimes our daily lives. Thanks and apologies to David Newkirk who dealt with many computer issues and subsequent hysterics. This research was supported by the National Sea Grant College Program, National Oceanic and Atmospheric Administration, and the University of North Carolina Sea Grant Program under grants R/MRD-49, R/MRD-51 and R/MER-55, with additional support for me through a Biotechnology Fellowship from the Graduate Assistance in Areas of National Need program and a Dissertation Completin Grant from the North Carolina State University Graduate School.

I would also like to thank Joe Dalrymple, Ryan Spidel, Tyler Averett, Randi Effland, Rachael Faison, Sally Petre, and Steven Polland for being wonderful techs and all around great people. Without their support and long hours of field work this research would not

have been possible. They also shared some great times and laughs and generally put up with me during the summers.

All of my work would not have been possible without the love and support of my family over the years; thank you Mom, Dad, Corey, Grandpa, and Nana. To the many friends I have made, Anne Geswender, Marybeth Brey, Steve and Dani Midway, Dana and Doug Sackett, Allison Leidner, Paula Gillikin, Patrick and Julie Cooney, Josh and Meredith Raabe, Michael and Martha Fisk, Bethany Galster, Amanda Boury, Dan Brown, Kelsey Lincoln, and all the many other "fish heads," thank you for being in my life and providing motivation, inspiration, support, and comedic relief. Also, though silly, I would like to thank my kitties Chester and Nibbit who were always around with unconditional love, purring, and would always listen to me ramble and make me feel better without questions. Last but not least I want to thank my husband, Bob Campbell, who met me during my project and still decided to hang around and marry me. Bob, without your love, patience, friendship, and unwavering support I do not think I could have made it through and kept as much sanity as I have.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Estuaries are vital nursery grounds for numerous finfish, shellfish, and invertebrate species. Juvenile fish use estuaries as nursery habitat during a critical life stage as a refuge from large predators, an area to find abundant food, and most importantly to grow. Rapid growth allows juvenile fish to reduce their susceptibility to size-selective predators and move into less vulnerable stages (Houde 1987). Nursery grounds help foster rapid growth and improve survival through a combination of factors, the most important being high-quality prey resources, refuge from predators, and suitable physicochemical conditions (e.g., temperature, salinity, dissolved oxygen) (Beck et al. 2001, Gibson 1994). Anthropogenic changes to estuarine habitat could have both direct and indirect effects on the growth and survival of juvenile fish and the value of the estuary as a nursery habitat (Beck et al. 2001). It is important to determine how changes to estuarine habitat affect fish and the function of the habitat as a nursery area (Eby & Crowder 2002, Stierhoff et al. 2009).

North Carolina estuaries are not only important to juvenile fishes and environmental sustainability but to the economy, culture and quality of life of coastal North Carolina residents as well. Most of the ecologically and economically important fish and shellfish species underpinning North Carolina's tourism and fishing industries depend on its estuarine system for their essential nursery habitat (Weinstein 1979, Ross & Epperly 1985, Able 1999). The Neuse River Estuary (NRE) is the end point of a watershed that stretches 402 kilometers

from the piedmont region of North Carolina through the coastal plain to Pamlico Sound. The watershed is composed of 5,472 kilometers of tributaries where a growing population of over 2 million people lives. Throughout the watershed are many urbanized and industrial areas, as well as extensive farmlands and confined animal feedlot operations. The Neuse River basin was listed in 2007 as one of America's Top Ten Endangered Rivers by the American Rivers Foundation due to effects on water quality from poor urban planning and expansion. The Neuse River also made the foundation's 1995 Threatened list due to the effects of agricultural practices in the watershed on the quality of the river system. One of the manifestations of reduced water quality in the NRE is the increase in duration and extent of hypoxia, dissolved oxygen (DO) less than 2.0 mg/L. Fish kills have been recorded in the NRE every summer since 1996 (the first year records were taken) and are mainly attributed to hypoxia (NCDWQ 2009).

Hypoxia is an increasing problem not only in North Carolina but in estuaries throughout the United States and globally (Breitburg et al. 2009). Estuaries are prone to hypoxic events due to physical and hydrologic characteristics such as being semi-enclosed and having water column stratification where fresh and saline water meet (Diaz & Rosenberg 2008). However, increased nutrient loading to rivers and estuaries from expanding urbanization, road run-off, and agriculture, to name a few sources, changes nutrient balance and alters the oxygen budget of estuaries, which can cause a shift in the extent and magnitude of hypoxia (Pinckney 2001). In coastal regions around the world there is serious concern that water quality problems due to anthropogenic eutrophication are having negative effects on

ecologically and economically important living resources (Caddy 1993, Anon. 1999, Schmitten 1999, Craig et al. 2001, Beck et al. 2001, Breitburg 2002). Scientists have hypothesized that poor water quality conditions reduce habitat quality and create a cascade of negative effects from benthic invertebrates up to higher predators (Peterson et al. 2000, Baird et al. 2004, Powers et al. 2005), but little empirical evidence from the field has been produced; negative effects are generally assumed but not tested.

The goal of this dissertation is to determine direct and indirect effects of hypoxia on juvenile fish growth, specifically in the NRE, by using approaches that directly assess impacts on growth rates in the field and evaluating linkages between changes in water quality and actual consequences to fish populations. Because fish are highly mobile and exhibit strong avoidance behavior to hypoxia (Wannamaker & Rice 2000, Eby & Crowder 2002, Bell & Eggleston 2005), indirect effects due to habitat compression and density-dependent processes in crowded oxygenated refuges may be important mechanisms by which hypoxia impacts juvenile cohorts (Pihl et al. 1991, Breitburg 1992). In Chapter 2, I start by evaluating the spatial and temporal dynamics of hypoxia across a section of the NRE. I further evaluate how fishes alter their spatial distribution in response to hypoxia, particularly how fish densities change in oxygenated refuges as they expand and contract. Then, I estimate the extent to which density-dependent processes in compressed refuges may result in indirect effects on cumulative growth of juvenile fish.

For the next sections of my dissertation, I evaluate the effects of hypoxia on growth rates of individual juvenile fish by using biological indicators (RNA:DNA ratio and RNA

concentration in muscle tissue, liver expression of insulin-like growth factor-I, circulating plasma levels of insulin-like growth factor-I, Hepatosomatic Index, and Fulton's K). A fish's growth integrates effects of all the conditions in its environment and could be considered a direct reflection of habitat suitability. To apply levels of indicators to growth rates of wild fish, calibration experiments must first be run to quantify values under specific conditions. In Chapter 3, I test the temporal sensitivity of biological indicators of growth to reductions in food and the relationship between each indicator and growth rates in the laboratory. I use juvenile spot *Leiostomus xanthurus* as my target fish since it is a representative estuarine-dependent species, benthically oriented (where hypoxia is most severe), and for which extensive background data are available on diet (Miltner et al 1995; Nemerson & Able 2004), effects of hypoxia on behavior, growth and survival (Wannamaker and Rice 2000, McNatt and Rice 2004, Shimps et al. 2005), and density-dependence of growth and survival (Craig et al. 2007).

In Chapter 4, I utilize information from the assessment of the growth indicators to produce a predictive model of growth rates based on a combination of indicators. Subsequently, I input measured indicator levels from juvenile spot collected in the wild into the model to estimate their individual growth rates. Finally, I relate these estimated recent growth rates of wild spot to recent DO conditions recorded at their site of capture to establish potential linkages between the frequency, duration and severity of hypoxia and juvenile spot growth rates.

Taken together, these studies provide valuable contributions to our knowledge of spatial and temporal dynamics of hypoxia, fish movement in relationship to those dynamics, calibration and evaluation of several biological indicators of recent growth for juvenile spot, and a quantitative estimate of the direct and indirect effects of hypoxia on growth of a juvenile estuarine-dependent fish that can inform scientists and fisheries managers.

REFERENCES

Able KW (1999) Measures of juvenile fish habitat quality: Examples from a national estuarine research reserve. In: Benaka, LR (ed) Fish habitat: Essential fish habitat and rehabilitation. American Fisheries Society, Bethesda, MD p 134-147

Anonymous (1999) National assessment of hypoxia. The National Science and Technology Council, Committee on Environment and Natural Resources, Task Force on Harmful Algal Blooms and Hypoxia.

Baird D, Christian RR, Peterson CH, Johnson GA (2004) Consequences of hypoxia on estuarine ecosystem function: Energy diversion from consumers to microbes. Ecol App 14(3):805-822

Beck MW, Heck KLJ, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern B, Hays CG, Hoshino K, Minello TJ, Orth RJ, Sheridan PF, Weinstein MP (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. BioScience 51(8):633-641

Bell GW, Eggleston DB (2005) Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia. Mar Biol 146:761-770

Breitburg DL (1992) Episodic hypoxia in Chesapeake Bay: Interacting effects of recruitment, behavior, and physical disturbance. Ecol Monogr 62:525-546

Breitburg DL (2002) Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. Estuaries 25:767-781

Breitburg DL, Hondorp DW, Davias LA, Diaz RJ (2009) Hypoxia, nitrogen, and fisheries: Integrating effects across local and global landscapes. Annu Rev Mar Sci 1:329-349

Caddy JF (1993) Toward a comparative evaluation of human impacts on fishery ecosystems of enclosed and semi-enclosed seas. Rev Fish Sci 1:57-95

Craig JK, Gray CD, McDaniel CM, Henwood TL, Hanifen JG (2001) Ecological effects of hypoxia on fish, sea turtles, and marine mammals in the northwestern Gulf of Mexico. In: Rabalais NN, Turner RE (eds) Coastal hypoxia: Consequences for living resources and ecosystems. American Geophysical Union, Washington, D.C. p 269-291

Craig JK, Rice JA, Crowder LB, Nadeau DA (2007) Density-dependent growth and mortality in an estuary-dependent fish: An experimental approach with juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 343:251-262

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321: 926-929

Eby LA, Crowder LB (2002) Hypoxia-based habitat compression in the Neuse river estuary: Context-dependent shifts in behavioral avoidance thresholds. Can J Fish Aquat Sci 59: 952-965

Gibson RN (1994) Impact of habitat and quality and quantity on the recruitment of juvenile flatfishes. Neth J Sea Res 32:191-206

Houde ED (1987) Fish early life dynamics and recruitment variability. Am Fish Soc Symp 2:17-29

McNatt RA, Rice JA (2004) Hypoxia-induced growth rate reduction in two juvenile estuary-dependent fishes. J Exp Mar Biol Ecol 311:147 -156

NCDWQ (2009) Neuse river basinwide water quality plan 2009. North Carolina Department of Environmental and Natural Resources, Division of Water Quality

Peterson C, Summerson H, Thomson E, Lenihan H, Grabowski J, Manning L, Micheli F, Johnson G (2000) Synthesis of linkages between benthic and fish communities as a key to protecting essential fish habitat. Bull Mar Sci 66:759-774

Pihl L, Baden SP, Diaz RJ (1991) Effects of periodic hypoxia on distribution of demersal fish and crustaceans. Mar Biol 108:349-360

Pinckney JL (2001) The role of nutrient loading and eutrophication in estuarine ecology. Environ Health Perspect 109:699-706

Powers SP, Peterson CH, Christian RR, Sullivan E, Powers MJ, Bishop MJ, Buzzelli CP (2005) Effects of eutrophication on bottom habitat and prey resources of demersal fishes. Mar Ecol Prog Ser 302:233-243

Ross SW, Epperly SP (1985) Utilization of shallow estuarine nursery areas by fishes in Pamlico Sound and adjacent tributaries, North Carolina. In: Yanez-Arancibia A (ed) Fish community ecology in estuaries and coastal lagoons: Towards and ecosystem integration. Universidad de Mexico, Mexico City, p 207-232

Schmitten RA (1999) Essential fish habitat: Opportunities and challenges for the next millennium. In: Benaka LR (ed) Fish habitat: Essential fish habitat and rehabilitation. American Fisheries Society, Bethesda, Maryland, p 3-10

Shimps EL, Rice JA, Osborne JA (2005) Hypoxia tolerance in two juvenile estuary-dependent fishes. J Exp Mar Biol Ecol 325:146-162

Stierhoff KL, Targett TE, Power JH (2009) Hypoxia-induced growth limitation of juvenile fishes in an estuarine nursery: assessment of small-scale temporal dynamics using RNA:DNA. Can J Fish Aquat Sci 66:1033-1047

Wannamaker CM, Rice JA (2000) Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern united states. J Exp Mar Biol Ecol 249:145-163

Weinstein MP (1979) Shallow marsh habitats as primary nurseries for fishes and shellfish, Cape Fear River, North Carolina. Fish Bull US Dep Comm 77:339-357

Chapter 2

Effects of hypoxia-induced habitat compression on juvenile fish in the Neuse River Estuary, North Carolina

INTRODUCTION

In coastal regions globally there is serious concern that water quality problems due to anthropogenic eutrophication are having negative effects on ecologically and economically important living resources (Caddy 1993, Anon. 1999, Schmitten 1999, Craig et al. 2001, Beck et al. 2001, Breitburg 2002). Increased nutrient loading to rivers and estuaries from sources such as expanding urbanization and agricultural runoff change nutrient balances and alter the oxygen budget of estuaries, which can increase the extent and magnitude of hypoxia (dissolved oxygen <2.0 mg l^{-1}) (Pinckney 2001). Increased frequency and duration of hypoxic events are commonly caused by high productivity due to excess nutrients added to warm water, coupled with reduced mixing rates due to salinity stratification. Patterns of increased hypoxia due to anthropogenic causes have been observed in Chesapeake Bay (Cooper 1995), the Gulf of Mexico (Rabalais et al. 2002), and elsewhere (Breitburg et al. 2009) including North Carolina (Luettich et al. 2000). Diaz and Rosenberg (2008) found that hypoxia can alter the state of the ecosystem in an estuary (increased nutrients and organic matter, diversion of nutrients to microbial pathways instead of higher trophic levels, decreased benthic fauna), and if hypoxia is a persistent, recurring event, recovery of that ecosystem may lag several years behind a reduction in nutrient loading.

Estuaries are important not only as a nursery and essential fish habitat for juvenile fish, but also to the economy, culture and quality of life of coastal residents. Most of the ecologically and economically important fish and shellfish species underpinning tourism and fishing industries in North Carolina and elsewhere, depend on estuarine systems for their essential nursery habitat (Weinstein 1979, Ross & Epperly 1985, Able 1999). In North Carolina, nearly 90% of commercially important fishes and 60% of recreationally important fishes are dependent on estuarine nursery areas at some point in their life cycle (Deaton et al. 2010). One of North Carolina's larger estuaries, the Neuse River Estuary (NRE), began receiving increased attention regarding eutrophication in the late 1970s (NCDWQ 2009), was placed on the North Carolina 303(d) list of impaired waters in 1994 (NCDWQ 2009), and a Neuse Nutrient Strategy was adopted by the North Carolina Environmental Management Commission in 1998 to reduce total nitrogen entering the estuary by 30%. Despite this, the Neuse River Basinwide Assessment (NCDWQ 2009) showed that there was no distinct reduction in overall nitrogen loading by 2006. An increasing number of fish kills in the NRE in the mid-1990s prompted detailed data collection on species and numbers of fish killed, water quality parameters, and pathology reports starting in 1997 that continues today. These data have shown peaks in fish kills in 2003 and 2008, and record highs in 2009 (North Carolina Division of Water Quality Environmental Sciences Section; annual reports available at http://portal.ncdenr.org/web/wq/ess/fishkills).

Fish kills are a highly visible indicator of water quality problems (e.g., Paerl et al. 1998) but generally have negligible population-level impacts, as even large fish kills

typically involve only a small fraction of the population and are relatively rare. Conversely, sublethal hypoxic conditions are common and widespread, and may affect a much larger portion of the fish population. Fish growth can be reduced by exposure to sublethal low or fluctuating hypoxia as shown in experimental trials with a variety of species (e.g., southern flounder *Paralichthys lethostigma*, Taylor & Miller 2001; spot *Leiostomus xanthurus* and menhaden *Brevoortia tyrannus*, McNatt & Rice 2004; summer flounder *Paralichthys dentatus* and winter flounder *Pseudopleuronectes americanus*, Stierhoff et al. 2006). However, fish can typically detect and avoid hypoxia, often at DO levels higher than those that would typically cause a reduction in growth (Wannamaker & Rice 2000, Eby & Crowder 2002, Bell & Eggleston 2005, Stierhoff et al. 2006).

Because fish can often avoid direct exposure to hypoxia, indirect effects of hypoxia on growth of individual fish may have greater consequences than direct effects. When hypoxic conditions expand, avoidance behavior by fish may increase densities in oxygenated refuges, typically near shore. Crowding in these oxygenated refuges may cause density-dependent effects on growth (Pihl et al. 1991, Breitburg 1992). Small-scale cage experiments in the field and larger-scale pond experiments have documented reductions in growth of juvenile estuary-dependent fish as densities increased, even in the absence of predation (Eby et al. 2005, Craig et al. 2007). In the field, reduced growth can prolong the period of vulnerability to gape-limited predators, increasing predation mortality (Sogard 1997). High densities of fish confined to a compressed oxygenated refuge may also attract

predators, or predators may be confined to the oxygenated refuge as well, resulting in higher encounter rates and increased mortality of juvenile fish (Hixon et al. 2002).

Hypoxia can also indirectly affect fish growth through its impact on prey resources. As hypoxic waters expand and contract repeatedly over a season, the abundance of benthic invertebrate prey can be reduced because these sessile invertebrates cannot avoid lethal DO levels (Peterson et al. 2000, Eby et al. 2005, Powers et al. 2005). Eby et al. (2005) concluded from field caging experiments with Atlantic croaker *Micropogonias undulatus* that reductions in growth due to reduced prey resources may surpass reductions in growth due to direct exposure to short-term hypoxic events. Thus, repeated episodic hypoxic disturbances affect the interactions of juvenile fish with both their predators and their prey as well as intraspecific competitive interactions (Breitburg 1992, Breitburg et al. 1997).

While substantial effort has been devoted to water quality monitoring and modeling, rarely are linkages between poor water quality and estuarine fish population responses specifically addressed (e.g., Borsuk et al. 2003, Bowen 2003, Wood et al. 2005). Scientists have hypothesized that poor water quality conditions reduce habitat quality and create a cascade of negative effects from benthic invertebrates to higher predators (Peterson et al. 2000, Powers et al. 2005), but little empirical evidence from the field has been produced; negative effects are generally assumed but not tested. Understanding these linkages and the magnitude of their potential sublethal and lethal effects on aquatic living resources would provide much-needed information to guide water quality and natural resource management. This understanding is particularly important for juvenile fish and estuarine habitats because

anthropogenic changes to estuarine habitats are increasing (Diaz & Rosenberg 2008) and the value of an estuarine habitat is often measured by its ability to serve as a nursery for juvenile fishes (Beck et al. 2001).

Because fish are highly mobile and exhibit strong hypoxia avoidance behavior, indirect effects due to habitat compression and density-dependent processes in oxygenated refuges may be important mechanisms by which hypoxia impacts juvenile cohorts. However, studying these processes is complicated because, unlike most other habitat features, water quality characteristics are spatially and temporally dynamic and the size and duration of refuges varies with wind and oxygen conditions in the system. In this study, we evaluated changes in the spatial distribution and density of juvenile fishes in response to seasonal and episodic hypoxia dynamics, and estimated the extent to which these changes may result in indirect effects of hypoxia on growth. We focused on juvenile spot and Atlantic croaker as these fish were abundant, occupy the full spectrum of impacted habitats, are benthically oriented (where hypoxia is most severe), and are representative estuarine-dependent fishes that support significant fisheries. We conducted our study on a cross section of the NRE that experiences frequent hypoxia and used a combination of water quality monitoring and fish sampling to address the following questions: How does the spatial distribution of hypoxia within a season and on an event basis influence fish densities? Does habitat compression due to hypoxic events result in significant increases in densities in oxygenated water refuges? Does feeding success change in response to fish densities or water quality condition? Does relative predator abundance change in oxygenated refuges as a response to habitat

compression? What potential changes in fish growth can be attributed to these indirect effects? Quantifying these effects could provide insight on the cumulative consequences of these indirect effects on fish growth and production.

METHODS

Study Area

The NRE is the end point of a watershed that stretches over 400 kilometers from the piedmont region of North Carolina through the coastal plain to Pamlico Sound, and is composed of 3,400 miles of tributaries. A population of over 2 million people live in the Neuse River basin and is expected to increase 44% from 2000-2020 (NCDWQ 2009). Throughout the watershed are many urbanized and industrial areas as well as extensive farmlands and numerous confined animal feedlot operations. The NRE is a relatively shallow estuary with circulation driven primarily by wind rather than tides, and slow flushing rates in the summer (63 days, NCDWQ 2009). Hypoxia develops on the river bottom as the processes decomposing organic materials uses up oxygen, and the water column becomes stratified due to low wind-driven mixing, particularly as water warms up during summer months. Hypoxia can quickly spread upward in the water column when no wind mixing occurs to bring oxygenated waters to the bottom. Strong, sustained winds can force downwelling of oxygenated waters on the down-wind side of the river, displacing the hypoxic layer into the shallow areas on the up-wind side of the basin creating a seiche event; when the winds relax the hypoxic layer can quickly flow back to the other side of the basin (Reynolds-Flemming & Luettich 2004). For example, winds from the southwest cause

hypoxic bottom water to upwell along the southwest shoreline, but after winds relax or turn from the northeast hypoxic water upwells on the northeast side of the river (Reynolds-Flemming & Luettich 2004). We selected a cross section of the NRE for our study transect known for hypoxia dynamics in other studies (Luettich et al. 2000, Bell et al. 2003), with areas that range from typically oxygenated to chronically hypoxic. While several studies have documented water quality along transects down the middle of the NRE (e.g., Luettich et al. 2000), no study has continuously monitored across the width of the river for an extended period. Our transect was located 19 river kilometers downstream of the Hwy 70 bridge in New Bern, North Carolina, and extended from just up-river of the mouth of Slocum Creek to just down-river of the mouth of Beard Creek (Figure 2.1). At this location the river is oriented from northwest (upstream) to southeast (downstream) so prevailing winds generally blow across the river, creating seiche events.

Water Quality Monitoring

During the summer of 2007, five YSI 600XLM data sondes were deployed at sites along the transect across the NRE (Figure 2.1) ~30 cm off the bottom and recorded temperature, salinity, dissolved oxygen and depth every 15 minutes, from early summer (May 25, 2007) to late summer (final retrieval September 24, 2007). Both nearshore stations (173 - 185 m from shore, ~1 m depth, south shore = site 1, north shore = site 5) where chosen to represent typically oxygenated areas with minimal severity of hypoxic conditions. The center station (midway between the two shores, ~4 m depth, site 3) was chosen to represent areas characterized by substantial, prolonged hypoxic events. Stations located between the

mid-river site and nearshore sites (621 m from south shore, site 2, and 366 m from north shore, site 4, ~2 - 3 m depth) represented areas that we expected would be more intermittently affected by hypoxia. Sondes were serviced weekly to clean off any accumulated debris and download data.

Fish Sampling

We conducted approximately weekly nighttime sampling trips (total 14, May to September) to estimate the density of spot and other fish species at five sites corresponding to the data sonde locations. We conducted nighttime sampling to reduce visual detection and gear avoidance in shallow waters, to target fish after potentially increased feeding activity at dusk, and to sample when occurrences of hypoxic events were more likely. Each site was sampled with an otter trawl (6 m head rope and foot rope with tickler chain attached, 20 mm bar mesh wings, 5 mm bar mesh cod end) pulled parallel to shore at constant speed for two minutes (average track length ~200 m). Sites were trawled once in order from south to north, then the same sequence was repeated two more times (for a total of three trawls at each site per sampling night). If the mid-river location (site 3) data sonde showed a pattern of continuous hypoxia, the water column was hypoxic to a depth beyond the height range of the trawl net (at least 1 m above the bottom), and no fish were caught during the first trawl set, it was not re-trawled during the other two sets. At the beginning and end of each trawl we recorded water quality readings (temperature, salinity, DO) ~10 cm below the surface and ~30 cm off the bottom using a hand-held YSI 600 QS. For samples with fewer than ~200 fish all individuals were identified to species, counted, and a subset of 20 individuals

measured. For larger samples, all individuals of rare species were identified, counted, and measured. The remaining sample was divided in half or quarters to obtain a subsample of ~200 fish, and the remaining fish were identified and counted. Total number of fish for a subsampled trawl was estimated by multiplying the number of fish in the subsample by the subsample's proportion of the total catch Fish density was calculated by dividing the total number of fish caught by the area trawled, then multiplying by 3.125 to account for estimated trawl efficiency of 32%, based on a study by Kjelson and Johnson (1978) using same net construction, similar sized spot, similar depths, and conducted in a nearby estuary. Trawled area was calculated by multiplying track length (determined from GPS points taken at the beginning and ending of each trawl) by the width of the net mouth while fishing (estimated as 5 m, based on Kjelson & Johnson 1978). Samples were assigned to three DO categories, based on the average of bottom DO readings at the beginning and end of the trawl: DO < 2 mg l^{-1}, DO between 2 - 4 mg l^{-1}, and DO > 4 mg l^{-1}. If DO readings at the beginning and end of a trawl indicated that the trawl track crossed the hypoxic boundary (one reading < 2 mg l^{-1}, one reading > 2 mg l^{-1}) the sample was not included in any of the above categories for analyses.

A subset of spot from each trawl (20 individuals, when available) was sealed in a plastic bag and flash frozen in dry ice-ethanol slurry; later fish were thawed, weighed (nearest 0.01 g) and measured (nearest mm standard length, SL), and stomachs were removed and placed in 10% formalin. Individual stomachs were later weighed to the nearest 0.001 g, and stomach fullness was categorized (on a scale from 0 - 6, completely empty to stomach

distended). An Index of Feeding was calculated by dividing the whole stomach wet weight by the wet weight of the fish.

To characterize the relative density of potential predators in relation to water quality, we conducted weekly nighttime gillnetting at each site. One experimental gill net of 2.44 m height with 50 m sections of 31.8 mm, 50.8 mm, 63.5 mm, and 76.2 mm bar mesh was set on the bottom parallel to shore at each station starting at sunset with a minimum soak time of 2.5 hours. Water quality readings (temperature, salinity, DO) were recorded ~10 cm below the surface and ~30 cm off the bottom when nets were set and picked up. All fish caught were identified to species and counted, and potential predators were measured to the nearest mm SL. Potential predators were determined as carnivorous fish with a gape that would allow ingestion of fish > 60 mm SL. Catch per unit effort (CPUE) was calculated as the number of fish caught per hour soak time of the gill net; a separate CPUE for potential predators only was calculated similarly.

Sites 1 and 5 were characterized as areas of oxygenated refuge from hypoxia which we then assigned to two different habitat compression states: refuge not compressed or refuge compressed. The refuge was defined as not compressed if both its DO reading and that of its adjacent site (site 2 or site 4) was above 2 mg l^{-1}; the refuge was compressed if its DO reading was above 2 mg l^{-1}, but DO at its adjacent site was below 2 mg l^{-1}. A special case was also considered when there was effectively no refuge because both DO readings were below 2 mg l^{-1}, so while not a true refuge this case is considered in our analysis (labeled by “hypoxic”) to give a representation of all conditions experienced.

Data Analysis

Records of temperature, salinity, and DO from the remotely-deployed data sondes were processed using Aquarius 2.0 software (Aquatic Informatics Inc., British Columbia, Canada) with corrections made for instrument drift based on comparison to simultaneous water quality readings taken with a calibrated, handheld YSI 600QS data sonde. We defined hypoxic events as follows: an event started when a dissolved oxygen reading was less than or equal to 2.0 mg l^{-1} and ended when two consecutive readings were above 2.5 mg l^{-1}. Then, we characterized frequency and duration of events at each site by month. Rate of onshore movement of the hypoxic front was estimated by dividing the time between the first hypoxic reading at site 2 (or 4) and the first hypoxic reading at site 1 (or 5) by the distance between the two data sondes. For ease of viewing in figures, water quality data are presented as a running average, such that, for a particular time point, readings from a specified interval (45 min or 3 h) both before and after that point are averaged and the resulting value is plotted on the figure. We examined patterns of DO and salinity in relationship to wind speed and direction over short term (1 week) and long term (3 months) time periods. Wind data was obtained from the State Climate Office of North Carolina for measurements taken at Cherry Point Marine Core Air Station (KNKT) in the form of hourly average wind speed and direction. Finally, we compared water quality readings from opposite sides of the NRE to evaluate if any relationship between the two could be detected.

The calculated value for fish density (fish m^{-2}) from each trawl sample was transformed ($\log_{10}$(density + 1)) to account for zeros and to meet the assumptions of

normality and equality of variance for statistical analysis, but we present untransformed data in the Results for ease of interpretation. Gillnet CPUE values were also transformed ($\log_{10}$(CPUE)) to meet the assumptions of normality and equality of variance, but we present untransformed data in the Results. The difference in densities and CPUE among water quality conditions was tested by Analysis of Variance (ANOVA). Comparisons were also tested among the different habitat compression categories for both densities of fish from trawl samples and gill net CPUE data. Index of Feeding values from the subsample of spot from each trawl were averaged to give a single Index of Feeding value for each trawl. When testing for fish length effects on Index of Feeding individual values were used. The Index of Feeding proportions were ArcSin-square root transformed and tested for differences in both water quality and habitat compression categories; untransformed values are presented in the results section. All analyses were performed in JMP 9 (SAS Institute, Inc., Cary, NC) with an alpha level of 0.05.

RESULTS

Dissolved Oxygen

Dissolved oxygen concentrations varied substantially over the short term (hours to days) and long term (weeks to months) on both temporal and spatial scales. Hypoxic events were dynamic with changes in dissolved oxygen at a site often dropping from above 6 mg l^{-1} to below 2 mg l^{-1} in less than an hour and recovering from hypoxia just as quickly. We often recorded many hypoxic events within a week at an individual site (Figure 2.2), and as many as four in a single day. During a typical 5-hour sampling period all three dissolved oxygen

categories could be recorded. Site 3 was hypoxic 75% of total recordings for the summer, with fewer individual events but each event longer (average 48.5 hours) and a single continuous event lasting 498 hours (Table 2.1). Intermediate sites (sites 2 and 4) had higher percentages of time spent hypoxic (16% and 27%, respectively) than their corresponding nearshore counterparts. August exhibited the highest occurrence of total individual hypoxic events. Site 4 had the highest number of events for the season at 83 with an average duration of 7.74 hours.

Consistent with observations reported by Bell et al. (2003), Reynolds-Flemming & Luettich (2004) and Eggleston et al. (2005), the hypoxia dynamics we observed were driven by wind, with winds from the southwest associated with hypoxic upwelling on the south side of the estuary and winds from the northeast with hypoxic upwelling on the north side of the estuary (Figure 2.2). The relationship between wind direction and speed, and the inverse patterns of hypoxic events on opposite sides of the estuary held throughout the whole June-August period (Figure 2.3). Because of the role wind plays in hypoxia dynamics, we observed an approximate inverse relationship between DO concentrations on opposite sides of estuary over the season (Figure 2.4). Generally, hypoxic conditions tended to occur between 1700 and 0700 hours on the southwest side of the estuary, and between 0300 and 1600 hours on the northeast side of the estuary (Figure 2.4). This pattern reflects the tendency for winds to increase in strength from the southwest during the afternoon, and then die down after sunset. The relaxation of wind strength reduces upwelling, allowing seiche events to take place, where previously upwelled hypoxic water would move to the opposite

side of the estuary before settling to the bottom of the basin, much like a pendulum swing. The average speed of the onshore movement of the hypoxic front on the southwest side of estuary was 7 cm s^{-1} with a maximum speed of 52 cm s^{-1}. On the northeast side of the estuary the average speed of the hypoxic front was 5 cm s^{-1} with a maximum speed of 24 cm s^{-1}.

In general, lower DO is associated with higher salinities due to stratification in the river. Thus, a decline in DO at a site due to wind-driven movement of bottom waters is also typically accompanied by an increase in salinity. Over the short term, salinity values can be coupled with DO, with a stronger relationship in some periods (e.g. Site 2: June 17 – 23, R^2 = 0.51, July 16 – 22, R^2 = 0.73) but a weaker relationship in others (e.g., Site 2: July 1 – 7, R^2 = 0.11, August 26 – 31, R^2 = 0.20) (Figure 2.2). However, on a longer time scale the relationship between DO and actual salinity values breaks down because bottom-water salinity can change seasonally. For example, in 2007 average bottom salinity at sites 2 - 4 changed from 9 - 13 ppt in June to 14 - 19 ppt in July and 15 - 17 ppt in August. As a result, though DO was significantly related to salinity, only a small portion of the variability in DO was explained by salinity at sites 1 - 5 when tested over June through August (linear regression, all $p < 0.0001$, R^2 values: site 1 = 0.14, site 2 = 0.28, site 3 = 0.26, site 4 = 0.13 and site 5 = 0.05). Even when regressions were run for data separated by month, the highest R^2 was 0.61 for site 3 in June but most R^2 values were less than 0.30, with linear regression of DO and salinity not significant at site 5 in June or July.

Fish Sampling

We conducted 149 trawls from May 29, 2007 to September 22, 2007. Our catch was comprised of 96% demersal species (in descending order of abundance, Atlantic croaker, spot, blue crab *Callinectes sapidus* , southern flounder, brown shrimp *Farfantepenaeus aztecus*, hogchoker *Trinectes maculatus* , summer flounder, bighead searobin *Prionotus tribulus*, and blackcheek tonguefish *Symphurus plagiusa*); of those, spot made up 42% and Atlantic croaker 56%. Non-demersal species included (in descending order of abundance) bay anchovy *Anchoa mitchilli*, pinfish *Lagodon rhomboides*, menhaden, harvestfish *Peprilus paru*, bluefish *Pomatomus saltatrix*, white mullet *Mugil curema*, silver perch *Bairdiella chrysoura*, and American eel *Anguilla rostrata*). Average size of spot increased from 70 ± 0.5 mm SL (mean ± SE) in June to 93 ± 1.3 mm SL in August; croaker were slightly larger, increasing from 82 ± 0.6 mm SL in June to 110 ± 1.2 mm SL in August.

Throughout the season density of demersal fish in trawl samples was strongly related to oxygen concentration. Since 96% of our trawls were made up of demersal species the densities we report are those only demersal of species only. As expected, overall fish densities declined over the season (ANOVA, $n = 105$, $p < 0.0001$, Student's t-test $t = 1.98$, June (a), July (b), August (b,c), September (c)). Mean density of fish from normoxic waters (DO > 4 mg l^{-1}) was almost twice that from waters with DO between 2 - 4 mg l^{-1}, and fifteen times greater than densities where DO was less than 2.0 mg l^{-1} (fish m^{-2}: 1.05, 0.62, 0.06 respectively; ANOVA, $df = 138$, $p < 0.0001$) (Figure 2.5). All samples from waters where

DO was greater than 2.0 mg l^{-1} contained fish; in contrast, 38% of samples from water with less than 2.0 mg l^{-1} DO had zero fish.

Densities of fish in oxygenated nearshore refuges were strongly affected by the extent to which hypoxia compacted these areas. When the nearshore oxygenated refuge was compressed (i.e., the adjacent site was hypoxic), mean density of fish (0.98 fish m^{-2}) was almost twice the mean density (0.55 fish m^{-2}) of samples in the same areas when the refuge was not compressed (i.e., the adjacent site was normoxic) and greater still than in the few cases when the whole area was hypoxic so there was no oxygenated refuge (0.13 fish m^{-2}) (ANOVA, df =65, p = 0.0247) (Figure 2.6).

Fish in compressed refuges had not been eating as much recently as fish in non-compressed areas during early summer. Index of Feeding in refuge areas did not vary significantly with time of day sampled (ANOVA, df = 56, p = 0.19), but did vary by Month (ANOVA, df = 3, p = 0.0071). Index of Feeding in the compressed refuge in June was about half (0.0063) of that when the refuge was not compressed (0.0108) (t-test, df = 18, p = 0.04); there was no difference in Index of Feeding in other months (t-tests: July, df = 25, p = 0.97; August, df = 2, p = 0.56; September, df = 4, p = 0.39) (Figure 2.7). Index of Feeding declined slightly as the season progressed (Figure 2.7). To investigate whether or not fish size had an allometric effect on Index of Feeding leading to decrease over the season and to evaluate whether the Index changed over the season (e.g., due to a seasonal change in food availability) we used values from individual collected at sites 1 and 5 when the refuge was not compressed (so as to not confound with refuge size), and ran an ANOVA to test for

effects of length (SL mm), Month, and their interaction. Only Month was a significant factor (ANOVA, df = 377, $p < 0.0001$; Effects tests: SL, df = 1, $p = 0.87$; Month, df = 3, $p < 0.0001$; Month*SL, df = 3, $p = 0.93$). Index of Feeding from spot collected during a prolonged hypoxic event August 8 – 10, 2007, when no refuge was available, was higher (1.4 ± 0.14) than average Index of Feeding during any other water condition (Figure 2.7).

We gillnetted on eight different nights from May 30 to September 23, 2007. Catches were comprised of 18% predators (bluefish, blue crab, longnose gar *Lepisosteus osseus*, Spanish mackerel *Scomberomorus maculatus*, southern flounder, red drum *Sciaenops ocellatus*, and spotted seatrout *Cynoscion nebulosus*), and 82% non-predators, which either did not eat demersal fish or were not of the size range to eat demersal fish (menhaden, cownose ray *Rhinoptera bonasus*, Atlantic croaker, harvest fish, silver perch, spot, Atlantic stingray *Dasyatis sabina*, pinfish, and pompano *Trachinotus carolinus*). Bluefish were the most common predator caught in our gill nets (n = 527), with a trend of increasing numbers as the season progressed. There was no difference in gillnet total fish CPUE (fish per hour) weather conditions were hypoxic during the net set (12.03 ± 5.19) or not (44.18 ± 7.34) (t-test, df = 42, $p = 0.054$), or whether the refuge was compressed (34.87 ± 23.3) or not (49.83 ± 9.84) (t-test, df =21, $p = 0.52$). Predator CPUE in gillnets was not different whether conditions were hypoxic (5.76 ± 1.04) or not (6.16 ± 1.82) (t-test, df = 41, $p = 0.93$) or whether the refuge was compressed (5.48 ± 1.18) or not (6.32 ± 1.45) (t-test, df = 20, $p = 0.66$). There was no difference in predator CPUE between nearshore sites (6.79 ± 1.53) and intermediate sites (4.84 ± 1.02) (ANOVA, df = 41, $p = 0.48$).

DISCUSSION

Our study shows that fish detect and avoid hypoxia in the field and can do so in response to rapidly changing conditions (minutes to hours). Throughout the season, fish were able to move away from incursions of hypoxic water and then rapidly redistribute into affected areas after these events passed; fish responded this way repeatedly over the summer instead of permanently emigrating from areas that frequently experienced hypoxic events. Eby et al. (2005) found that early in the season benthic prey were more abundant in deeper sites (similar to our intermediate sites), which may explain why fish return to these areas despite frequent hypoxic events. Our results supplement findings by others that fish move away from hypoxia (e.g., Pihl et al 1991, Tyler & Targett 2007, Keller et al 2010), but are unique in that we documented this behavior in response to hypoxic events occurring on the scale of hours and recurring repeatedly over several months.

The added movement and interactions associated with repeatedly avoiding hypoxic waters may have greater cumulative energetic and mortality consequences than evading only a few, stable, long-term events over the course of a season. Energetic costs of swimming increase exponentially with swimming speed (Fry 1971). The average on-shore movement rate of hypoxic waters we observed (5 - 7 cm s^{-1}) was similar to previous calculations by Luettich et al. (2000) of 6 cm s^{-1}; however, several hypoxic events greatly exceeded the average on-shore speed (26 - 52 cm s^{-1}), illustrating how quickly hypoxic waters can move and fish must promptly evade. Telemetry tracking of juvenile spot in relation to oxygen conditions documented spot swimming at speeds up to 3.5 body lengths per second when

avoiding hypoxia (J. Kevin Craig, pers. comm.). Based on the average sizes of fish in our samples, spot could swim up to 28 cm s^{-1} and croaker 33 cm s^{-1} to avoid hypoxic waters, making it possible for them to outswim the average on-shore movement of hypoxia but unlikely to outswim our fastest calculated hypoxic movement of 52 cm s^{-1}. Some fish will likely be caught in hypoxic waters during their escape and will be subject to greater direct hypoxia consequences during this time. Many benthic species can move not only horizontally away from hypoxia, but also vertically out of the more dense hypoxic waters into overlaying normoxic waters. Moving vertically a few meters or less would require negligible energy expenditure compared to prolonged, rapid, lateral movement. However, this behavior would separate demersal fish like spot and croaker from their normal benthic food sources and their vulnerability to predators may be elevated while they remain in this less familiar pelagic environment. Even if spot and croaker remain near the bottom during avoidance movement, their ability to feed on benthic prey would likely also be reduced when swimming at elevated speeds. Thus, successful avoidance behavior may still result in negative indirect effects due to increased activity costs, decreased feeding, and potentially increased predation mortality.

If the hypoxic front is moving rapidly there could be a "snow plow" effect of fish accumulating on the hypoxic edge while moving to get out of the way of advancing hypoxia, increasing localized densities on the hypoxic front. Craig (2011) found evidence of fish aggregating on the edge of the hypoxic zone in the Gulf of Mexico. Two of our trawls that crossed the hypoxic boundary (i.e., at one end of the trawl DO was > 2 mg l^{-1}, at the other

end DO was < 2 mg l^{-1}) had very high densities of fish, in the top 10 highest of the study. One trawl was conducted at site 2 when the end of this trawl went into slightly deeper waters, encountering the hypoxic edge. The second trawl that crossed the boundary occurred at site 1 at the start of a prolonged hypoxic event as the hypoxic front advanced towards shore. The data sonde at this site showed a rapid decline in DO and then several hours of hypoxia. We visibly observed fish aggregating near shore and then migrating laterally toward the mouth of a nearby tributary. These incidents show fish aggregating on the edge of the hypoxic zone similar to the behavior observed by Craig (2011), but on a much smaller scale. If fish are staying in these edge areas with increased density, then even more inter- and intra-species interactions could take place than we can accurately describe from the samples we collected.

Because 38% of trawls under hypoxic conditions collected no fish and the remainder collected very low numbers of fish, it is worth considering why fish were located in hypoxic waters at all. The presence of at least some of the fish in our trawls under hypoxic conditions might be an artifact of the mouth of the net extending up into normoxic waters at times, catching fish that were not truly in hypoxic water. Though we did not get many fish in our trawls when sampling at intermediate sites when the bottom was hypoxic, we noted that the depth finder sonar image showed a layer of fish just above upper boundary of the hypoxic layer. Pihl et al (1992) suggested that fish take advantage of invertebrate prey temporarily stunned by hypoxia, and others have noted that benthic prey migrate vertically to the sediment surface during hypoxia exposure (Diaz & Rosenberg 1995, Taylor & Eggleston 2000), so the fish in these areas could be exploiting this resource (as reported by Roberts et

al. 2012). We observed one instance in our study when spot may have been employing this strategy, as the Index of Feeding (0.013 ± 0.002) of spot collected in low DO waters during a prolonged hypoxic event on August 8 - 10 was two times higher than of spot collected during normoxia (0.0058 ± 0.001). However, prolonged events such as this did not frequently occur at our nearshore and intermediate sites. Some researchers suggest short hypoxic events (less than 6 hours), such as those observed in our study, might not last long enough for infauna to migrate to the sediment surface, reducing the potential profitability of foraging as a behavioral response to hypoxia (Bell et al. 2003). Fish may also choose to stay in hypoxic waters to avoid predators; Froeschke and Stunz (2012) found through tank experiments that pinfish and Atlantic croaker would not go into hypoxic waters for food, but would do so to avoid predators. We did not find evidence of highly increased predator density in oxygenated refuges that would drive fish into hypoxic waters. Despite the myriad reasons that could drive fish to occupy hypoxic waters, fish rarely did so in our study.

Our results showed that hypoxia has the potential to indirectly reduce growth of juvenile fish through increased densities and reduced feeding success in oxygenated refuges during periods of habitat compression. Though we only detected a decrease in Index of Feeding in June. That we were able to detect a decrease in Index of Feeding at all was surprising considering that, on average, fish were collected after only three hours of habitat compression; therefore, more prolonged periods of habitat compression may result in an even greater reduction in feeding success. Since prey densities are reported to be higher earlier in the summer (Buzzelli et al. 2002, Eby et al. 2005), a lack of differences between the two

groups later in the summer may simply be due to insufficient resources being available for a difference to be manifest in such a short period. Reductions in feeding or a shift in diet to less nutritional prey items could result in reduced growth (Eby et al. 2005, Powers et al. 2005). Intermittent hypoxia altered prey availability to croaker in Eby et al.'s (2005) study; they cited a decrease in food as the main cause for decreased croaker growth rates. Reduced food resources and increased competition not only for food but also space leads some species, such as Atlantic croaker, to escalate aggressive behaviors towards conspecifics (Gibbard et al. 1979).

The nearly two-fold increase in demersal fish densities in nearshore oxygenated refuges during periods of habitat compression has the potential to cause density-dependent effects on mortality and growth. Given that 98% of demersal fish caught were a combination of spot and croaker, it is not unreasonable to use total demersal fish densities when considering density-dependent effects on either species. Spot and Atlantic croaker distributions overlap strongly in the NRE (Eby & Crowder 2002; this study) and these species share similar diets (Stickney et al. 1975). Thus, a spot would likely experience the same density-dependent effect from both other spot and croaker. Density-dependent effects are common and can occur over multiple spatial and temporal scales (Hixon & Jones 2005). Several studies have concluded that density-dependence acts most intensely on growth at low to moderate abundance levels, such as those seen in our study (Jenkins et al 1999, Grant & Imre 2005, Martino & Houde 2012). Craig et al. (2007) measured spot growth at varying densities in experimental ponds and found a non-linear relationship between density and

growth (growth= $\alpha e^{(\beta * \text{density})}$ where $\alpha = 0.719 \pm 0.042$, $\beta = -0.426 \pm 0.056$). When we apply this equation to the fish densities we observed, the estimated growth rate of fish in compressed refuge areas (0.47 mm d^{-1}) is 17% lower than the estimated growth of fish in refuge areas that were not compressed (0.57 mm d^{-1}). These growth rates fall into the range of growth observed in wild spot (Weinstein 1983, Weinstein et al. 1984). It should be noted that because prey densities and other conditions in the ponds may have been different than in the field, the relative differences between the two estimates are more reliable than the actual growth rates estimates for fish in the field. In a field cage study Eby et al. (2005) found that croaker growth rates at low densities (0.67 fish m^{-2}) were twice those at high densities (2.67 fish m^{-2}); hence, increased densities in compressed refuges would similarly affect croaker growth.

However, over the course of the summer, nearshore, oxygenated refuge areas were only compressed an average of 21.5% of the time. Thus, the cumulative effect over the season would be much smaller: only a 4% reduction in growth (0.785*0.57 mm d^{-1} + 0.215*0.47 mm d^{-1} = 0.55 mm d^{-1}). Such a modest decrease in growth may seem to indicate that these effects are not worth considering, but 2007 was a mild year of hypoxia; during years with more severe hypoxia, growth reduction could be more substantial. For example, if refuges were compressed 50% of the time then estimated reductions in growth would be 9%. In all likelihood, wild fish will have lower growth than fish in ponds or cages at similar densities, as the costs of hypoxia exposure can be exacerbated in the field where fish increase swimming speeds and distances during exposure (Craig & Rice, unpub. data), food

availability can be limited, fish must actively forage and avoid predators, and hypoxia avoidance activity may reduce foraging time. We consider the degree of indirect effects on growth calculated above to be conservative. To draw more accurate comparisons we recommend measures of actual fish growth in the field.

Levin and Stunz (2005) found that even small changes in the quantity or quality of essential fish habitat will have large effects on population dynamics, and Myers and Cadigan (1993) concluded after a study of density-dependent mortality that the juvenile stage is very important for population regulation of most species. Reduced growth rates can prolong the period that fish remain vulnerable to size-dependent predation, increasing overall mortality of the population (Houde 1987, Sogard 1997). While our methods were unable to conclusively detect an increase in predators in compressed refuges (though the trend of increased predator density was observed), others have shown that increased density of spot increased mortality from predators (Wright et al. 1993) and speculated that species compaction enhances predation risk from blue crabs via harassment and agonism (Eby & Crowder 2002, Bell & Eggleston 2005). Craig et al (2007) also found that even in the absence of predators, mortality of spot increased with increasing densities.

Decline in the Index of Feeding over the summer is consistent with evidence produced by other researchers that benthic prey composition is altered and reduced over the summer by repeated hypoxic events in the NRE (Bell et al. 2003, Eby et al. 2005, Powers et al. 2005). Allometric effects could possibly contribute to a decline in Index of Feeding as spot sizes increased over the summer, but we found no evidence of that over the time period

and size range of fish we sampled. Decline in available food over the summer would hamper potential compensatory growth of fewer late-summer and fall survivors as pressure from hypoxia decreases (Sogard 1997). Reduced growth can result in smaller size at the beginning of winter, which can lead to reduced survival over the winter (Sogard 1997). Martino and Houde (2012) found that slower growth of age-0 striped bass was linked to a smaller age-1 year class the following spring. Using population modeling, Eby et al. (2005) estimated that reduced growth of Atlantic croaker due to hypoxia in the NRE caused a 4% decline in their population. All the cascading effects from reduced growth due to hypoxia dynamics could lead to smaller population size and disruption of prey base for larger predators, among many other possible disturbances of vital ecosystem and trophic links.

Hypoxic events in our study proved to be more spatially and temporally dynamic than we anticipated, especially at our intermediate sites. Other studies of water quality in the NRE (Luettich et al. 2000, Eby & Crowder 2002, Eby et al. 2005, Powers et al. 2005) have typically sampled areas once a day, during day light hours, and generally at the same time of day (but see Bell et al. 2003 as another example of using fixed water quality instruments). As our data indicate, this sampling approach can detect some hypoxic events, but miss the intervening dynamic behavior of hypoxic waters generally driven by prevailing wind patterns. Buzzelli et al. (2002) developed a model to estimate the cross-river extent of hypoxia based on water quality profiles taken down the middle of the NRE. While this model provides useful predictions for some applications, it was developed assuming a level oxycline. Our more detailed cross-river data show that the oxycline is rarely level, as

evidenced by the out-of-phase pattern of DO and salinity readings on opposite sides of the NRE. Water quality readings taken at locations deeper than our intermediate sites (more toward the middle of the NRE or middle river, e.g., Reynolds-Flemming & Luettich 2004, Eby et al. 2005) show more stable and longer hypoxic events consistent with our mid-river observations, as they are not on the dynamic edge of the hypoxic water lens. While deeper sites might have less variability in hypoxic events, they are also less likely to be frequented by demersal fish (as demonstrated by small and zero catches at our site 3 in the middle of the estuary; see also Bell et al. 2005); consequently, examining and extrapolating behavior, movement, and consequences of hypoxia from these locations would not reflect the highly variable conditions experienced by the majority of the fish population that inhabits shallower habitats. Water quality projections based on fewer measurements can be useful for large-scale applications and when predicting generalities for a system (e.g., Buzzelli et al. 2002, Bowen et al. 2003), but scientists need to be aware that these models do not accurately depict conditions at the small spatial and temporal scale fish must respond to.

Defining and quantifying the consequences of hypoxia for fish and other living resources via either direct or indirect effects is increasingly important as fisheries managers must employ ecosystem-based approaches to manage changing environments (Myers et al. 2003). Many studies on hypoxia focus on the direct effects of fish caught in hypoxia, but as our study and others have demonstrated, fish are usually successful in avoiding hypoxia, so more attention needs to be focused on the potential indirect effects of hypoxia and how these effects on the individual level aggregate to the population level. Our study helps to

understand sub-daily movement patterns in relation to hypoxia which are crucial to predict ecological consequences of hypoxia (Roberts et al. 2012). The highly dynamic aspect of hypoxia in the NRE can lead to many indirect effects that cannot be fully measured and taken into account by conventional means; despite this we were able to see some coarse effects of hypoxia on fish densities and potential density-dependent effects. When trying to quantify the impact of hypoxia on fish we must first comprehend the response to fine-scale spatiotemporal changes due to dynamic hypoxia before we can resolve the consequences of environmental quality changes for fish (Rose 2000). Measuring recent growth of fish responding to hypoxic environments will more accurately depict the toll of direct and indirect costs of hypoxia as a cumulative measure of all the stressors on fish that effect growth.

REFERENCES

Able KW (1999) Measures of juvenile fish habitat quality: Examples from a national estuarine research reserve. In: Benaka, LR (ed) Fish habitat: Essential fish habitat and rehabilitation. American Fisheries Society, Bethesda, MD p 134-147

Anonymous (1999) National assessment of hypoxia. The National Science and Technology Council, Committee on Environment and Natural Resources, Task Force on Harmful Algal Blooms and Hypoxia.

Beck MW, Heck KLJ, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern B, Hays CG, Hoshino K, Minello TJ, Orth RJ, Sheridan PF, Weinstein MP (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. BioScience 51(8):633

Bell, GW (2002) Behavioral response of free-ranging blue crabs to episodic hypoxia. MS thesis, North Carolina State University, Raleigh, NC

Bell GW, Eggleston DB (2005) Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia. Mar Biol 146:761-770

Bell GW, Eggleston DB, Wolcott DL (2003) Behavioral responses of free-ranging blue crabs to episodic hypoxia. II. Feeding. Mar Ecol Prog Ser 259:227-235

Borsuk ME, Stow CA, Reckhow KH (2003) An integrated approach to TMDL development for the Neuse river estuary using a Bayesian probability network model (Neu-BERN). J Water Resour Plann Manage 129:271-282

Borsuk ME, Powers SP, Peterson CH (2002) A survival model of the effects of bottom-water hypoxia on the population density of an estuarine clam (*macoma balthica*). Can J Fish Aquat Sci 59:1266-1274

Bowen JD, Asce AM, Hieronymus JW (2003) A CE-QUAL-W 2 model of Neuse estuary for total maximum daily load development. J Water Resour Plann Manage 129:283-294

Breitburg DL (1992) Episodic hypoxia in Chesapeake Bay: Interacting effects of recruitment, behavior, and physical disturbance. Ecol Monogr 62:525-546

Breitburg DL (2002) Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. Estuaries 25:767-781

Breitburg DL, Hondorp DW, Davias LA, Diaz RJ (2009) Hypoxia, nitrogen, and fisheries: Integrating effects across local and global landscapes. Annu Rev Mar Sci 1:329-349

Breitburg DL, Loher T, Pacey CA, Gerstein A. (1997) Varying effects of low dissolved oxygen on trophic interactions in an estuarine food web. Ecol Monogr 67:489-507

Buzzelli CP, Luettich Jr RA, Powers SP, Peterson CH, McNinch JE, Pinckney JL, Paerl HW (2002) Estimating the spatial extent of bottom-water hypoxia and habitat degradation in a shallow estuary. Mar Ecol Prog Ser 230:103-112

Caddy JF (1993) Toward a comparative evaluation of human impacts on fishery ecosystems of enclosed and semi-enclosed seas. Rev Fish Sci 1:57-95

Cooper SR (1995) Chesapeake Bay watershed historical land use: Impact on water quality and diatom communities. Ecol Appl 5:703-723

Craig JK (2011) Aggregation on the edge: effects of hypoxia avoidance on the spatial distribution of brown shrimp and demersal fishes in the Northern Gulf of Mexico. Mar Ecol Prog Ser 445:75-95

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

Craig JK, Gray CD, McDaniel CM, Henwood TL, Hanifen JG (2001) Ecological effects of hypoxia on fish, sea turtles, and marine mammals in the northwestern Gulf of Mexico. In: Rabalais NN, Turner RE (eds) Coastal hypoxia: Consequences for living resources and ecosystems. American Geophysical Union, Washington, D.C. p 269-291

Craig JK, Rice JA, Crowder LB, Nadeau DA (2007) Density-dependent growth and mortality in an estuary-dependent fish: An experimental approach with juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 343:251-262

Deaton AS, Chappell WS, Hart K, O'Neal J, Boutin B (2010) North Carolina coastal habitat protection plan. North Carolina Department of Environmental and Natural Resources. Division of Marine Fisheries, NC

Diaz RJ, Rosenberg R (1995) Marine benthic hypoxia: a review of its ecological effects and the behavioural reponses of benthic macrofaunna. Oceanogr Mar Biol Annu Rev 33:245-303

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321: 926-929

Eby LA, Crowder LB (2002) Hypoxia-based habitat compression in the Neuse river estuary: Context-dependent shifts in behavioral avoidance thresholds. Can J Fish Aquat Sci 59: 952-965

Eby LA, Crowder LB, McClellan CM, Peterson CH, Powers MJ (2005) Habitat degradation from intermittent hypoxia: Impacts on demersal fishes. Mar Ecol Prog Ser 291:249-262

Froeschke JT, Stunz GW (2012) Hierarchical and interactive habitat selection in response to abiotic and biotic factors: The effect of hypoxia on habitat selection of juvenile estuarine fishes. Environ Biol Fish 93:31-41

Fry FEJ (1971) The Effect of Environmental Factors on the Physiology of Fish. In: Hoar WS, Randall DJ (eds) Fish Physiology. Academic Press, New York, p 1-98

General Assembly of North Carolina. 1995 Session, Chapter 572, House Bill 1339.

Gibbard GL, Strawn K, Aldrich DV (1979) Feeding and aggressive behavior of Atlantic croaker, black drum, and striped mullet in monoculture and polyculture. Proc World Maric Soc 10:241-248

Grant JWA, Imre I (2005) Patterns of density-dependent growth in juvenile stream-dwelling salmonids. J Fish Biol 67:100-110

Hixon MA, Pacala SW, Sandin SA (2002) Population regulation: Historical context and contemporary challenges of open vs. closed systems. Ecology 83:1490-1508

Houde ED (1987) Fish early life dynamics and recruitment variability. Am Fish Soc Symp 2:17-29

Jenkins TM, Diehl S, Kratz KW, Cooper SD (1999) Effects of population density on individual growth of brown trout in streams. Ecology 80:941-956

JMP, Version 9. SAS Institute Inc., Cary, NC, 1989-2012

Keller AA, Simon V, Chan F, Wakefield WW, Clarke ME, Barth JA, Kamikawa D, Fruh EL (2010) Demersal fish and invertebrate biomass in relation to an offshore hypoxic zone along the US West Coast. Fish Oceanogr 19:76-87

Kjelson MA, Johnson GN (1978) Catch Efficiencies of a 6.1-meter otter trawl for estuarine fish populations. Trans Am Fish Soc 107:246-254

Levin PS, Stunz GW (2005) Habitat triage for exploited fishes: Can we identify essential "Essential Fish Habitat?" Estuar Coast Shelf S 64:70-78

Luettich RA Jr, McNinch JE, Pinckney JL, Alperin MJ, Martens CS, Paerl HW, Peterson CH, Wells JT (2000) Neuse River Estuary modeling and monitoring project, Final Report: Monitoring Phase. UNC Water Resources Research Institute, Technical Report

Martino EJ, Houde ED (2012) Density-dependent regulation of year-class strength in age-0 juvenile striped bass (*Morone saxatilis*). Can J Fish Aquat Sci 69:430-446

McNatt RA, Rice JA (2004) Hypoxia-induced growth rate reduction in two juvenile estuary-dependent fishes. J Exp Mar Biol Ecol 311:147 -156

Myers RA, Cadigan NG (1993) Density-dependent juvenile mortality in marine demersal fish. Can J Fish Aquat Sci 50(8):1576-1590

Myers N, Mittermeier RA, Mittermeier CG, da Fonesca GAB, Kent J (2003) Biodiversity hotspots for conservation priorities. Nature 403:853-858

NCDWQ (2009) Neuse river basinwide water quality plan 2009. North Carolina Department of Environmental and Natural Resources, Division of Water Quality

Paerl HW, Pinckney JL, Fear JM, Peierls BL (1998) Ecosystem responses to internal and watershed organic matter loading: Consequences for hypoxia in the eutrophying Neuse river estuary, North Carolina, USA. Mar Ecol Prog Ser 166:17-25

Peterson C, Summerson H, Thomson E, Lenihan H, Grabowski J, Manning L, Micheli F, Johnson G (2000) Synthesis of linkages between benthic and fish communities as a key to protecting essential fish habitat. Bull Mar Sci 66:759-774

Pihl L, Baden SP, Diaz RJ (1991) Effects of periodic hypoxia on distribution of demersal fish and crustaceans. Mar Biol 108:349-360

Pihl L, Baden SP, Diaz RJ, Schaffner LC (1992) Hypoxia-induced structural changes in the diet of bottom-feeding fish and crustacean. Mar Biol 112:349-361

Pinckney JL (2001) The role of nutrient loading and eutrophication in estuarine ecology. Environ Health Perspect 109:699-706

Powers SP, Peterson CH, Christian RR, Sullivan E, Powers MJ, Bishop MJ, Buzzelli CP (2005) Effects of eutrophication on bottom habitat and prey resources of demersal fishes. Mar Ecol Prog Ser 302:233-243

Rabalais NN, Turner RE, Scavia D (2002) Beyond science into policy: Gulf of Mexico hypoxia and the Mississippi River. BioScience 52:129-142

Reynolds-Fleming JV, Luettich RA Jr (2004) Wind-driven lateral variability in a partially mixed estuary. Estuar Coast Shelf S 60:395-407

Roberts JJ, Grecay PA, Ludsin SA, Pothoven SA, Vanderploeg HA, Hook TO (2012) Evidence of hypoxic foraging forays by yellow perch (*Perca flavescens*) and potential consequences for prey consumption. Freshw Biol 57:922-937

Rose KA (2000) Why are quantitative relationships between environmental quality and fish populations so elusive? Ecol Appl 10:367-385

Ross SW, Epperly SP (1985) Utilization of shallow estuarine nursery areas by fishes in Pamlico Sound and adjacent tributaries, North Carolina. In: Yanez-Arancibia A (ed) Fish community ecology in estuaries and coastal lagoons: Towards and ecosystem integration. Universidad de Mexico, Mexico City, p 207-232

Schmitten RA (1999) Essential fish habitat: Opportunities and challenges for the next millennium. In: Benaka LR (ed) Fish habitat: Essential fish habitat and rehabilitation. American Fisheries Society, Bethesda, Maryland, p 3-10

Sogard S (1997) Size-selective mortality in the juvenile stage of teleost fishes: A review. Bull Mar Sci 60:1129-1157

Stickney RR, Taylor GL, White DB (1975) Food habits of five species of young southeastern United States estuarine Sciaenidae. Chesap Sci 16:104–114

Stierhoff KL, Targett TE, Miller K (2006) Ecophysiological responses of juvenile summer and winter flounder to hypoxia: Experimental and modeling analyses of effects on estuarine nursery quality. Mar Ecol Prog Ser 325:255-266

Taylor JC, Eggleston DB (2000) Effects of hypoxia on an estuarine predator-prey interaction: foraging behavior and mutual interference in the blue crab *Callinectes sapidus* and the infaunal clam prey *Mya arenaria*. Mar Ecol Progr Ser 196:179-191

Taylor JC, Miller JM (2001) Physiological performance of juvenile southern flounder, *Paralichthys lethostigma* (Jordan and Gilbert, 1884), in chronic and episodic hypoxia. J Exp Mar Biol Ecol 258:195-214

Tyler RM, Targett TE (2007) Juvenile weakfish Cynoscion regalis distribution in relation to diel-cycling dissolved oxygen in an estuarine tributary. Mar Ecol Progr Ser 333:257-269

Wannamaker CM, Rice JA (2000) Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern united states. J Exp Mar Biol Ecol 249:145-163

Weinstein MP (1979) Shallow marsh habitats as primary nurseries for fishes and shellfish, Cape Fear River, North Carolina. Fish Bull US Dep Comm 77:339-357

Weinstein MP (1983) Population dynamics of an estuarine-dependent fish, the spot (*Leiostomus xanthurus*), along a tidal creak-seagrass meadow coenocline. Can J Fish Aquat Sci 40:1633-1638

Weinstein MP, Scott L, O'Neil SP, Siegfried RC, Szedlmayer ST (1984) Population dynamics of spot, *Leiostomus xanthurus*, in polyhaline tidal creeks of the York River estuary, Virginia. Estuaries 7:444-450

Wool TA, Davie SR, Rodriquez HN (2003) Development of Three-Dimensional Hydrodynamic and Water Quality Models to Support Total Maximum Daily Load Decision Process for the Neuse River Estuary, North Carolina. J Water Resour Plann Manag 129:295-306

Wright RA, Crowder LB, Martin TH (1993) The effects of predation on the survival and size-distribution of estuarine fishes: an experimental approach. Environ Biol Fish 36:291-300

Table 2.1 Number of hypoxic events, average duration, and maximum duration in hours by month and site.

	June			July			August			Overall		
Site	Number of Events	Average Duration (hours)	Max Duration (hours)	Number of Events	Average Duration (hours)	Max Duration (hours)	Number of Events	Average Duration (hours)	Max Duration (hours)	Number of Events	Average Duration (hours)	Time <2mg l^{-1}
1	9	5.3	14.25	11	5	17.5	19	5.1	16	39	5.1	11%
2	17	5.2	15	22	5.6	17.75	26	6.7	23.8	65	5.9	16%
3	10	52.2	304.3	8	77.1	498	12	27	136.5	30	48.5	75%
4	27	5.7	29.5	33	4.4	14.5	23	14.9	93.3	83	7.7	27%
5	2	2.3	3.3	8	4.3	13.75	21	10.3	85.8	31	8.2	22%

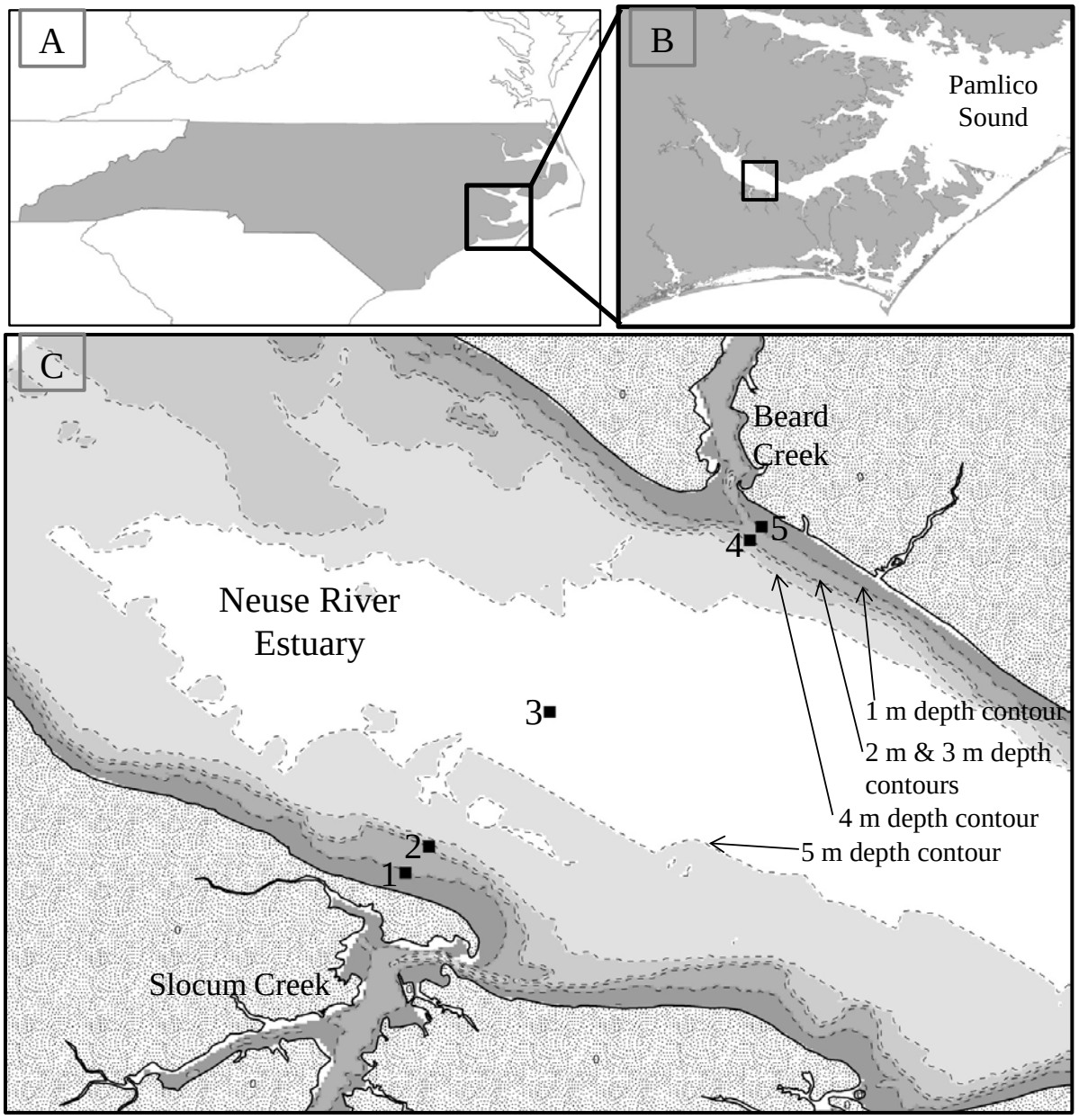


Figure 2.1. Map of study location in relation to North Carolina (A), and section of state showing Pamlico Sound and square of a portion of NRE where study sites located (B). Location of study sites in NRE with depth contours (C). Site 1 is a near-shore, typically oxygenated site on the Southeast side of the estuary, ~1 m depth, ~173 m from shore. Site 2 is an intermediate hypoxic site on the South-East side of the estuary, ~2-3 m depth, ~621 m from shore. Site 3 is a chronically hypoxic site in the middle of the estuary, ~4 m depth. Site 4 is an intermediate hypoxic site on the North-West side of the estuary, ~2-3 m depth, ~366

m from shore. Site 5 is a typically oxygenated site on the North-West side of the estuary, ~1 m depth, ~185 m from shore.

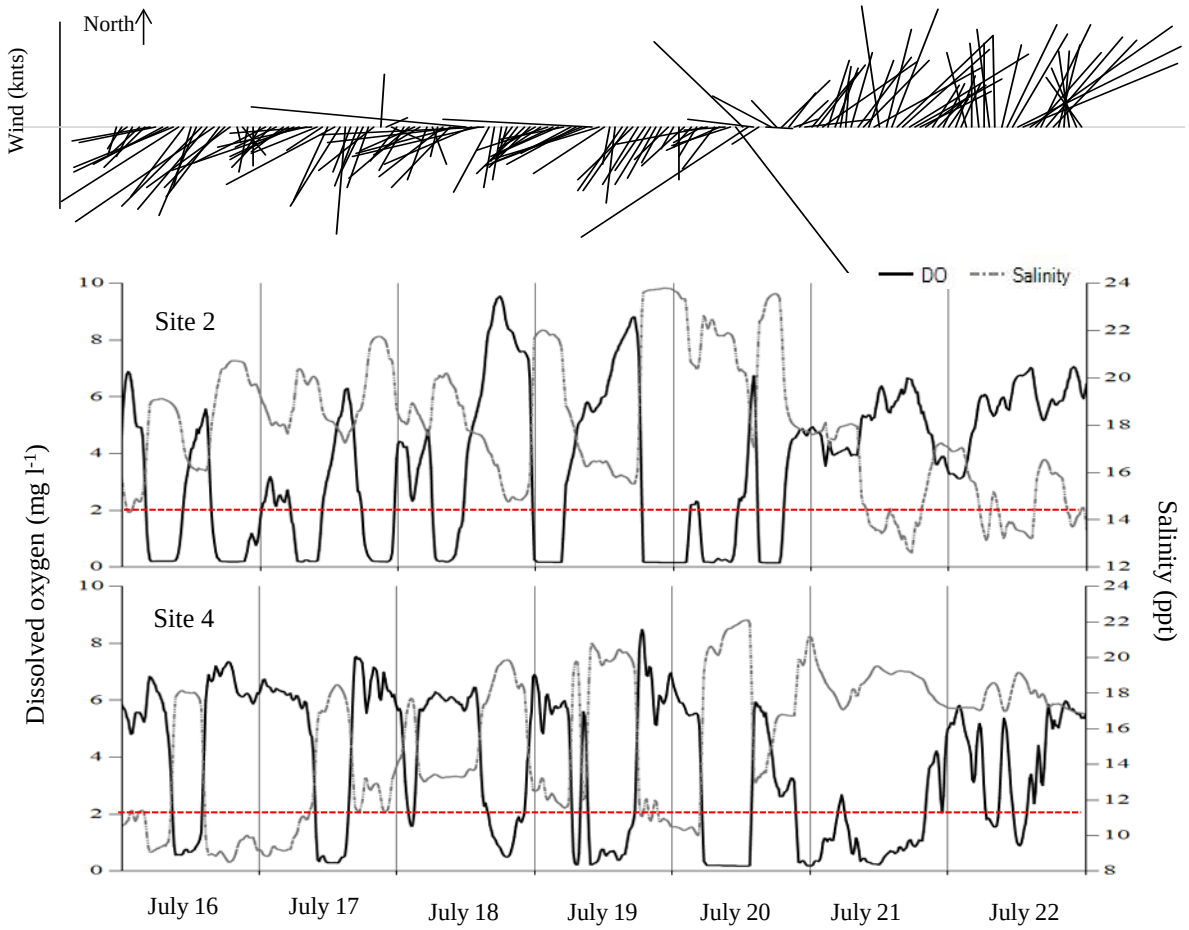
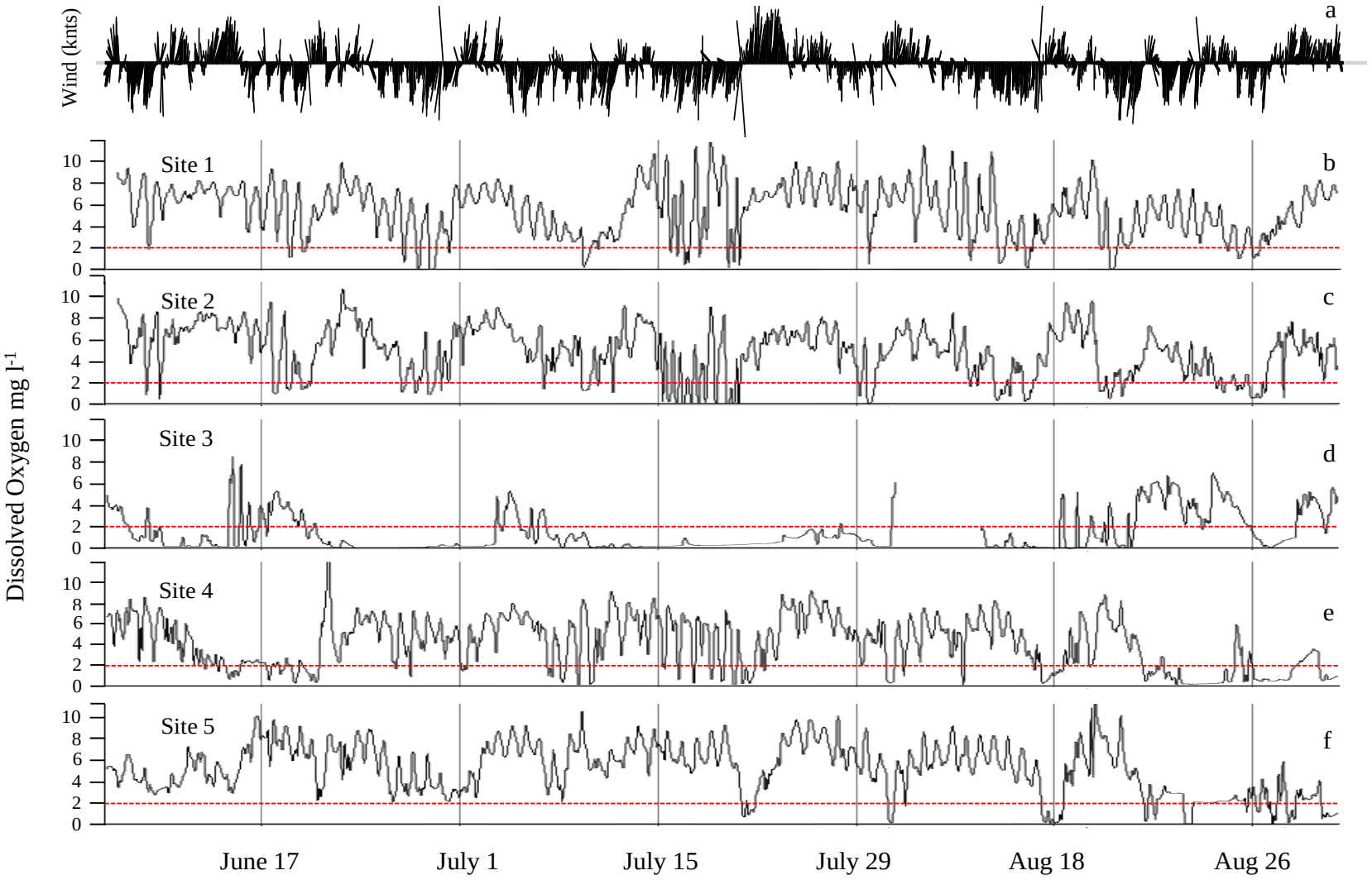


Figure 2.2 Feather plot of wind speed (longer lines indicate increased speed) and direction (a straight up line would indicate winds from the North) (top) at hourly intervals from July 16-July 22, 2007. Average wind speed 8 knots and max speed 21 knots over time period shown. Dissolved oxygen and salinity values (45-min running averages) for site 2 (middle) and site 4 (bottom) over the same time period.

Figure 2.3 Feather plot of wind speed (longer lines indicate increased speed) and direction (a straight up line indicates winds from the North; much of the Easterly and Westerly slant is compressed due to the long time period shown as an artifact from plotting program) from June 6-August 31, 2007 (a). Average wind speed 6 knots and max wind speed 21 knots over time period shown. Dissolved oxygen concentration (plotted as a 3-hour running average for clarity) for sites 1 (b), 2 (c), 3 (d), 4 (e), and 5 (f). Sites are ordered from south (top) shore to north shore (bottom).


Wind (knts)
a
Site 1
b
Site 2
c
Site 3
d
Site 4
e
Site 5
f
Dissolved Oxygen mg l-1
0
2
4
6
8
10
June 17
July 1
July 15
July 29
Aug 18
Aug 26

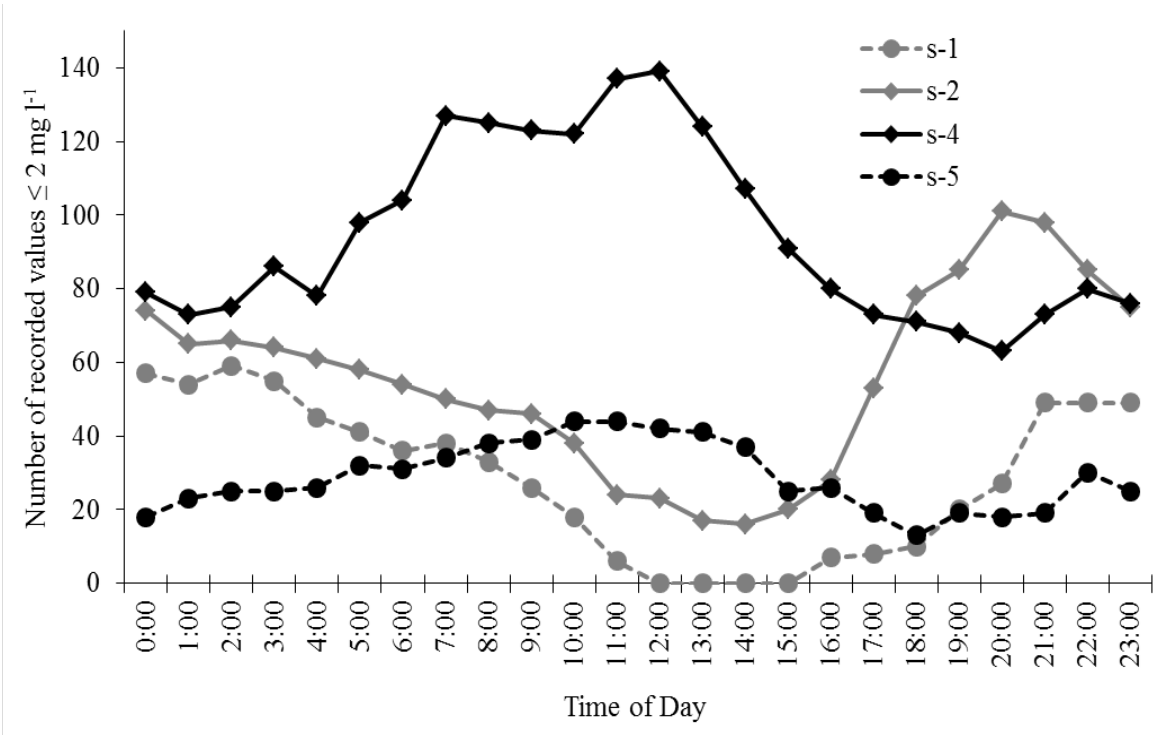


Figure 2.4 Number of dissolved oxygen measurements ≤ 2 mg l^{-1}, by time of day (sum of readings taken during the hour) for sites 1, 2, 4, and 5 June through August, 2007.

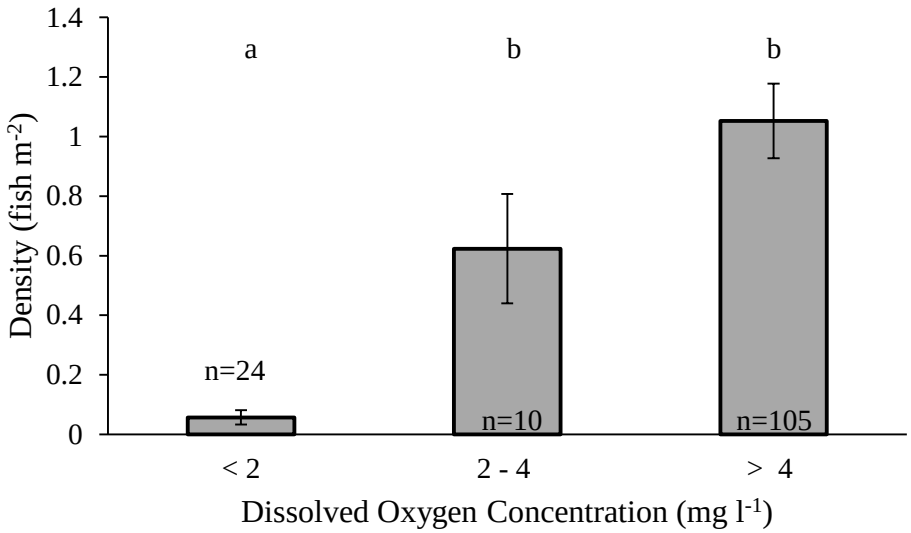


Figure 2.5 Mean density ± SE of fish collected in trawls conducted in different water quality conditions: DO < 2 mg l^{-1}, DO 2-4 mg l^{-1}, and DO > 4 mg l^{-1}; different letters above error bars denote statistically significant differences denoted by Tukey's HSD.

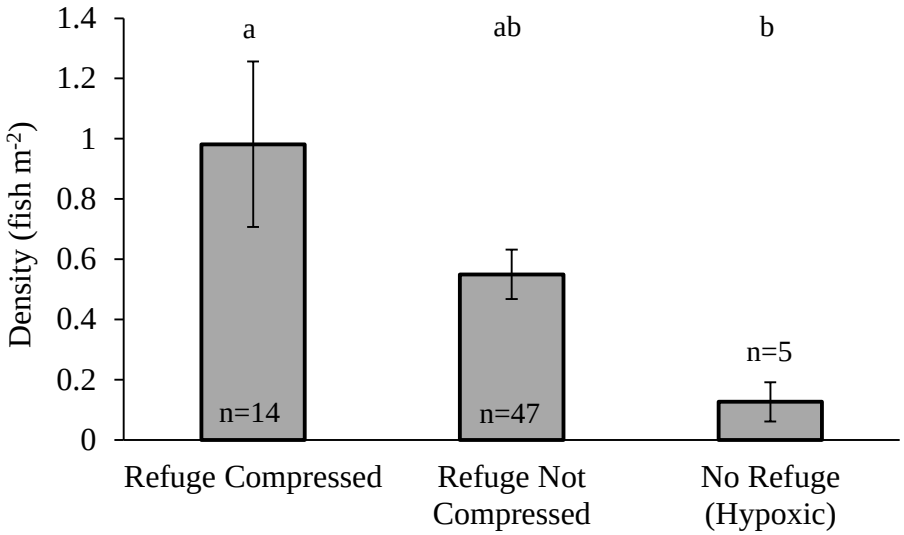


Figure 2.6 Mean densities ± SE of fish in trawls conducted at nearshore sites when the refuge was compressed, not compressed, or no refuge (hypoxic); different letters above error bars denote statistically significant differences determined by Tukey's HSD.

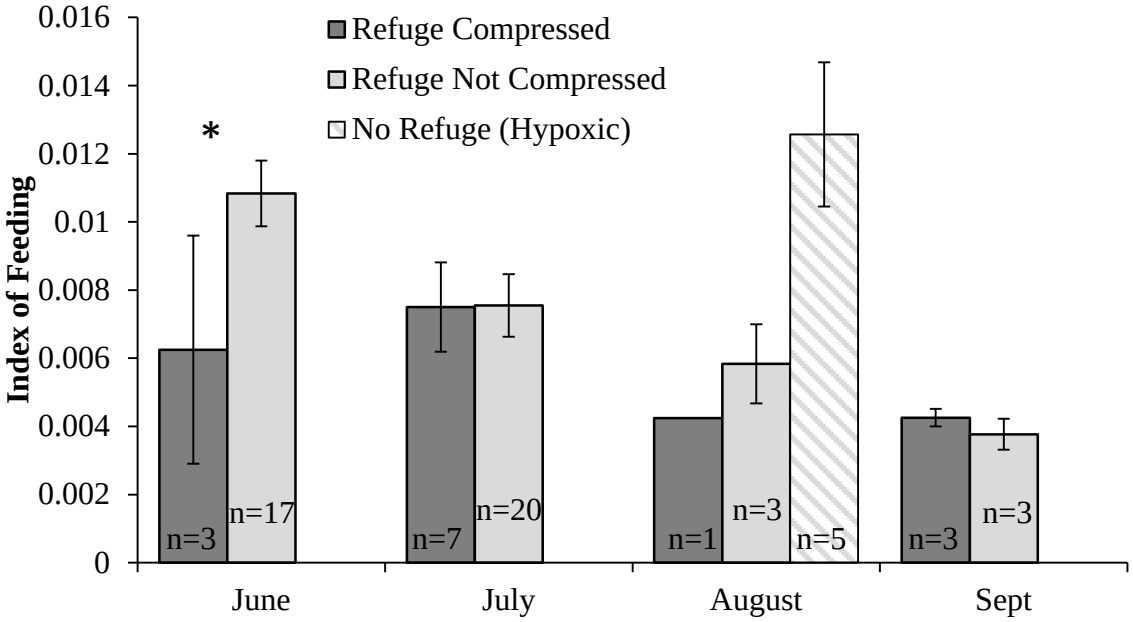


Figure 2.7. Mean Index of Feeding ± SE in refuges that were not compressed and compressed. Asterisk indicates significant difference in the month of June. The special case of no refuge (hypoxic) is shown for August (the only month such cases occurred) but was not tested against other values. Values of n in each bar indicate the number of trawls from which fish were collected.

Chapter 3

Magnitude and timing of changes in bioindicators of recent growth in relation to changes in growth rate for juvenile spot *Leiostomus xanthurus*

INTRODUCTION

Most fish species exhibit the highest mortality rates during the critical developmental stages of early life history. Small reductions in juvenile growth rates can prolong vulnerable life stages, in some estimates reducing recruitment rates 3- to 10-fold, while increasing susceptibility to predation (Houde 1987, Rice et al. 1993, Scharf 2000). Limited knowledge of growth rates in relation to environmental conditions reduces our ability to understand how changes in the environment can affect fishes (Rooker & Holt 1996, Searcy et al. 2007a,b). The magnitude and relative importance of both direct and indirect effects of environmental conditions on fish growth are elusive, but are a pressing question for both scientists and fisheries managers (Rose 2000). The ability to produce reliable estimates of growth rates and condition of fish collected from the field would increase our understanding of factors that influence successful recruitment to the next life history stage (Peck et al. 2003), however few tools are available to measure recent growth rate in the field. Mark-recapture methods can provide useful measure of individual growth for some applications, but are not a feasible way to repeatedly obtain numerous estimates at sufficiently fine spatial and temporal scales in open environments. Researchers need effective methods to assess changes in growth of individual fish over the short-term (e.g., one to several days) in response to recent habitat

conditions. The ability to assess recent growth rates of fishes in their natural environment has broad appeal for application to ecological and fisheries questions. Because the inability to measure short-term weight gain of numerous individual fish in the wild precludes direct calculation of specific growth rate, researchers have been seeking alternative means of assessing recent growth using morphological, biochemical, physiological and endocrine indicators (Ferron & Leggett 1994).

Much research has been carried out on nucleic acids and their relationship to growth (e.g., Bulow 1987, Houlihan et al. 1993, Malloy & Targett 1994, Buckley et al. 1999, Peck et al. 2003). Because growth is achieved through protein synthesis, the relationship between the quantity of DNA in a cell, which is constant, and the quantity of RNA, which fluctuates with protein synthesis rate, can be linked to growth (Buckley et al. 1984, Ferron & Leggett 1994). Numerous studies suggest that RNA:DNA ratios of larval and juvenile fishes reflect recent growth conditions over periods ranging from 1-3 days up to 2 weeks preceding capture, and thus can be related to environmental measures taken concurrent with sampling (e.g., Bulow 1987, Malloy & Targett 1994, Stierhoff et al. 2009). Other studies have expressed RNA content in relation to tissue sample weight and found that this approach better correlated to growth rate than comparing RNA to DNA in fishes (Houlihan et al. 1993, Mathers et al. 1993, Foster et al. 1993).

A relatively new entry into the field of growth rate indicators is the endocrine hormone, insulin-like growth factor-I (IGF-I). IGF-I is responsible for cell differentiation and proliferation, the stimulation of processes related to skeletal elongation, and ultimately

for body growth (see Duan 1997, LeBail et al. 1998 for reviews). Due to the causal relationship that IGF-I plays in directly regulating somatic growth, IGF-I is a promising candidate for measuring recent growth of fish in the field. A collective body of evidence in controlled tank trials and within aquaculture studies suggests that plasma levels of IGF-I may provide an accurate reflection of recent, variable growth rates in a number of fish species (reviewed by Picha et al. 2008, Beckman 2011). Furthermore, it appears that levels of IGF-I correspond to both positive and negative growth rates regardless of which biotic (e.g., feeding level) or abiotic (e.g., temperature, photoperiod, stress, water quality) factors are influencing growth (Beckman 2011). While many studies establish important precedent for the use of IGF-I as a growth bioindicator in controlled aquaculture settings (Beckman et al. 2004, Picha et al. 2006, Vera Cruz et al. 2006, Picha et al. 2008a,b, Beckman 2011), few studies have applied IGF-Ito wild fish (though see Stefansson et al. 2011 for Atlantic salmon), and to our knowledge no studies have evaluated IGF-I in a wild, estuarine, non-aquacultured fish.

Two morphometric variables that have been used in the past to evaluate fish condition and are relatively easy to obtain are the hepatosomatic index (HSI) and Fulton's condition factor (Fulton's K). In most fish species, stores of liver glycogen are the first energy source used to meet short-term energy demands (Picha et al. 2006, Perez-Jimenz et al 2007). HSI, an expression of relative liver weight as a percentage of body weight, could thus be used as a measure reflecting short-term energy reserves or condition of fish, though this would likely be a less sensitive measure of growth. Fulton's K for fish has been used for many years

(Bolger & Connolly 1989, Stevenson & Woods 2006) and is based on the assumption that individuals within a group with higher K values (plumper fish) would contain more energy reserves, most likely in the form of fat and protein (Caldaron et al. 2012). The main draw to Fulton's K is the fact that it can be obtained non-lethally, however it more likely reflects longer term growth rate in fish or overall condition, and may not be as sensitive to recent growth.

Given the variety of biochemical and morphological means to assess growth over a short period and the ability to evaluate these indicators through experimental processes, we examined which of several indicators mentioned above could best be used to develop methods to estimate short-term growth of fish in the field. Even though the relationship between growth rate and short-term growth indicators has been studied for many species, there is variation in response by species (e.g., Buckley et al. 1999, Stierhoff et al. 2009), so assessments must be done for each species before data can be interpreted and applied. We quantified levels of growth and condition indicators in juvenile spot *Leiostomus xanthurus*, a representative estuarine-dependent fish for which extensive background data are available on diet (Miltner et al 1995; Nemerson & Able 2004), effects of hypoxia on behavior, growth and survival (Wannamaker and Rice 2000, McNatt and Rice 2004, Shimps et al. 2005), and density-dependence of growth and survival (Craig et al. 2007), but to our knowledge no studies evaluating nucleic acids or IGF-I as growth rate indicators. We examine several indicators (RNA:DNA ratio, RNA concentration, expression of IGF-I mRNA in the liver, circulating levels of IGF-I in plasma, HSI, and Fulton's K) in relation to growth of juvenile

spot to determine if, and how quickly, each indicator responds when fish are switched from high feeding levels to either starvation or reduced feeding rates. Changing between extreme food conditions (high feeding to starvation) will elicit the strongest response of the indicators. We also examine the effects of reduced feeding, as long periods of reduced feeding levels are more likely to occur in the wild than long periods of no feeding. Given that feeding rates of individual fish in the wild may fluctuate substantially over short periods (on the order of days), we also compared growth rates and responses of the indicators between groups of fish that were fed constantly or intermittently, but received the same amount of food on average. This study will help identify potential methods of ascertaining short-term growth in juvenile spot. It is also part of a larger effort to describe estimated growth rates of juvenile spot in relation to hypoxic conditions in estuarine nursery areas (see Campbell 2012 Chapter 4) and sets the foundation for further work on wild-caught juvenile spot.

METHODS

2009 Trials

Juvenile spot (mean SL = 74 mm, range = 56 - 96 mm; mean weight = 8.26 g, range = 3.85 - 21.03 g) were collected by trawl from the Neuse River Estuary, North Carolina, in May and June 2009, transported back to the Fisheries Research Laboratory located at the University of North Carolina's Institute of Marine Sciences in Morehead City, North Carolina, and placed in recirculating holding tanks. Water was recirculated through a bubble-washed bead biofilter with a 20% water change daily with a mix of filtered sea water

from Bogue Sound, North Carolina and filtered freshwater. Spot were individually marked with visible implant elastomer (Northwest Marine Technologies, www.nmt.us) and randomly placed into two 791-l round tanks (at a stocking density of ~0.95 g l^{-1}). Fish were acclimated to experimental conditions for two weeks (temperature 25 - 27°C, salinity 22-27 ppt) with feedings to apparent satiation twice a day of thawed freshwater *Mysis* shrimp (Piscine Energetics Inc., British Columbia, Canada). Uneaten food and feces were removed from the tanks prior to morning feeding, and any buildup of algae on the sides of tanks was periodically removed. Daily measurements of temperatures were recorded for each tank, and dissolved oxygen and salinity were monitored daily for each tank. Other parameters (e.g., nitrites, pH) were monitored intermittently to insure that water quality remained in appropriate ranges.

We were interested in evaluating the response time of each indicator to a major shift in fish growth rate (initiated by a major change in feeding regimen), and to see if there was a difference in magnitude or timing of response of each indicator between a shift from *ad libitum* feeding to starvation or a low ration of 2% body weight per day. Fish were randomly sampled from each tank (one tank per feeding regimen) so all fish in each group experienced the same holding conditions and to eliminate differences due to keeping fish in multiple tanks. In this design individual fish were the experimental units, each providing an independent estimate (replicate) of bioindicator responses to growth rate at specific time points. We related the individual measures of bioindicators to growth rate of the individual;

we then estimated the temporal response of the bioindicators after the initiation of a change in growth rate, resulting from the change in feeding levels.

At the start of the experiment all fish were weighed (nearest 0.01 g) and measured (nearest 1 mm SL) then returned to tanks. There were some mortalities during the acclimation period, so fish were randomly divided so that one tank would have enough for 10 individuals per sampling event and the other 8 individuals per sampling event, plus 2 - 3 extra fish in each tank in case of mortalities during the experiment). Feeding to apparent satiation twice a day (at least 4% body weight at each feeding) was continued for a week and then all fish were again weighed, measured, and returned to their respective tanks (except fish that were sampled). One tank was then randomly assigned to the starvation feeding level (No Food, 78 individuals in this tank, stocking density 0.92 g l^{-1}) and fish in the other tank were fed 2% body weight per day (2% Food, 65 individuals in this tank, stocking density 0.63 g l^{-1}), split between two feedings: one in the morning ~9 am and one in the afternoon ~4 pm). Sixteen days after the change in food levels, all remaining fish were again reweighed and measured and then food was restored to satiation levels twice a day for eight more days. Fish were sampled seven times during the experiment (10 from the starvation group and 8 from the 2% food group on each sample date): after the initial seven days at apparent satiation, 4, 8, 12, and 16 days after the feeding regimens were changed to starvation or 2% body weight per day, and 4 and 8 days after satiation feeding was resumed for both groups. Food at the 2% feeding level was adjusted for the removal of individuals. All weights and measurements were taken in the morning before the first daily feeding (at least 12 hours after

last feeding). At the time of sampling, each fish was killed with an overdose of tricaine methanesulfonate (MS-222; Argent, Redmond, WA, USA) then was weighed (nearest 0.01 g) and measured (nearest 1 mm), a ~0.5 ml blood sample was collected, ~0.029 g of dorsal muscle tissue was excised, and the whole liver was removed. Muscle tissue (for RNA and DNA analysis) and liver (for HSI and IGF-I mRNA expression) samples were placed in vials containing RNAlater (Ambion, Austin, TX), stored at 4°C for 24 to 48 hours, and then placed in a -80°C freezer until later analysis. Blood was collected from the caudal vein using heparinized 1-cc syringes with 22-gauge needles. Blood was then dispensed into 1.5 ml tubes and stored on ice until all sampling was done. Plasma was separated by centrifugation at 8,000 *g* for 15 minutes at 4°C, removed by pipette, placed in new 1.5 ml tubes, and then stored at -80°C until further analysis. For each fish we calculated specific growth rate (SGR), HSI, and Fulton's K values.

SGR was calculated as:

$$\text{SGR} = \left(\frac{(\ln bw_2 - \ln bw_1)}{time}\right) \times 100$$

where bw_2 is the body weight in grams of the fish at the end of the growth interval, bw_1 is weight at the beginning of the growth interval, and *time* is the interval in days between the two measurements expressed as percent body weight per day (% bw d^{-1}). We also calculated the percent weight gained or lost in grams for each measurement interval.

HSI was calculated as:

$$HSI = \left(\frac{liver\ Wgt\ (g)}{body\ Wgt\ (g)}\right) \times 100$$

Fulton's K condition factor was calculated as:

$$K = \left(\frac{W}{L^3}\right) \times 100$$

where W is weight in grams and L is standard length in cm.

2010 Trials

This second experiment was conducted similarly to the 2009 experiment, but the design was modified to allow us to: 1) evaluate responses of the various indicators over a longer time, 2) compare responses at an even lower feeding rate (1%) to those at starvation, and 3) see if patterns of growth and indicator responses would differ between fish fed at a constant 1% ration and fish that received the same 1% ration on average, but were intermittently fed 2% ration alternating with equal periods of starvation.

Juvenile spot (mean SL = 115 mm, range = 84 - 160 mm; mean weight = 26.16 g, range = 12.12 - 52.24 g) were collected by trawl from the Neuse River Estuary in September 2010. Fish were then transported to the Lake Wheeler Multispecies Building located on North Carolina State University campus, Raleigh, North Carolina, and placed in recirculating holding tanks. Spot were individually marked with visible implant elastomer (Northwest Marine Technologies, www.nmt.us) and randomly placed into three 1,019-l round tanks at a stocking density of ~1.9 g l^{-1}. Fish were acclimated to experimental conditions for two weeks (temperature 23 - 25°C, average salinity 12 - 15 ppt) with feedings to apparent satiation twice a day of thawed freshwater *Mysis* shrimp. Fish were maintained in a recirculating system equipped with biofiltration, UV-sterilization, and aeration; salinity was maintained by adding Crystal Sea Marine Mix (Marien Enterprises Inc., Baltimore, MD) to

well water. Tank maintenance and water quality monitoring were conducted as in the 2009 experiment.

At the start of the experiment all fish were weighed and measured, then returned to their respective tanks (79 individuals in the starvation regimen tank, 79 individuals in the 1% food regimen tank, and 21 individuals in the variable feed regimen tank). We continued to feed to apparent satiation twice daily for a week and then all fish were again weighed, measured, and returned to their respective tanks. Feeding was then stopped for fish in the No Food regime and reduced to 1% body weight per day level for fish in the constant ration regime (1% Food), divided into a morning and late afternoon feeding. Three weeks after the change in feeding regime all remaining fish in both regimes were again reweighed and measured, and food was restored to feeding to apparent satiation twice daily for eight more days. The No Food and 1% Food fish were sampled (10 individuals per sample) after the initial seven days at apparent satiation, then 4, 8, 12, 16 and 21 days after the feeding regimens were changed, and then 4 and 8 days after satiation feeding was. Food at the 1% feeding level was adjusted for the removal of individuals.

For the intermittent feeding regime (Variable Food) no individuals were sampled for tissues or blood after the initial seven days of feeding to apparent satiation, as we had fewer fish to work with in this group and up to this point they had experienced the same conditions as the other two groups. For these fish the feeding regimen was then changed to two days fasting, two days of 2% body weight per day, two days fasting, and then two days 2% body weight per day. Thus, over the eight-day period, the fish on average received 1% body

weight food per day, as in the 1% constant food regime. Because of the limited number of fish in this group five individuals were sampled after each two-day segment, and this regime ended eight days after the initial reduction in feeding rate.

For each of the three groups, at the time of sampling each fish was killed with an overdose of tricaine methansulfonate, then weighed and measured. All weights and measurements were taken in the morning before first daily feeding (at least 12 hours after last feeding). Dorsal muscle tissue, whole liver and blood samples were collected and treated in the same manner as for the 2009 experiment and SGR, HSI and Fulton's K were also calculated the same way.

Laboratory Work

RNA and DNA Nucleic Acid Quantification from Muscle Tissue

RNA and DNA concentrations in white muscle samples were quantified using a one-dye, two-enzyme (ethidium bromide (EB) plus RNase and DNase) fluorometric microplate assay following Caldarone et al. (2001). Tissue samples were removed from RNAlater solution, rinsed with deionized water, patted dry with new tissue wipes, and weighed (nearest 0.0001 g). Samples were placed in vials with a 2% N-lauroylsarcosine solution with RNase free 0.5 mm Zirconium Oxide beads, vortexed for 30 minutes and then placed in a cooled Bullet Blender (Next Advance, Inc., New York) for five minutes. If tissues were not completely digested, samples were again vortexed and placed in the Bullet Blender. Once tissues were digested, 1.35 ml TE buffer was added and then centrifuged at 14,000 rpm for 15 minutes to separate cell debris. Supernatants of samples were removed and diluted to a

working concentration on 0.1% N-lauroylsarcosine before quantification of nucleic acids. Triplicate aliquots (0.075 ml) of each sample were loaded into flat-bottom, non-binding surface96-well microplates (Corning, USA), along with serial-diluted RNA and DNA standards (18S + 28S ribosomal RNA from calf liver, Sigma R-0889; DNA from calf thymus, Sigma D-4764), and stained with 0.075 ml of 2.0 μg ml^{-1} EB. Total fluorescence at 520 nm excitation and 612 nm emission wavelengths was measured using a fluorometer (Fusion Universal Microplate Analyzer, Packard BioScience Company). After initial fluorometric reading, RNA and DNA fluorescence were measured as the decrease in fluorescence after the sequential addition of RNase and DNase, respectively. The slope ratio of DNA to RNA standard curves was 2.41 ± 0.57. Tissue nucleic acids were expressed as the ratio of RNA:DNA (μg RNA μg^{-1} DNA) and RNA concentration per gram wet tissue weight (RNA:tissue weight; μg g^{-1}). Tissue weights inadvertently were not retained for the 2009 starvation group, so for these samples we used the average weight of tissue samples collected in the same manner for the 2009 2% body weight per day food group (mean ± SE, 0.029 ± 0.001 g).

Radioimmuno assay (RIA) for plasma IGF-I

Circulating levels of IGF-I were measured from acid/ethanol extracted plasma by RIA using recombinant barramundi IGF-I as a tracer and standard, rabbit anti-barramundi IGF-I primary antibody (Novozymes Gro Pep, Adelaide, Australia) and goat anti-rabbit secondary antibody (Sigma), according to previously described methods (Shimizu et al. 2000, Picha et al. 2006). Barramundi IGF-I was iodinated using the chloramine-T method

and purified by column chromatography. Tracer (^{125}I-barramundi IGF-I) was diluted to 20,000 cpm for each assay tube. Samples were run in triplicate and only samples with values that fell on the linear part of the barramundi standard curve were evaluated. Many samples were lost in a freezer malfunction, yielding limited plasma IGF-I analysis from the 2009 experiment and no samples for analysis from the 2010 experiment.

IGF-I Gene Expression in Liver Tissue

As an alternative to measuring IGF-I levels in plasma, many of the livers we sampled and stored in RNAlater were suitable for IGF-I gene expression analysis using real-time quantitative PCR. Liver tissue total RNA was isolated by TRI reagent extraction (Molecular Research Center, Cincinnati, OH), with an added glycogen removal step using Plant RNA Isolation Aid (Ambion, Austin, TX) (Picha et al. 2008). The Isolation Aid (polyvinypyrrolidone) binds glycogen in aqueous solutions, forming a pellet after incubation and centrifugation, allowing for total RNA to be reprecipitated from the drawn aqueous phase and improve accuracy of RNA quantification by spectroscopy. Total RNA was then suspended in RNase-free water and checked in triplicate for concentration and purity by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE). Absorbance values ($Abs_{260/280}$) in samples were 1.71 - 2.08. Gel electrophoresis (1% agarose, 0.4 μg ml^{-1} ethidium bromide) confirmed quality of RNA. Samples were then treated with DNase (Turbo DNA-free, Ambion, Austin, TX) and final quantification and integrity was measured by spectrophotometry using a Nanodrop 1000. One microgram of

DNase-treated total RNA was reverse transcribed to cDNA using a High Capacity cDNA Synthesis kit (Applied Biosystems, Foster City, CA).

Liver IGF-I mRNA was measured in triplicate by SYBR Green Real-Time PCR assay (Qiagen, Valencia, CA) using a gene-specific primer pair designed using ABI Primer Express (v 3.0) software (Forward: TGC TGC TTC CAA AGC TGT GA; Reverse: TCT TGG CAG GTG CAC AGT ACA). We first partial sequenced IGF-I by PCR of genomic DNA to design gene-specific primers. A 608 bp gene fragment was amplified with degenerate primers designed from a multiple sequence alignment of known fish IGF-I. The PCR product was purified with QIAquick PCR Purification Kit (Qiagen) and sent for sequencing at the University of Chicago Cancer Research Center DNA Sequence facility. We ran our spot IGF-I sequence through the National Center for Biotechnology Information GenBank BLAST search and found it was identified as identical to that of red drum *Scianops ocellatus* IGF-I (see Faulk et al. 2010). Real-time PCR analysis was performed using 20-ng total RNA sample with Brilliant II SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA) containing 1.5 µM primer concentrations on an ABI 7300 96-well detection system. After a 10 minute denaturation at 95°C, 40 cycles of PCR were performed consisting of a denaturing step at 95°C for 30 seconds and an annealing and extension step at 60°C for 60 seconds, followed by a dissociation step of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. Real-time measurements were made during the extension step. Melting curve analysis denoted a single gene product. Pooled cDNA samples were used to account for assays using multiple plates (across-plate normalization), with negative controls

run on each plate. Cycle threshold (C_t) values for samples were analyzed by absolute quantification, using standard curves of 10-fold diluted copy number cDNA (dilution ranged from 10 to 10^{10} copies per µl; R^2=0.99). Sample mRNA data were normalized to total RNA concentration, liver weight, and body weight (Bustin 2000, Picha et al. 2008) and are presented as IGF-I mRNA total copy number for the entire liver relative to body weight ((ng total liver RNA X copy number of gene per ng RNA)/(g body weight)). We expressed hepatic IGF mRNA levels this way to account for gene expression output for the entire liver, to take into account the dramatic size fluctuations of the liver relative to body weight during feeding manipulations, and finally dividing by body weight to put mRNA expression in an organismal context (Picha et al. 2008).

Data Analysis

Relationships between SGR and indicator values from each fish were analyzed by regression analysis. Values of RNA:DNA ratios, IGF-I mRNA expression, and IGF-I plasma levels were $\log_{10}$ transformed before regression analysis to meet the assumptions of normality and equality of variance for statistical analysis. We used two-factor analysis of variance (ANOVA) tests to evaluate bioindicator values over time and to compare fish from the different feeding regimes on each sample day. As noted above, individual fish provided independent estimates of an indicator's response at a specific time since the change in growth rate was initiated. Post-hoc linear pairwise comparisons were made for individual sample days and comparisons of fish within feeding regime were made using Tukey's HSD. All

analyses were conducted using JMP 9 (SAS Institute, Inc., Cary, NC) with an alpha level of 0.05.

RESULTS

2009 Trials

As expected, specific growth rates of juvenile spot dropped to markedly negative values for both the No Food regimen and 2% Food regimen after the change in ration on day 7 (Figure 3.1). Two-factor ANOVA showed no significant interaction between day sampled and fish from the different feeding regimes ($df = 6,125$, $p = 0.59$) indicating that SGR of fish responded in a similar manner over time. The main effect of day sampled was significant ($df = 6, 125$, $p < 0.001$), but linear comparisons showed no difference between the SGRs of two feeding regimes on specific days sampled. Fish in the No Food regimen incurred over half of their total percent weight lost in the first four days after feeding ceased. No interaction was found for cumulative percent weight lost between day sampled and fish from different feeding regimes (ANOVA, $df = 6, 125$, $p = 0.15$), but the main effect of day sampled was significant ($df = 6, 125$, $p < 0.0001$). Linear comparisons showed that SGR was significantly higher 16 days after the change in feeding for fish in the 2% Food regimen than in the No Food regimen ($df = 1, 114$, $p = 0.04$) (Figure 3.1). The cumulative percentage weight lost of fish in the No Food regime was not significantly different from day 4 to day 16 after feeding ceased (Figure 3.1), indicating that most of the change in growth rate and weight lost happened during the first week and then the fish reached a new, lower steady metabolic state. For fish with 2% Food ration, virtually all the weight loss occurred in the first four days after

ration was reduced; there was no significant difference in cumulative percentage weight lost during the whole feed restriction time (Figure 3.1). For both regimens, the apparent improvement in SGR (i.e., becoming less negative) from day 4 to 16 over the restricted feeding period is largely an artifact of the initial sharp drop in growth rate being averaged with progressively longer periods of nearly neutral SGR.

Four days after reinstatement of feeding to satiation, fish from the No Food regimen still had not achieved an average positive SGR (mean ± SE, -0.30 ± 0.54% *bw* d^{-1}), while fish from 2% Food regimen did (0.12 ± 0.15% *bw* d^{-1}); however, after eight days fish from the No Food regimen had higher SGR (0.54 ± 0.06% *bw* d^{-1}) than fish from the 2% Food regimen (0.28 ± 0.10% *bw* d^{-1}). However, growth rates of both groups after 8 days of satiation feeding following 16 days of starvation or low ration were still much lower than growth rates after the initial satiation feeding period.

In both regimens values of HSI decreased significantly over the first eight days after feeding was restricted, and then did not decline further (Figure 3.2) The two-factor ANOVA found significant main effects of day sampled ($df = 6, 123$, $p < 0.0001$) and fish feeding regime ($df = 1, 123$, $p = 0.001$), but their interaction was not significant ($df = 6, 123$, $p = 0.14$). However, linear comparisons found fish in the 2% Food regimen had higher HSI values than fish in the No Food regimen 16 days after feeding was restricted ($df = 1, 110$, $p = 0.008$) and after seven days of satiation feeding ($df = 1, 110$, $p = 0.001$) (Figure 3.2). After the change back to satiation feeding, HSI increased for both groups, but not to the levels exhibited by fish sampled after the initial seven days of satiation feeding. The HSI values of

fish in the No Food regimen only decreased 30% after four days of starvation, with a 62% percent decrease after 16 days. After return to feeding, HSI levels increased, but were still 29% less than HSI after the first feeding period (Figure 3.2). The 2% Food regimen HSI levels followed a similar pattern with less of a drop in HSI values over the period of food restriction, but final HSI values after eight days of return to satiation feeding were still 46% less than values after the initial satiation feeding period (Figure 3.2).

Fulton's K changed less in response to the changes in feeding regime than other indicators; there was a significant interaction between day sampled and fish feeding regime (ANOVA, df = 6, 125, $p = 0.0049$) in Fulton's K response driven by the fact that Fulton's K values in the No Food regimen did not increase as quickly after return to satiation feeding as those of the fish in the 2% Food regimen; day sampled was also a significant main effect (df = 6, 125, $p < 0.0001$). Values were slightly lower in both regimens after 12 and 16 days of restricted feeding and both groups showed modest increases eight days after the return to satiation feeding (Figure 3.2).

Logistical problems reduced somewhat the data available for our analyses of RNA:DNA ratio and RNA concentration. Inconsistencies among replicates forced us to eliminate some individual samples, resulting in low sample sizes for some samples, and the final sample from the No Food group was lost as a result of a freezer malfunction. The high RNA:DNA ratio for the No Food regimen after the initial satiation period was driven by lower measured DNA levels than in other samples, rather than much higher levels of RNA

(Figure 3.3). This value is based on data from only three fish, but the replicates of each individual sample were consistent so we retained them in our analysis.

Despite these limitations, the available data indicate relatively modest and slow responses by both RNA:DNA ratio and RNA concentration to changes in feeding regime and SGR (Figure 3.3). For RNA:DNA ratio there was a significant interaction between day sampled and feeding regime (ANOVA, $df = 5, 83$, $p = 0.0003$) with both main effects also significant (feeding regime $df = 1, 83$, $p = 0.002$; day sampled $df = 5, 83$, $p < 0.0001$). In the No Food group, RNA:DNA ratio declined over the first 4-8 days of no feeding, then changed little over the remainder of the starvation period and did not increase after four days of satiation feeding. In the 2% Food regimen group RNA:DNA ratios four days after the reduction in feeding rate were similar to those following the initial seven days of satiation feeding, but were significantly lower eight days after the decline in feeding, then remained constant for the remainder of the reduced feeding period. Linear comparisons sowed a significant difference in RNA:DNA ratios between fish from the two feeding regimes eight days after feeding was reduced ($df = 1, 71$, $p = 0.0005$). When satiation feeding was resumed in the 2% Food regimen group, the rate of response in RNA:DNA ratio was similar to that following the initial decline in feeding rate; there was no change after four days of satiation feeding, but an increase after eight days, though not to the level observed after the first satiation feeding period (Figure 3.3). There was a significant interaction between day sampled and fish from different feeding regimes for RNA concentration (ANOVA, $df = 5, 82$, $p < 0.0001$), along with the significant main effect of day sampled ($df = 5, 82$, $p <$

0.0001). RNA concentrations in fish from the No Food regimen did not decline until eight days of starvation (Figure 3.3). RNA concentrations of fish in the 2% Food did not decline until 12 days after food reduction, and while RNA concentration did increase 8 days after return to satiation feeding (692.1 ± 69.3 μg g^{-1}), it did not reach the same level as after the first satiation period (955.0 ± 114.2 μg g^{-1}) (Figure 3.3).

In fish from both feeding groups, IGF-I mRNA expression in the liver had a generally similar, though less pronounced, pattern as HSI in response to feeding regime over time with no interaction between sampling day and feeding regime (ANOVA, df = 6, 112, p = 0.07). IGF-I mRNA expression decreased in during the first 4-8 days of starvation or restricted feeding, had no change during the remainder of the reduced food period or four days after return to satiation feeding, but a significant increase eight days after return to high feeding level (feeding regime df = 1, 112, p = 0.003; day sampled (df = 6, 112, p < 0.0001). After seven days satiation feeding and on days 8, 12 and 16 after food restriction fish in the 2% Food regimen had significantly higher IGF-I mRNA expression levels than fish in the No Food regimen in linear comparisons (7 days satiation df = 1, 99, p = 0.003; 8 days df = 1, 99, p = 0.02; 12 days df = 1, 99, p = 0.0005; 16 days df = 1, 99, p = 0.02) (Figure 3.4). Expression levels of IGF-I mRNA eight days after the return to satiation feeding matched (2% Food fish) or exceeded (No Food fish) expression levels after the initial seven days of satiation feeding (Figure 3.4).

Circulating IGF-I plasma levels showed no significant main effects (ANOVA, feeding regime df = 1, 35, p = 0.14; day sampled df = 5, 35, p = 0.28) or interaction (feeding

regime*day sampled df = 5, 35, p = 0.09) (Figure 3.4). In the 2% Food regimen levels appeared to decline over the first eight days of reduced feeding rate, but sample size was too low to interpret this with confidence. There was no significant relationship between IGF-I mRNA expression and IGF-I plasma levels in either of the feeding regimes (regression analysis, No Food: df = 22, p = 0.26; 2% Food: df = 6, p = 0.36), but the low number of observations for plasma levels limited the sample sizes for these comparisons.

To assess the potential each of the indicators had for explaining SGR, we ran regression analyses between SGR and each individual indicator. Even though most of the indicators showed some pattern in relation to changes in food regime over time (Figures 3.1-3.4), their values were not tightly coupled with SGR when analyzed over the whole trial; many relationships were not significant, and those that were explained a modest amount of variation in SGR at best (Table 3.1). In the No Food regimen, RNA:DNA ratio ($R^2 = 0.30$) and HSI ($R^2 = 0.29$) explained the most variation in SGR. In the 2% Food regimen HSI was the only variable that was significantly related to SGR with a comparable R^2 value (0.34); IGF-I liver expression was also significantly related to SGR, but only with $R^2 = 0.10$ (Table 3.1).

Because the sharpest changes in SGR and most indicators occurred in the first 4-8 days following the shift from satiation feeding to restricted feeding, and to a lesser extent in the 4-8 days following the return to satiation feeding at the end of the trials, we focused more specifically on these two periods to further explore the SGR-indicator relationship. One set of regression analyses was conducted using data from fish sampled at the end of the initial 7-

day satiation feeding period and 4 and 8 days after food reduction; the second set used data from fish collected on day 16 of the reduced food period and 4 and 8 days after satiation feeding resumed. In these analyses each indicator that exhibited a significant relationship to SGR explained a higher proportion of the variation in SGR than it did using all the data combined, but R^2 values were still generally modest (Table 3.1). In the No Food regimen RNA:DNA ratio explained the most variation in SGR following the shift from satiation feeding to starvation ($R^2 = 0.41$) and HSI explained the most variation in SGR following the shift back to satiation feeding ($R^2 = 0.33$). In the 2% Food regimen, $\log_{10}$(IGF-I plasma) explained 80% of the variation in SGR following the initial drop in feeding level, but this should be interpreted with caution as the sample size was small (n = 6) and this indicator was not a significant predictor of SGR in any of the other regressions. The next best predictor of SGR following the initial drop in feeding level was HSI ($R^2 = 0.52$). Following the return to satiation feeding only one indicator, RNA:DNA ratio, was significantly related to SGR, but it only explained a small portion of variation ($R^2 = 0.17$) (Table 3.1).

Since most studies relating IGF-I levels to growth rate test for changes after longer periods of time (typically weeks or months, see Beckman 2011), we also compared IGF-I mRNA expression levels between fish collected after 7 days of satiation and after 16 days of starvation or reduced feeding. In both of these regression analyses IGF-I mRNA expression was positively related to SGR and explained over half of the variation in SGR (No Food, $R^2 = 0.63$, $p < 0.0001$; 2% Food, $R^2 = 0.64$, $p = 0.001$); further ANCOVA analysis indicated

they had the same slope (m = 3.84), but the No Food regimen had a higher intercept value (No Food = -27.29 vs. 2% Food = -27.86).

Tank Trial 2010

As in 2009 we analyzed differences in response of bioindicators between fish in the No Food and 1% Food feeding regimes with a two-factor ANOVA with day sampled and feeding regime as factors. However, since fish in the Variable Food regime were sampled on a different frequency and over a shorter period than fish in the other regimes, we analyzed these fish in a separate one-factor ANOVA to discern changes over time. On days 4 and 8 after change in food levels, when we had samples from all three regimes, we tested for differences in indicator response among fish in all three feeding regimes using an ANOVA for each of those days.

Growth rates of juvenile spot during the initial satiation feed period were lower in 2010 than in 2009, with several fish exhibiting negative SGR during the first week. Distribution of growth rates for this period was non-normal and skewed towards lower growth rates in 2010, unlike the normal distribution in 2009. While social hierarchy behavior was not observed (e.g., fish chasing each other around the tank, nipping at fins), the variation of growth rates within the tanks could be due to more dominant individuals consuming a larger share of food than others. In 2010, during the satiation week, fish were fed at least 8% of their body weight per day (often more), divided into two feedings a day, but this obviously did not provide satiation levels of food to all fish or some fish were not eating due to other stressors. Since we wanted to examine how the indicators responded when fish with high

SGR switched to negative SGR, we eliminated fish that had a negative SGR during the satiation feeding week from our analysis (but not from the tanks), reducing the sample size from 70 to 36 in the No Food regimen and from 70 to 51 in the 1% Food regimen; no fish were eliminated from the Variable Food regimen.

The Variable Food group had lower SGR after periods of fasting than after periods of 2% *bw* d^{-1} variable feeding (ANOVA, df = 41, $p < 0.0001$), but on days 4 and 8 after the initial satiation feeding period, when the Variable Food group had received on average the same amount of food as the 1% Food group, growth rates of fish in the Variable Food group did not differ from those of fish in the 1% Food or No Food groups (ANOVAs; 4 days post satiation, df = 16, $p = 0.625$; 8 days post satiation df = 17, $p = 0.3135$) (Figure 3.5). Cumulative percent weight loss by fish in the Variable Food regimen was similar for days 2 (-1.63% ± 0.27) and 4 (-1.79% ± 0.30) after food reduction, indicating that after this weight loss during the first two days of fasting fish weight remained ~constant during the next two days of 2%. On day 6, after two more days of fasting, cumulative weight loss for Variable Food regimen fish had roughly doubled (-3.70% ± 0.39) and remained similar on day 8 (-3.18% ± 0.25) after two more days of 2% food (Figure 3.5).

Fish from the No Food and 1% Food regimes showed similar patterns in change of cumulative percent weight loss over time (ANOVA, fish food regime*day sampled df = 6, 86, $p = 0.60$), but did have a significant main effect of day sampled (day sampled df = 6, 86, $p < 0.0001$). The 1% Food regimen group had similar but slightly increasing cumulative percent weight loss on days 4, 8, and 16 after food reduction, indicating that most of this

weight loss had occurred in the first four days, but that some minor weight loss was continuing over this period. However, between days 16 and 21weight declined more with the cumulative change in weight going from -4.0% (± 0.96%) on day 16 to -6.3% (± 0.60%) on day 21 (Figure 3.5). Fish in the No Food regimen also continued to lose weight over the whole starvation period, but as in the 2009 experiment the rate of weight loss decelerated over the course of the starvation period. Over half of the total percent weight loss by day 21 (-7.9% ± 0.70) had occurred by day 8 (-4.7% ± 1.05) and nearly all had occurred by day 16 (-7.0% ± 0.25%) (Figure 3.5). The only significant differences in SGR and cumulative percent weight loss between fish in the No Food and 1% Food regimens occurred 16 days after food reduction ($df = 1, 73$, $p = 0.02$) (Figure 3.5).

In contrast to the 2009 experiment that had a 16-d restricted feeding period, in the 2010 experiment spot exhibited compensatory growth rates after a 21-d restricted feeding period in both the No Food and 1% Food regimens. In No Food regime fish's SGR values after 4 and 8 days of refeeding were 101% and 232%, respectively, of SGR during the initial 7-d satiation feeding period; and, in the 1% Food regimen fish SGR values after 4 and 8 days of refeeding were 156% and 297%, respectively, of SGR during the initial 7-d feeding period. Day sampled had a significant effect on SGR (ANOVA, $df = 6, 83$, $p < 0.0001$) but the interaction between day sampled and fish feeding regime was not significant ($df = 6, 83$, $p = 0.96$) (Figure 3.5).

In fish both in the No Food and 1% Food regimens, HSI decreased after food reduction in a pattern similar to that observed in the 2009 experiment; however, unlike in the

2009 experiment, HSI levels of fish increased substantially during the refeeding period, surpassing the levels observed immediately after the initial 7-day period of satiation feeding. The significant increase of HSI of fish in the No Food regime 8 days after refeeding (linear comparison df = 1,72, p = 0.01) drives the interaction between fish feeding regimes and day sampled (ANOVA, df = 86, fish feeding regime p = 0.20, day sampled p < 0.0001, fish feeding regime*day sampled p = 0.04) (Figure 3.6). By 8 days after return to satiation feeding, HSI of fish in the No Food regimen (2.06 ± 0.08) was 61% greater than their HSI values after the initial 7-day satiation period (1.28 ± 0.22) and HSI of fish from the 1% Food regimen (1.76 ± 0.14) was 59% greater than after the initial satiation period (1.10 ± 0.07). A (non-significant) greater decrease in HSI was detected four days after food restriction in fish from the No Food regimen (45%) than in fish from the 1% Food regimen (27%), but by 8 days after food restriction began, all fish's HSI levels had decreased by ~54% (Figure 3.6). HSI values in the Variable Food regimen declined slightly over the restricted food period, but were not significantly different from one another (ANOVA, df = 20, p = 0.1352). However, HSI was significantly higher in the Variable Food regime than in the 1% Food regime 8 days after feed restriction, with HSI of fish in the No Food regime similar to both groups (ANOVA, df = 16, p = 0.02).

There was a small but significant decline in Fulton's K over the first eight days after food restriction in all feeding regimes. Values remained at this lower level for the remainder of the restricted feeding period, then increased significantly eight days after resumption of satiation feeding in the 1% Food and No Food fish (ANOVA, df = 86, fish feeding regime p

= 0.07, day sampled $p < 0.0001$, fish feeding regime*day sampled $p = 0.08$) (Figure 3.6). Fulton's K was greater in the 1% Food regimen four days after return to satiation feeding (linear comparison df = 1, 73, $p = 0.004$), but after eight days refeeding, there was no difference between groups, and Fulton's K levels were equal to those after the initial 7-day satiation feeding period (Figure 3.6). The Variable Food fish had a significant effect of day sampled in Fulton's K response and exhibited a decline over the time period (though less pronounced than fish from other feeding regimes), but Tukey's HSD post-hoc test assigned no differences in levels among days sampled (ANOVA, Variable Food: df = 41, $p = 0.0384$) (Figure 3.6).

Similar patterns of RNA:DNA ratio decrease occurred in fish from both the No Food and 1% Food regimes (ANOVA, fish feeding regime*day sampled df = 6, 53, $p = 0.21$), ratios declined within four days after feeding ceased, then did not increase until food was again made available to the fish (day sampled df = 6, 53, $p = 0.0003$, Tukey's HSD showed differences by day in No Food regime fish) (Figure 3.7). As in the 2009 experiment, sample sizes were reduced due to some samples being rejected because of lack of agreement between replicates. In the No Food regimen, RNA:DNA ratios, after removal of food, averaged 48% lower than immediately after the initial seven days of satiation feeding. When satiation feeding resumed, RNA:DNA ratios began climbing and reached levels similar to those after the initial seven days of satiation feeding, which is unlike the pattern seen in 2009 and may have to do with the compensatory growth that occurred during the refeeding period in this experiment (Figure 3.7).

As for the 2009 experiment, we ran regression analyses on the relationship between SGR and each indicator for the 2010 No Food and 1% Food regimen groups to explore how well each of the indicators explained variability in SGR. Generally the indicators explained more variability in SGR in the 2010 experiment than in the 2009 trials. Over the whole experiment, the positive relationship of HSI to SGR explained the most variability in SGR in both the No Food regimen ($R^2 = 0.65$) and the 1% Food regimen ($R^2 = 0.56$) (Table 3.2). RNA:DNA ratio also explained a substantial amount of variation ($R^2 = 0.46$) in the No Food regimen and all indicators except Fulton's K explained significant amounts of variation in SGR in both groups. In this experiment we again looked for differences after a prolonged period of reduced food as has been done in previously published studies, as well as for more rapid responses. In the analyses using data from fish collected on day 21 of the reduced food period and 4 and 8 days after satiation feeding resumed, all the indicators exhibited significant, positive relationships with SGR, and the amount of SGR variation explained by indicators was much higher than for the overall experiment, apparently due to the substantial increase in SGR and indicator values in response to satiation refeeding. Again, HSI explained the greatest amount of variability in both the No Food group ($R^2 = 0.74$) and the 1% Food group ($R^2 = 0.61$); RNA:DNA ratio also explained a substantial amount of the variation in SGR in these groups (0.66 and 0.46, respectively) and even Fulton's K performed relatively well (Table 3.2). In contrast, analyses using data from fish sampled at the end of the initial 7-day satiation feeding period through the first eight days of food reduction showed that relationships between most indicators and SGR were either not

significant, or explained even less variation in SGR than in analyses based on all data from the trials. The one exception was RNA:DNA ratio ($R^2 = 0.80$), though the sample size for this analysis was quite low (n = 10).

DISCUSSION

The indicators we examined in juvenile spot responded to pronounced changes in feeding levels, but not as rapidly as we anticipated. There was a lag of at least four days, and sometimes eight days, between the drop in SGR and the full drop in indicator values after feeding was reduced. Similarly, upon refeeding, indicators did not fully respond after four days. Hence, indicators did not change as rapidly as exogenous factors (on a time scale of four day intervals) (in this case, feeding level) caused changes in growth rates. Fulton's K did not show a large nor quick response to reduction in feeding, but did gradually decrease towards the end of food restriction and increase eight days after refeed. Therefore, Fulton's K could be used as a convenient, non-lethal tool to quickly and coarsely evaluate condition and long term growth. In contrast, other studies have reported more rapid responses of RNA:DNA ratios to reduction in feeding levels, ranging from four-to-six days in larval cod *Gadus morhua* (Clemmesen & Doan 1996) and one to two days in larval red drum *Sciaenops ocellatus* (Rooker & Holt 1996). However, values of RNA:DNA ratios and RNA concentration and their rate of response to changes in growth rates vary among species, with some species responding more quickly than others, as seen in response times of RNA:DNA ratios in summer flounder and weakfish *Cynoscion regalis* (Stierhoff et al. 2009). The quick response time of these indicators in larval and very young juvenile fish may not be

achievable in older fish, and some question the applicability of RNA:DNA ratios in older juveniles (Houlihan et al 1993; Buckley et al 1999). Moreover, before moving forward with RNA and DNA indices to estimate growth rates of wild spot, the effect of water temperature on the relationship between growth rate and RNA:DNA ratio needs to be calibrated, as other studies have shown this relationship to be temperature-dependent (Buckley et al 1999). We encourage evaluation across a larger range of feeding levels and intermittent feeding patterns, as these will be more applicable to the field than just fed-starved extremes.

Each individual indicator did not have consistent explanatory value across feeding regimes and feeding periods. In specific cases, RNA:DNA ratio (2010 food change from high food to no food) and IGF-I plasma (2009 food change from high food to 2% food) each explained 80% of the variation in SGR, but as these relationships were not evident in other instances, (and sample size was quite small for the 2009 IGF-I plasma data), we suggest caution in using relationships over specific time periods to predict growth rates over periods of different durations. The indicators we evaluated generally showed a stronger relationship with SGR over a relatively short (4-8 days) period following a change in feeding level (either high to low or low to high) than over longer periods of starvation or low ration, implying the response of these indicators does not track the change in SGR as it becomes less negative over prolonged periods of restricted feeding. As in other studies that have observed a significant decline of IGF-I in fish that had been fed *ad libitum* and then starved for a prolonged period (see Picha et al. 2008 and Beckman 2011 for reviews), we also saw a strong decline in IGF-I mRNA expression between the end of the initial 7-day satiation feeding

period and after 16 days of starvation or 2% feeding, which accounted for much of the variability in SGR between these extremes (R^2 = 0.63 - 0.64). For applications that can predict short-term growth rates of fish in relation to environmental conditions in the wild, however, we need resolution over a shorter time period (scale of 1-2 days). While individual indicators might not consistently provide high explanatory power, using multiple indicators in concert with each other may allow us to more effectively estimate recent growth of juvenile fishes.

Given that IGF-I plays a direct role in regulating somatic growth (Duan 1997, LeBail et al. 1998), we expected it to better predict SGR than the other indicators we tested. Some studies have found no correlation between IGF-I and growth rates (Silverstein et al. 1998, Nankervis et al. 2000), though results across research studies range from no evident relationship, to weak but significant, to robust positive relationships (Beckman 2011). Our results do indicate that changes in IGF-I are related to growth of spot, but its effectiveness for predicting short-term growth rates appears limited. However, the high variation in response and little pattern in relation to feeding to IGF-I plasma levels that we observed may due, in part, to small sample sizes. Many of the plasma samples we analyzed were not in the linear detection range of the radioimmunoassay technique we used, which could be due to lower levels of circulating IGF-I in blood of juvenile spot than have been found in older hybrid striped bass (Picha et al. 2006). Simply increasing the amount of plasma used in the analysis was not feasible, as the spot were small and only a limited amount of blood could be extracted from each fish. In future studies of IGF-I plasma levels in small fish, we

recommend using the fluoroimmunoassay method of evaluating IGF-I plasma that requires less plasma per sample and may be more sensitive to detecting lower IGF-I levels (Small & Peterson 2005).

Because most circulating IGF-I is produced in the liver (Duan 1997, LeBail et al. 1998), measures of IGF-I mRNA expression in the liver are used as commonly as circulating IGF-I plasma levels in studies of fish. Studies with several fish species have found significant positive relationships between IGF-I plasma levels and hepatic IGF-I mRNA levels (Pierce et al. 2005, Vera Cruz et al. 2006, Luckenbach et al. 2007, Picha et al. 2008a, b), suggesting that hepatic IGF-I mRNA levels could serve as a growth indicator. The lack of relationship in our study may have been due to low sample size, however, Uchida et al. (2003) also did not observe a positive relationship between IGF-I plasma levels and IGF-I mRNA expression for Mozambique tilapia *Oreochromis mossambicus*. Most research evaluating the response of IGF-I to changes in growth rate in fish, and how environmental conditions might affect that relationship, is quite recent (Beckman 2011). So while IGF-I may correspond well to growth rates in some fishes and conditions, it might not serve as well as a growth index for all species (Beckman 2011), including spot. However, as this was the first study of IGF-I in spot, we encourage further evaluation of its utility as a growth indicator for this species.

Because feeding rates in the field may vary from day to day, we examined how growth rate, and several potential indicators of recent growth, responded to intermittent and constant feeding rates with the same average feeding level. Growth rates of fish in the

intermittently fed group were generally equivalent to those of fish in the group fed at a constant rate when compared on days 4 and 8 of sampling. However more negative spikes in SGR occurred after the two-day fasting periods, so results might have differed somewhat if the order of fasting and fed periods were switched and we compared SGRs after period of fasting. Both HSI and Fulton's K remained high in the Variable Food group, while the constantly fed 1% Food group exhibited modest but significant declines in both of these metrics. This difference in response suggests that pulses of food consumption following short periods of fasting may be sufficient to replenish energy stores in the liver and maintain body condition in spot. Further investigations into the responses of growth rate and growth rate indicators to intermittent feeding is warranted, given that irregular feeding patterns are more likely for fish in the wild than constant feeding rates or long periods of starvation. Further, examination of how intermittent fasting and feeding over longer periods might change biochemical, endocrine or metabolic processes in fish would be interesting, to see if these processes continue to respond to short-term changes in feeding and growth, or become insensitive to fluctuations after a time and reach a new steady state.

With this research we gained valuable insight into how HSI, Fulton's K, RNA:DNA ratio, and IGF-I mRNA expression in the liver of juvenile spot respond over time to sharp changes in SGR. The time lag between change in SGR and responses of the indicators suggests that the shortest period over which juvenile spot growth rates can reasonably be evaluated with these indicators is around eight days. Individual indicators alone might not be able to predict individual SGR with great confidence, but could possibly be used in a

threshold manner as a biomarker for growth. Our findings that IGF-I levels would not be able to provide a precise estimate of SGR or differentiate between small changes in growth rates are consistent with conclusions made by Beckman et al. (2004) for coho salmon *Oncorhynchus kisutch* and Luckenbach et al. (2007) for southern flounder *Paralichthys lethostigma*. Yet, both RNA:DNA ratios and IGF-I mRNA expression could be used to detect relatively large differences in growth rates, and larger divergences in growth rates might be more informative in applications to fish in the wild, as their biological consequences would be greater than smaller changes. We encourage more research to evaluate the responses of these indicators at a variety of growth rates, and to explore whether combinations of indicators may prove more effective for estimating growth rates over a range of conditions.

REFERENCES

Beckman BR (2011) Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. Gen Comp Endocrinol 170:233-252

Beckman BR, Fairgrieve W, Cooper KA, Mahnken CVW, Beamish RJ (2004) Evaluation of endocrine indices of growth in individual postsmolt Coho salmon. Trans Am Fish Soc 133:1057-1067

Bolger T, Connolly PL (1989) The selection of suitable indices for the measurement and analysis of fish condition. J Fish Biol 34:171-182

Buckley L, Caldarone E, Ong TL (1999) RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. Hydrobiologia 401:265-277

Buckley LJ, Turner SI, Halavik TA, Smigielski AS, Drew SM, Laurence GC (1984) Effects of temperature and food availability on growth, survival and RNA-DNA ratio of larval and sand lance (*Ammodytes americanus*). Mar Ecol Prog Ser 15:91-97

Bulow FJ (1987) RNA-DNA ratios as indicators of growth in fish: A review. In: Summerfelt RC, Hall GE (eds) The Age and Growth of Fish. The Iowa State University Press, Ames, Iowa p 45-64

Caldaron EM, MacLean SA, Sharak B (2012) Evaluation of bioelectrical impedance analysis and Fulton's condition factor as nonlethal techniques for estimating short-term responses in postsmolt Atlantic salmon (*Salmo salar*). Fish Bull 110:257-270

Caldarone EM, Wagner M, St. Onge-Burns J, Buckley LJ (2001) Protocol and guide for estimating nucleic acids in larval fish using a fluorescence microplate reader. Ref

Doc 01-11:1-22, National Marine Fisheries Service, Woods Hole, MA. Also available at www.nefsc.noaa.gov/nefsc/publications/crd/crd0111/0111.htm

Campbell LA (2012) Direct and indirect effects of hypoxia on juvenile fish in the Neuse River Estuary, North Carolina. PhD dissertation, North Carolina State University, Raleigh, NC

Clemmesen C, Doan T (1996) Does otolith structure reflect the nutritional condition of a fish larva? Comparison of otolith structure and biochemical index (RNA/DNA ratio) determined on cod larvae. Mar Ecol Prog Ser 138:33-39

Duan C (1997) The insulin-like growth factor system and its biological actions in fish. Am Zool 37:491-503

Faulk CK, Pérez-Domínguez R, Webb Jr KA, Holt GJ (2010) the novel finding of four distinct prepro-IGF-I domains in a perciform fish, Sciaenops ocellatus, during ontogeny. Gen Comp Endocrinol 169:75-81

Ferron A, Leggett WC (1994) An appraisal of condition measures for marine fish larvae. Adv Mar Biol 30:217-303

Foster AR, Houlihan DF, Hall SJ (1993) Effects of nutritional regime on correlates of growth rate in juvenile Atlantic cod (*Gadus morhua*): comparison of morphological and biochemical measurements. Can J Fish Aquat Sci 50**:**502-512

Houde ED (1987) Fish early life dynamics and recruitment variability. Am Fish Soc Symp 2:17-29

Houlihan DF, Mathers EM, Foster A (1993) Biochemical correlates of growth rate in fish. In: Rankin JC, Jensen FB (eds) Fish Ecophysiology. Chapman & Hall, London p 45-71

Le Bail PY, Gentil V, Noel O, Gomez JM, Carre F, Le Goff P, Weil C (1998) Structure, function, and regulation of insulin-like growth factors in fish. In: Vandry H, Tonon MC, Roubos EW, de Loof A (eds) Trends in Comparative Endocrinology and Neurobiology: From Molecular to Integrative Biology. The New York Academy of Sciences, New York p 157-161

Luckenbach JA, Murashige R, Daniels HV, Godwin J, Borski RJ (2007) Temperature affects insulin-like growth factor I and growth of juvenile southern flounder, *Paralichthys lethostigma*. Comp Biochem Physiol A 146:95-104

Malloy KD, Targett TE (1994) The use of RNA:DNA ratios to predict growth limitation of juvenile summer flounder (*Paralichthys dentatus*) from Delaware and North Carolina estuaries. Mar Biol 118:367-375

Mathers EM, Houlihan DF, McCarthy ID, Burren LJ (1993) Rates of growth and protein synthesis correlated with nucleic acid content in fry of rainbow trout, *Oncorhynchus mykiss*: effects of age and temperature. J Fish Biol 43:245-263

Nankervis L, Matthews SJ, Appleford P (2000) Effect of dietary non-protein energy source on growth, nutrient retention and circulating insulin-like growth factor I and triiodothyronine levels in juvenile barramundi, Lates calcarifer. Aquaculture 191:323-335

Peck MA, Buckley LJ, Caldarone EM, Bengston DA (2003) Effects of food consumption and temperature on growth rate and biochemical-based indicators of growth in early juvenile Atlantic cod (*Gadus morhau*) and haddock (*Melanogrammus aeglefinus*). Mar Ecol Prog Ser 251:233-243

Perez-Jimenez A, Guedes MJ, Morales AE, Oliva-Teles A (2007) Metabolic responses to short starvation and refeeding in *Dicentrarchus labrax* effect of dietary composition. Aquaculture 265:325-335

Picha ME, Silverstein JT, Borski RJ (2006) Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops x Monrone saxatilis*). Gen Comp Endocrinol 147:196-205

Picha ME, Turano MJ, Beckman BR, Borski RJ (2008a) Endocrine biomarkers of growth and applications to aquaculture: a mini-review of growth hormone, Insulin-like Growth Factor-I and IGF binding proteins as growth indicators in fishes. N Am J Aquacult 70:196-211

Picha ME, Turano MJ, Tipsmark CK, Borski RJ (2008b) Regulation of endocrine and paracrine sources of Igfs and Gh receptor during compensatory growth in hybrid striped bass (*Morone chrysops* X *Morone saxatilis*). J Endorin 199:81-94

Pierce AL, Beckman BR, Shearer KD, Larsen DA, Dickhoff WW (2001) Effects of ration on somatotropic hormones and growth in coho salmon. Comp Biochem Physiol B 128:255-264

Rice JA, Miller TJ, Rose KA, Crowder LB, Marschall EA, Trebitz AS, DeAngelis DL (1993) Growth rate variation and larval survival: Inferences from an individual-based size-dependent predation model. Can J Fish Aquat Sci 50:133-142

Rooker JR, Holt GJ (1996) Application of RNA:DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). Mar Freshw Res 47:283-290

Rose KA (2000) Why are quantitative relationships between environmental quality and fish populations so elusive? Ecol Appl 10:367-385

Scharf FS (2000) Patterns in abundance, growth, and mortality of juvenile red drum across estuaries on the Texas coast with implications for recruitment and stock enhancement. Trans Am Fish Soc 129:1207-1222

Searcy SP, Eggleston DB, Hare JA (2007a) Is growth a reliable indicator of habitat quality and essential fish habitat for a juvenile estuarine fish? Can J Fish Aquat Sci 64:681-691

Searcy SP, Eggleston DB, Hare JA (2007b) Environmental influences on the relationship between juvenile and larval growth for Atlantic croaker, *Micropogonias undulatus*. Mar Ecol Prog Ser 349:81-88

Silverstein JT, Shearer KA, Dickhoff WW, Plisetskaya EM (1998) Effects of growth and fatness on sexual development of Chinook salmon (Oncorhynchus tshawytscha) parr. Can J Fish Aquat Sci 55:2376-2382

Small BC, Peterson BC (2005) Establishment of a time-resolved fluoroimmunoassay for measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on plasma concentration and tissue mRNA expression of IGF-I and growth hormone (GH) in channel catfish (*Ictalurus punctatus*). Domest Anim Endocrin 28:202-215

Stefansson SO, Hauglan M, Björnsson BT, McCormick SD, Holm M, Ebbesson LOE, Holst JC, Nilsen TO (2011) Growth, osmoregulation and endocrine changes in wild Atlantic salmon smolts and post-smolts during marine migration. Aquaculture, in press, doi:10.1016/j.aquaculture.2011.07.002

Stevenson RD, Woods WA Jr (2006) Condition indices for conservation: new uses for evolving tools. Integr Comp Biol 46:1169-1190

Stierhoff KL, Targett TE, Power JH (2009) Hypoxia-induced growth limitation of juvenile fishes in an estuarine nursery: assessment of small-scale temporal dynamics using RNA:DNA. Can J Fish Aquat Sci 66:1033-1047

Uchida K, Kajimura S, Riley LG, Hirano T, Aida K, Grau EG (2003) Effects of fasting on growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*. Comp Biochem Physiol A 134:429-439

Vera Cruz EM, Brown CL, Luckenbach JA, Picha ME, Bolivar RB, Borski RJ (2006) Insulin-like growth factor-I cDNA cloning, gene expression and potential use as a growth rate indicator in Nile tilapia, *Oreochromis niloticus*. Aquaculture 251:585-595

Table 3.1 R^2 values for linear regression analyses from the 2009 Trials between specific growth rate (SGR) and HSI, Fulton's K, $\log_{10}$(RNA:DNA ratio), RNA concentration, $\log_{10}$ (IGF-I liver mRNA expression), and $\log_{10}$(IGF-I plasma levels) for each of the two feeding regimens. Values are presented from analyses based on all individuals in the trial (Overall), just individuals sampled at the end of the initial satiation feeding period and during the first eight days of reduced feeding, and just individuals sampled at the end of the reduced feeding period and during the eight days of refeeding to satiation. For each relationship the R^2 value is presented with the sample size in parentheses and a symbol indicating whether the relationship is positive (+) or negative (-). Significance levels are indicated by * for $p < 0.05$ and *** for $p < 0.0001$; non-significant values are in gray, -- indicates insufficient data.

	No Food regimen		
	Overall	7 d satiation, 4 & 8 d reduced food	16 d reduced food, 4 & 8 d satiation
HSI	+ 0.29 (68)***	+ 0.34 (28)*	+ 0.33 (30)*
Fulton's K	+ 0.09 (68)*	+ 0.34 (28)*	+ 0.09 (30)
$\log_{10}$(RNA:DNA ratio)	+ 0.30 (35)*	+ 0.41 (18)*	+ 0.05 (9)
RNA concentration	+ 0.11 (35)*	+ 0.15 (18)	+ 0.01 (9)
$\log_{10}$(IGF-I liver expression)	+ 0.12 (64)*	+ 0.04 (24)	+ 0.29 (30)*
$\log_{10}$(IGF-I plasma levels)	+ 0.07 (25)	+ 0.06 (13)	+ 0.00 (9)
	2% Food regimen		
	Overall	7 d satiation, 4 & 8 d reduced food	16 d reduced food, 4 & 8 d satiation
HSI	+ 0.34 (56)***	+ 0.52 (23)*	+ 0.10 (26)
Fulton's K	+ 0.04 (58)	+ 0.13 (23)	+ 0.14 (27)
$\log_{10}$(RNA:DNA ratio)	+ 0.04 (49)	+ 0.18 (17)	+ 0.17 (25)*
RNA concentration	- 0.03 (48)	- 0.01 (16)	+ 0.00 (25)
$\log_{10}$(IGF-I liver expression)	+ 0.10 (49)*	+ 0.21 (21)*	+ 0.12 (25)
$\log_{10}$(IGF-I plasma levels)	+ 0.27 (11)	+ 0.80 (6)*	--

Table 3.2 R^2 values for linear regression analyses from the 2010 Trials between specific growth rate (SGR) and HSI, Fulton's K, and RNA:DNA ratio for the No Food and 1% Food regimens. Values are presented from analyses based on all individuals in the trial (Overall), just individuals sampled at the end of the initial satiation feeding period and during the first eight days of reduced feeding, and just individuals sampled at the end of the reduced feeding period and during the eight days of refeeding to satiation. For each relationship the R^2 value is presented with the sample size in parentheses and a symbol indicating whether the relationship is positive (+) or negative (-). Significance levels are indicated by * for $p < 0.05$ and *** for $p < 0.0001$; relationships that were not significant are in gray.

	No Food regimen		
	Overall	7 d satiation, 4 & 8 d reduced food	21 d reduced food, 4 & 8 d satiation
HSI	+ 0.65 (36)***	+ 0.39 (15)*	+ 0.74 (15)***
Fulton's K	+ 0.02 (36)	- 0.00 (15)	+ 0.30 (15)*
$\log_{10}$(RNA:DNA ratio)	+ 0.46 (23)*	+ 0.80 (10)*	+ 0.66 (8)*

	1% Food regimen		
	Overall	7 d satiation, 4 & 8 d reduced food	21 d reduced food, 4 & 8 d satiation
HSI	+ 0.56 (50)***	+ 0.20 (19)	+ 0.61 (25)***
Fulton's K	+ 0.29 (51)***	+ 0.20 (20)*	+ 0.48 (25)*
$\log_{10}$(RNA:DNA ratio)	+ 0.19 (31)*	- 0.03 (13)	+ 0.46 (15)*

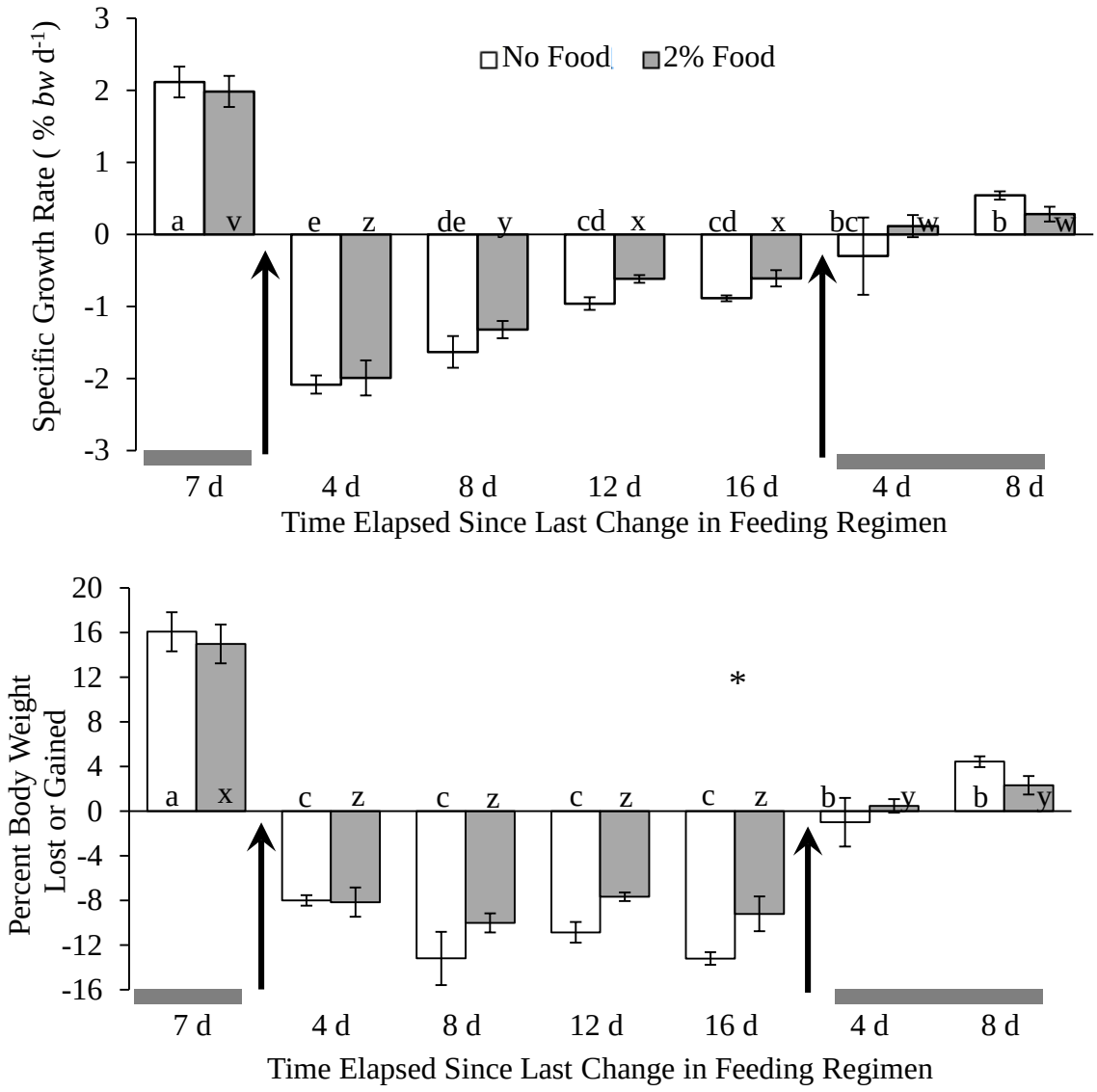


Figure 3.1 Comparison of specific growth rate (top) and percent weight loss or gain (bottom) between the 2009 No Food and 2% Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 2% Food group) and linear comparisons between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Time periods when fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Values on the horizontal axis are days since the last feeding regimen change. No Food regimen samples had n values of 9-10; 2% Food regimen samples had n values of 8, except after initial seven days of satiation n was 7, and eight days after satiation feeding resumed n was 11.

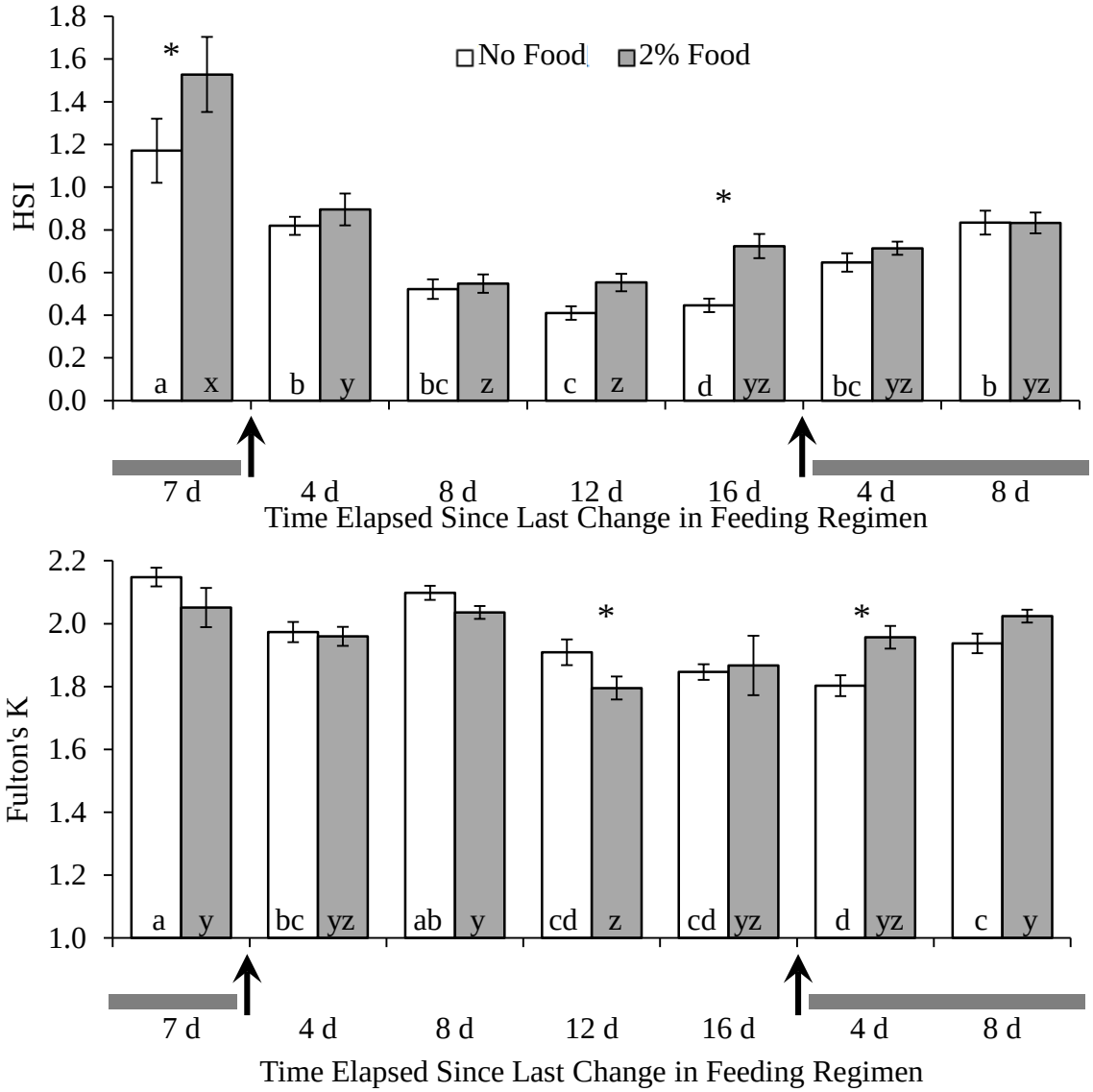


Figure 3.2 Comparison of HSI (top) and Fulton's K (bottom) between the 2009 No Food and 2% Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 2% Food group) and linear comparisons between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Time periods when fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Values on the horizontal axis are days since the last feeding regimen change. No Food regimen samples had n values of 9-10; 2% Food regimen samples had n values of 8, except after initial seven days of satiation n was 7, and eight days after satiation feeding resumed n was 10.

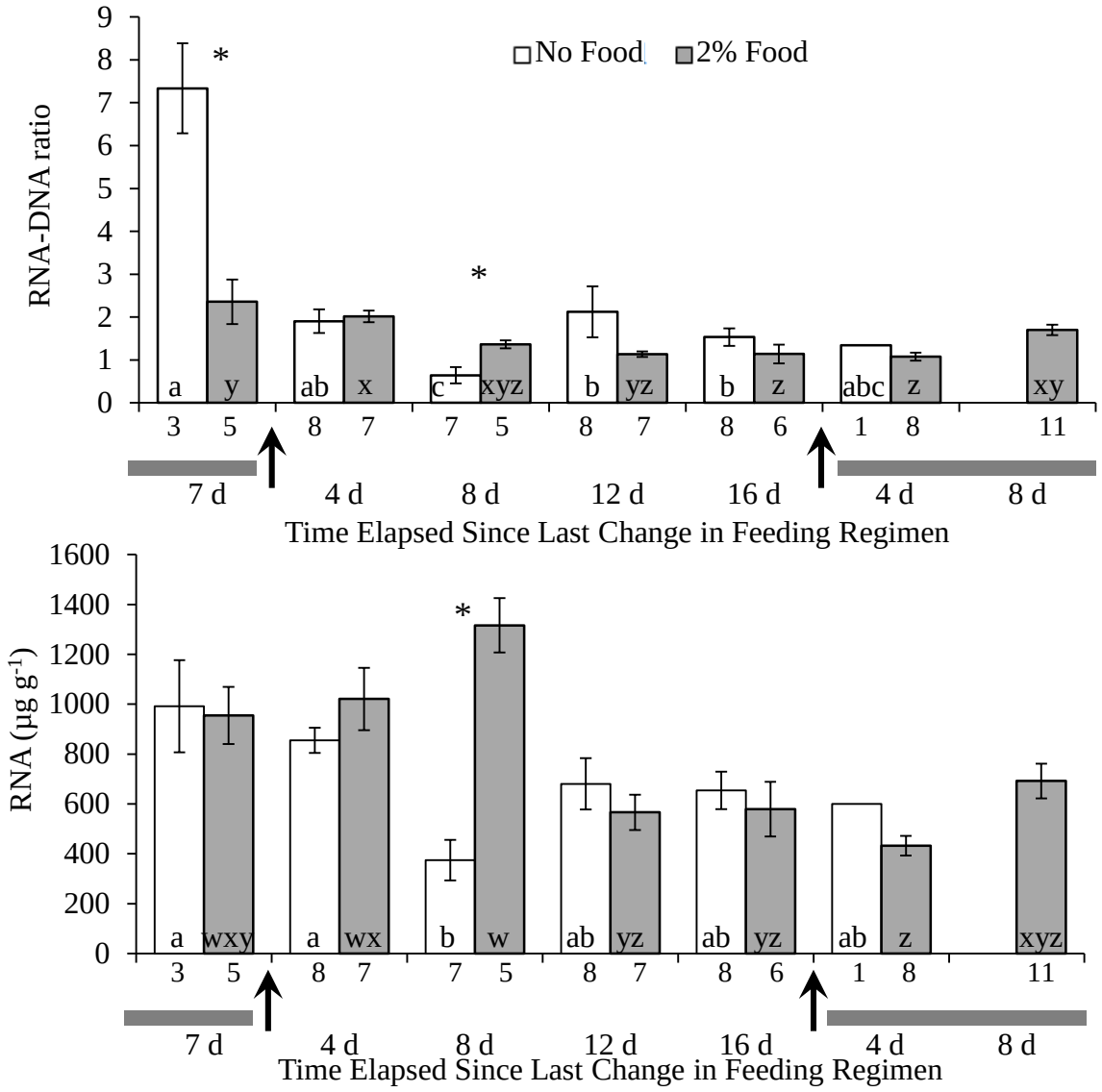


Figure 3.3 Comparison of RNA:DNA ratio (top) and RNA concentration (bottom) between the 2009 No Food and 2% Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 2% Food group) and linear comparisons between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Numbers below each bar indicate sample size. Time periods when fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Values on the horizontal axis are days since the last feeding regimen change.

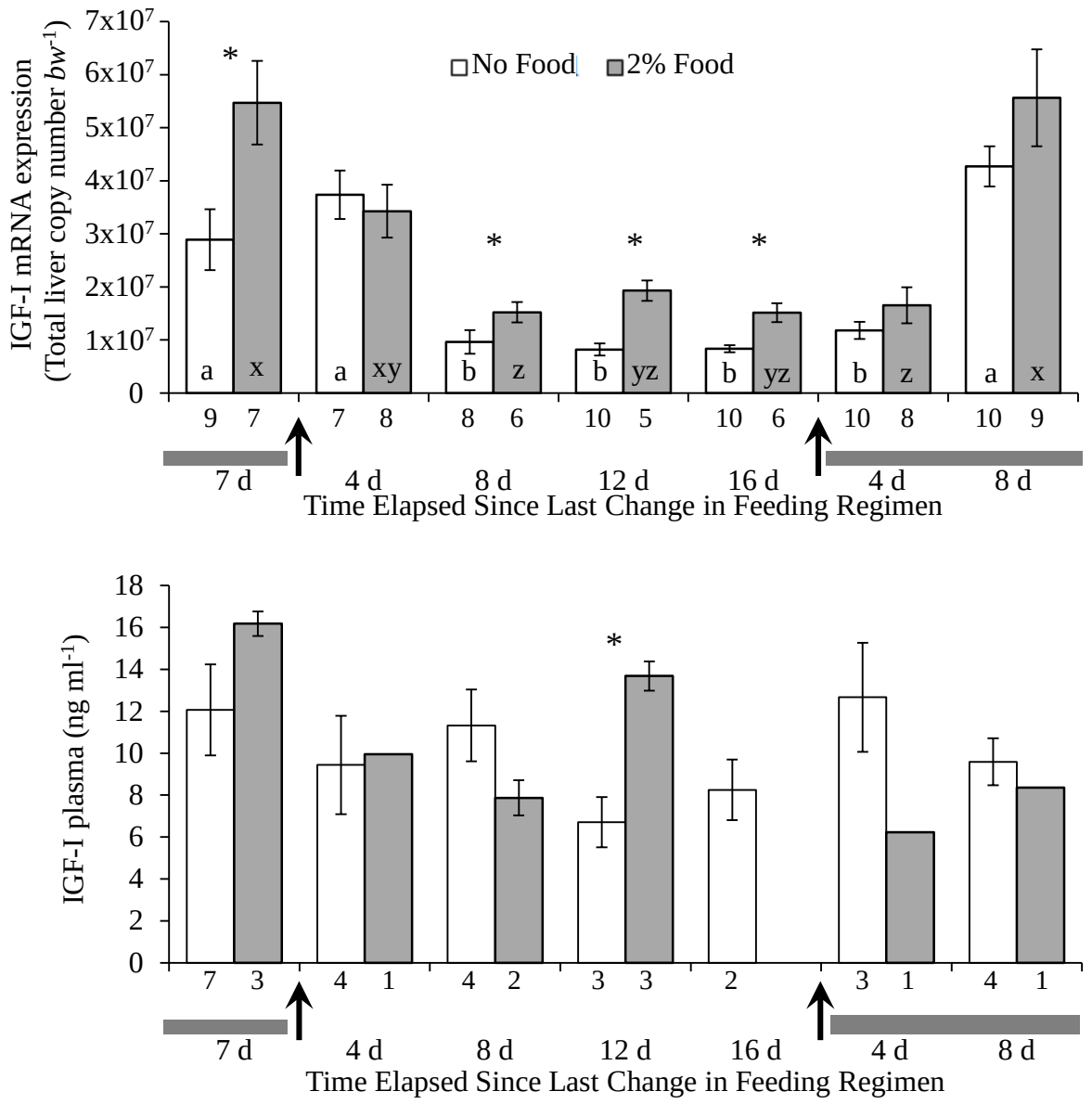


Figure 3.4 Comparison of IGF-I mRNA expression (top) and IGF-I plasma levels (bottom) between the 2009 No Food and 2% Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 2% Food group) and linear comparisons between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Numbers below each bar indicate sample size. Time periods where fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Values on the horizontal axis are days since the last feeding regimen change.

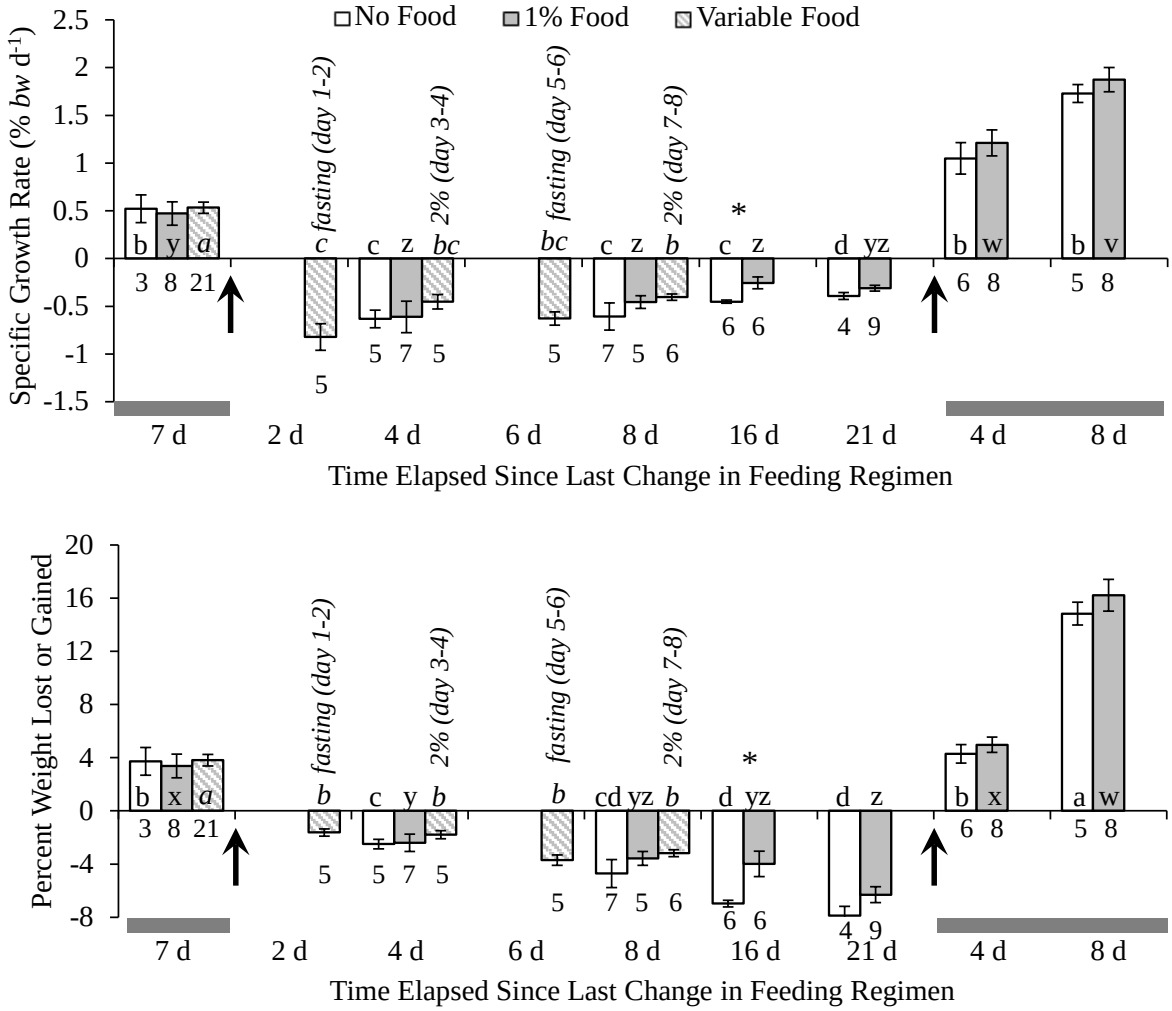


Figure 3.5 Comparison of specific growth rate (top) and percent weight loss or gain (bottom) within the 2010 No Food, 1% Food and Variable Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 1% Food group, italicized *abc* for Variable Food group) and between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Numbers below each bar indicate sample size. Time periods when fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Above the Variable Food regimen bars is the type of feeding that was occurring in the two days prior to that particular sample: fasting = no food given, 2% = 2% *bw* d^{-1}. Values on the horizontal axis are days since the last feeding regimen change.

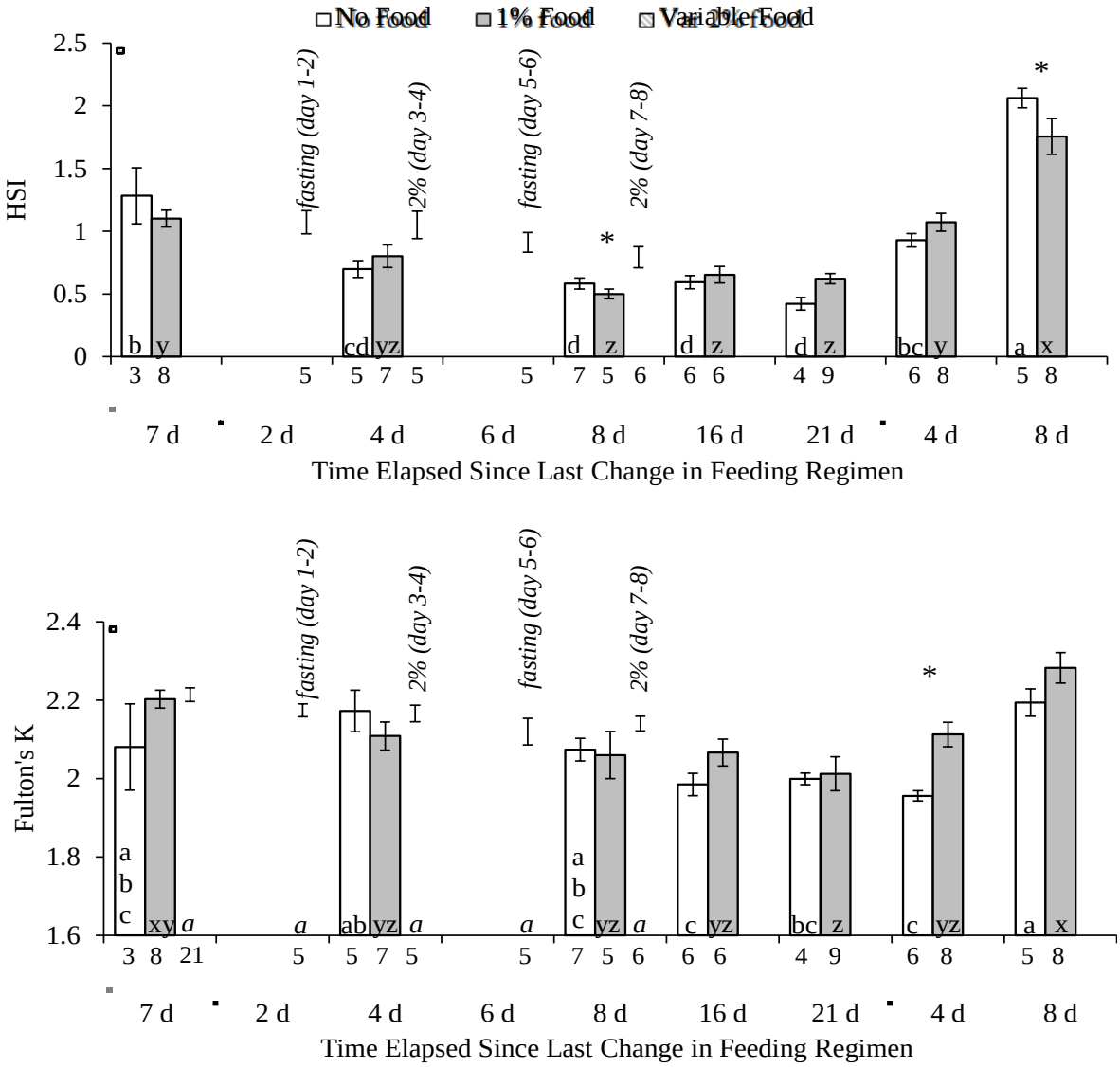


Figure 3.6 Comparison of HSI (top) and Fulton's K (bottom) within the 2010 No Food, 1% Food and Variable Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 1% Food group, italicized *abc* for Variable Food group) and between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Numbers below each bar indicate sample size. Time periods when fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Above the Variable Food regimen bars is the type of feeding that was occurring in the two days prior to that particular sampling point; fasting = no food given, 2% = 2% *bw* d^{-1}. Values on the horizontal axis are days since the last feeding regimen change.

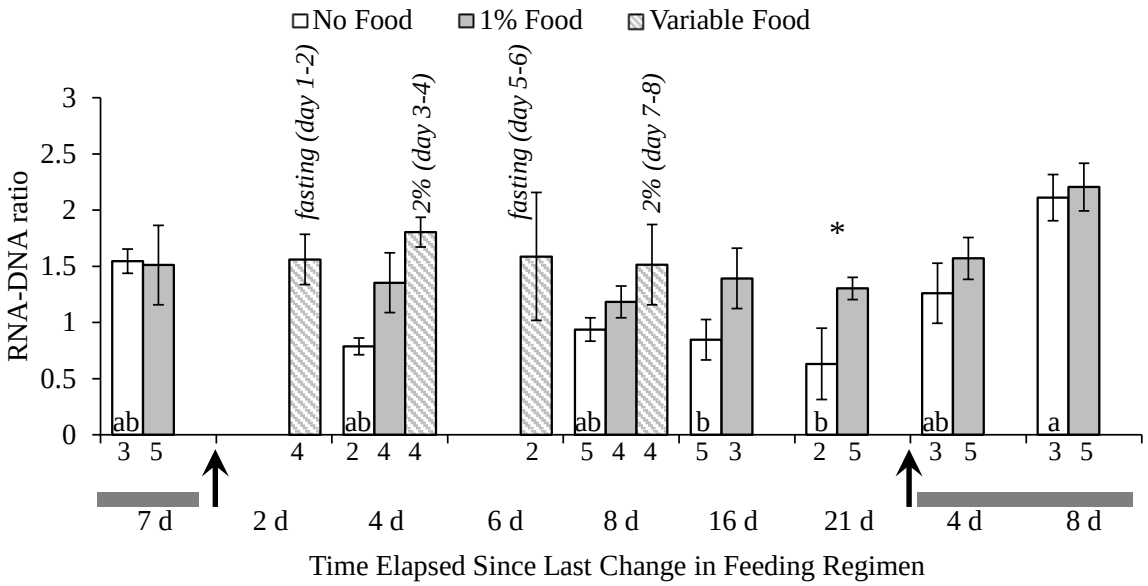


Figure 3.7 Comparison of RNA:DNA ratios within the 2010 No Food, 1% Food and Variable Food trials, by day sampled (Tukey's HSD different levels are indicated by letters: abc for No Food group, xyz for 1% Food group, italicized *abc* for Variable Food group; if there are no letters, no significant differences were found) and between groups on day sampled (significant differences indicated by asterisks). Error bars indicate ± SE. Numbers below each bar indicate sample size. Time periods when fish were fed to satiation are signified by gray bars. Points in time when feeding regimen was changed are shown by arrows. Above the Variable Food regimen bars is the type of feeding that was occurring in the two days prior to that particular sampling point; fasting = no food given, 2% = 2% *bw* d^{-1}. Values on the horizontal axis are days since the last feeding regimen change.

Chapter 4

Development and field application of a predictive model for short-term growth of spot *Leiostomus xanthurus* in response to episodic hypoxia

INTRODUCTION

Processes affecting growth of juvenile fish in nursery habitats can be important in regulating year class strength (e.g., Scharf 2000). Even minor decreases in growth can prolong stage duration (Houde 1987), which can increase size-selective predation; in addition, slower growth can increase overwinter mortality (Sogard 1997). At the juvenile stage, habitats that promote growth and survival are critical for the success of fish (Beck et al. 2001). Juvenile fish use estuaries as nursery habitat during this critical life stage to help foster rapid growth and improve survival through a combination of factors including refuge from predators, increased prey resources, and suitable physiochemical conditions (e.g., temperature, salinity, dissolved oxygen; Beck et al. 2001; Gibson 1994). Because the productivity and quality of a nursery habitat is linked to many factors that vary spatially and temporally, it is important to understand how these dynamics affect the function of estuarine nursery habitat and their consequences for fish growth and year class strength (Beck et al. 2001, Searcy et al. 2007a,b).

In particular, there is growing concern that hypoxia (low dissolved oxygen, DO) due to excessive anthropogenic nutrient loading is having negative impacts on fishes and their habitats in North Carolina (Luettich et al 2000, McClellen et al. 2002), across the United States (Diaz 2001, Diaz and Rosenberg 2008), and globally (Breitburg 2002, Breitburg et al.

2009). Increases in the frequency or severity of hypoxia in estuarine habitats could have both direct and indirect effects on growth and survival of juvenile fish (Eby and Crowder 2002, Stierhoff et al. 2006). Chronic, stable zones of hypoxia in deep waters have become increasingly common (Chesapeake Bay, Breitburg 1990; Neuse River Estuary, NC, Luettich et al. 2000; Gulf of Mexico Dead Zone, Rabalais et al. 2002), but in shallower habitats more often occupied by juvenile fish, DO levels can change rapidly over a 24 hour period creating a more dynamic environment that fish must respond to quickly and continuously (Stierhoff et al 2009a, Campbell 2012 Chapter 2). The peak of juvenile fish abundance and occupancy in estuaries generally occurs during summer months (Weinstein 1979, Able and Fahay 1998), which corresponds with the establishment and spread of hypoxic conditions (Diaz and Rosenberg 2008); as a result estuary-dependent juvenile fish are likely to be affected by hypoxia in many ways (Stierhoff et al. 2009a). With frequency of hypoxia on the rise (Diaz & Rosenberg 2008), quantifying the effects of hypoxia on fish in the field is crucial to understanding the impacts of habitat change.

Researchers have provided much information about the direct effects of hypoxia on fish through laboratory and field enclosure studies. Direct exposure to various low DO levels caused reductions in growth or survival for several species of fish (McNatt and Rice 2004, Shimps et al. 2005, Stierhoff et al. 2006). But laboratory experiments have also documented the ability of fish to detect and avoid low DO (Wannamaker and Rice 2000, Tyler and Targett 2007); in addition, telemetry studies (McClellan et al. 2002, J.K. Craig pers. comm.) and trawling studies (Eby and Crowder 2002, Bell and Eggleston 2005, Campbell 2012

Chapter 2) illustrate that fish generally avoid direct exposure to severe hypoxia. Furthermore, recent studies have shown that measurement of relationships between growth and direct exposure to hypoxia in the laboratory underestimate DO concentrations at which growth is reduced in the field (see Stierhoff et al 2009 a,b). Clearly, findings from the laboratory do not encompass all effects on free ranging fish in the wild, so we need a way to measure the cumulative effects of hypoxia on fish in the field.

A fish's growth integrates effects of all the environmental conditions and stressors it experiences and could be considered a direct reflection of habitat suitability, as reduced growth rate is the obvious effect of sublethal stressors (Rose 2000). Even if fish can minimize or eliminate direct effects of hypoxia on growth, indirect effects may still be important. Movement associated with hypoxia avoidance behavior may increase metabolic costs and reduce foraging efficiency or the time available for feeding. Fish may also experience a reduction in prey availability both from physical separation and negative effects of hypoxia on prey resources. As fish crowd into oxygenated refuges, densities can double (Campbell 2012 Chapter 2) leading to density-dependent effects that reduce growth and survival, as shown in cage and pond experiments (Eby and Crowder 2005, Craig et al. 2007). Even if it were possible to tease out each of these individual effects, the response of a fish to the suitability of its environment could be better assessed by evaluating how well its recent environmental history fostered growth. Levels of DO in nearshore habitats can change from normoxia to hypoxia and back in a matter of minutes to hours, with many episodes of hypoxia within a week, to which fish must continuously respond and adjust (Stierhoff et al.

2009a, Campbell 2012 Chapter 2). Due to the temporal and spatial dynamics of DO concentration in the field we need to be able to capture how a fish is affected by changes on a physiologically and ecologically relevant time scale of days to weeks. Identifying appropriate tools to quantify the magnitude and relative importance of effects of stressors on fish growth remains a pressing need for both scientists and fisheries managers seeking to assess and monitor overall system health (Rose 2000, North Carolina Division of Water Quality 2009).

Few tools are available to measure short-term growth rate in the field. Because individual fish in the wild cannot readily be recaptured, the traditional approach to calculating individual growth from change in size over a given time period is not applicable. The use of otolith daily growth increments to calculate growth rates may be useful (Maillet & Checkley 1991, Fey 2005, Rakocinski et al. 2006, Searcy et al. 2007a,b), but typically does not provide adequate resolution over short time periods immediately prior to capture. Therefore, researchers have been seeking alternative means of assessing recent growth using morphological, biochemical, physiological and endocrine indicators that can be measured from a fish captured just once (Ferron & Leggett 1994).

Biological indicators (bioindicators) have the potential to provide integrated measures of the effects of DO and other environmental variables on growth, serving as a "bioassay" of how recent (days to weeks) habitat conditions translate directly into biological impacts on fish. Biological indicators have been used as a proxy for physiological condition (feeding and growth) in larval and small juvenile fishes (Westerman & Holt 1988, Ferron & Leggett

1994, Rooker et al. 1997, Buckley et al. 1999, Glass et al. 2008, Ciotti et al. 2010). In this study, we estimate short-term growth of juvenile estuary-dependent fish in the Neuse River Estuary, North Carolina to directly link changes in DO with impacts on recent growth due to the sublethal effects of hypoxia. To estimate growth, we evaluated a suite of bioindicators: two primary indicators based on RNA and DNA concentrations and insulin-like growth factor-I (IGF-I), and two secondary indicators, hepatosomatic index (HSI) and Fulton's K. Because growth is achieved through protein synthesis, the relationship between the quantity of DNA in a cell, which is constant, and the quantity of RNA, which fluctuates with protein synthesis rate, can be linked to growth (Buckley et al. 1984, Ferron & Leggett 1994). This technique has previously been applied using muscle tissue from larval and small juvenile fish to relate recent growth to habitat conditions over the previous 1-3 days to two weeks (Rooker & Holt 1996, Malloy & Targett 1994, Stiefhoff et al. 2009a,b). Since RNA and DNA analysis has been successfully applied in a variety of field applications, we used it in this study with the expectation that it would provide interpretable results and also serve as a benchmark to evaluate the relative sensitivity of our other indicators. The second bioindicator, IGF-I, is a hormone responsible for cell differentiation and proliferation, the stimulation of processes related to skeletal elongation, and ultimately for somatic growth (see Duan 1997, LeBail et al. 1998 for reviews). Evidence in controlled tank trials from aquaculture studies suggests that levels of IGF-I may provide an accurate reflection of variable growth rates in a number of fish species (reviewed by Picha et al. 2008a, Beckman 2011). However, its utility in the variable context of the natural environment has yet to be

ascertained. In addition to these two primary indicators, we use two basic metrics of condition that are easy to obtain without laboratory processing. The first, HSI or relative liver weight expressed as a percentage of body weight, is a simple but likely less sensitive measure reflecting the amount of short-term energy reserves. Because these reserves are the first to be mobilized to meet short-term energy demands and are restored as soon as energy intake is sufficient, HSI can serve as a basic indicator of energetic status (Picha et al. 2006, Perez-Jimenez et al. 2007). Finally, Fulton's K is a morphometric condition index that provides a way to examine overall growth, but may not be as useful for recent growth (Suthers 1998).

We experimentally tested responses of these various indicators to changes in recent growth using spot *Leiostomus xanthurus*, a representative estuary-dependent fish which is benthically oriented (where hypoxia is the worst), as our model organism. Previous studies have assessed how spot behaviorally respond to hypoxia (Wannamaker & Rice 2000), how chronic exposure to hypoxia reduces growth (McNatt & Rice 2000), probability of mortality as a function of acute hypoxia exposure (Shimps et al. 2005), and the effects of density dependence on spot growth and survival (Craig et al. 2007). Weinstein (1983) and Weinstein et al. (1984) found that spot of similar size range (48.6 – 96.0 mm SL and 45.3 – 97.3 mm SL respectively) to our collected fish (57 – 131 mm SL, mean 85 mm SL) showed site fidelity to an area with an average residence time of 91 and 81 days (from each study respectively); which suggests that responses of spot in our study could be linked to recent environmental

conditions where they were collected. Spot are found in estuarine nursery areas throughout North Carolina, which allows comparisons among habitats.

Using lab experiments, we evaluated the sensitivity and utility of these bioindicators in quantifying recent growth of juvenile spot. Based on these results, we developed a set of predictive statistical models relating the indicators (alone and in concert) and environmental variables to growth rate. We then use an information theoretic approach (Burnham & Anderson 2002) to examine the relative strengths of the candidate models and guide in selection of the model that best predicts recent spot growth. We apply the best predictive growth model from our laboratory study to bioindicator values measured from spot collected in the field from habitats experiencing a range of temporal patterns in hypoxia. Comparing the resulting estimates of spot growth under differing hypoxic conditions allowed us to evaluate the extent to which increased severity, frequency and duration of hypoxia reduces spot growth either directly due to suppression of food consumption, or indirectly via impacts of hypoxia on prey resources, cost of movement, and density-dependent effects. Rose (2000) suggested that modeling individual growth provides a straightforward way to link sublethal effects of hypoxia to population responses, so we also simulated cumulative seasonal growth under various hypoxia scenarios. Quantifying the relationship between spatially and temporally dynamic hypoxia and fish growth will provide insights that scientists and fishery managers need when moving forward in understanding ecosystem level effects of episodic and chronic hypoxia.

METHODS

Tank Trials

Juvenile spot (mean SL = 80.5 mm, range = 67 - 102 mm) were collected by trawl from the Neuse River Estuary, NC, in May and June 2009, then transported back to the Fisheries Research Laboratory located at the University of North Carolina's Institute of Marine Science in Morehead City, North Carolina, and placed in recirculating holding tanks. Spot were individually marked with visible implant elastomer (Northwest Marine Technologies, www.nmt.us) and eight individuals were randomly placed into each of 18 75.7-l treatment tanks. Fish were acclimated to two treatment temperatures (nine tanks each, 24°C and 28°C) and ~20 ppt salinity for two weeks and fed *ad libitum* twice a day with thawed freshwater mysis shrimp (Piscine Energetics Inc., British Columbia, Canada). Water was recirculated through a bubble-washed bead biofilter (one filter for each temperature treatment) with a 20% water change daily. Uneaten food and feces were removed from the tanks prior to morning feeding, and any buildup of algae on the sides of tanks was periodically removed. Daily measurements of minimum and maximum temperatures were recorded for each tank, and dissolved oxygen and salinity were monitored daily for each tank group; other parameters (e.g., nitrites, pH) were monitored intermittently to insure that water quality remained good.

Evidence indicates that hypoxia reduces growth primarily by reducing feeding rather than by altering the growth process directly (e.g., Stierfhoff et al. 2006). Thus, we divided fish into five feeding levels to produce a variety of growth rates at two different temperatures

for the purpose of evaluating bioindicators over a wide range of growth rates (not to test for differences between the feeding levels). Feeding levels were: no food, minimum food (2-3% body weight d^{-1} total), intermediate food (4-5% body weight d^{-1} total), high food (8-10% body weight d^{-1} total), and *ad libitum*. Fish were fed half their food ration in the morning and half in the late afternoon. Each food treatment level had two randomly assigned tanks per temperature treatment, except intermediate food, which had only one tank per temperature. On day 0 fish changed from *ad libitum* acclimation feeding to their assigned feeding treatment. On day 0, one fish from each tank was sampled (n = 18), on sample days 7 and 14 two fish from each tank were sampled (n = 36 each day), and on day 21, the last sampling day, all remaining fish in each tank were sampled (n = 52). Each individual was weighed (nearest 0.01 g) and measured (nearest 1 mm, both SL and TL) at the start of the experimental period (Day 0) and at the time it was sampled (Day 7, 14, or 21). All weights and measurements were taken in the morning before first daily feeding (at least 12 hrs after last feeding). No mortalities due to ration level occurred during our experiment, though two fish jumped from tanks and died. At the time of sampling a white muscle tissue sample (for RNA and DNA analysis) and the whole liver (for IGF-I expression analysis and HSI) were taken from each fish; both tissues were placed in separate vials of RNAlater (Ambion, Austin, TX), stored at 4°C for 24-48 hours, and then stored at -80°C until analysis. For each fish we calculated specific growth rate (SGR, percent body weight per day), HSI, and Fulton's K values.

SGR was calculated as:

$$SGR = \left(\frac{(\ln bw_2 - \ln bw_1)}{time}\right) \times 100$$

where bw_2 is the body weight in grams of the fish at the end of the growth interval and bw_1 is weight at the beginning of the growth interval and *time* is the interval in days between the two measurements. Since fish were only weighed and measured at the start of the experiment and when they were removed for analysis, growth rates measured for fish sampled on day 7 accurately reflect their growth over the week before they were sampled, but growth rates estimated for fish sampled on day 14 include growth during the first week as well as the second week, and growth rates estimated for fish sampled on day 21 are averaged over all three weeks. Because growth rate might change over time we wanted to estimate growth just during the week prior to sampling for fish sampled on days 14 and 21 as well. To do this, we first determined the average percent weight change for fish in a feeding treatment during week 1. We then multiplied the initial weight (on day 0) of each fish sampled on day 14 by that percentage to get its estimated weight at the end of week 1. This weight served as the estimated initial weight at the start of week 2 for each fish in that treatment sampled on day 14, and was used along with their actual weight on day 14 to estimate their SGR from day 7 to day 14. For example, consider a fish that initially weighed 10g on day 0 and weighed 8.5g on day 14; its SGR over the whole 2-week period would be -1.16% *bw* d^{-1} ($bw_1 = 10$, $bw_2 = 8.5$, $t = 14$); however, within its feeding group fish sampled on

day 7 had lost an average of 9.75% body weight during week 1, so the estimated weight of the fish at the end of week 1 would be 9.02g (10g - (10*0.0975). Thus, its estimated SGR for week 2 would be -0.85% bw d^{-1} (bw_1 = 9.02, bw_2 = 8.5, t = 7). The average weight of fish sampled on day 14 from a given feeding treatment was used as the initial weight of fish sampled on day 21 to estimate their growth rate over the previous week in the same manner. HSI was calculated as:

$$\text{HSI} = \left(\frac{\text{liver wgt (g)}}{\text{body wgt (g)}}\right) \times 100$$

Fulton's K was calculated as:

$$\text{K} = \left(\frac{\text{W}}{\text{L}^3}\right) \times 100$$

where W is final weight in grams and L is final standard length in cm. Multiple linear regression was used to describe the relationship between SGR and each of the individual bioindicators (RNA:DNA ratio, RNA concentration, IGF-I liver yield expression, IGF-I expression fold change, HSI, Fulton's K) along with temperature and day sampled and all potential interactions; non-significant terms were dropped from regressions.

Field Collection

Our field sites included both impacted and reference areas differing substantially in the severity, frequency and duration of hypoxia, in different types of nursery habitat: the

mainstem of the Neuse River estuary (NRE), North Carolina, where hypoxia dynamics are generally large-scale and episodic, a similar but smaller estuary less impacted by hypoxia, and small tidal creeks where diel fluctuations in DO dominate. This sampling design enabled us to assess both intraseasonal dynamics within sites and broader differences between impacted and relatively pristine areas, in both major habitat types. We selected two sites in the NRE; the first site (Nearshore) in shallow (~1 m depth), nearshore waters to represent an area that was typically oxygenated with minimal impacts of hypoxia, and the second site (Intermediate) further from shore and deeper (~2-3 m) to represent greater impacts of hypoxic events (usually daily hypoxic episodes, but not continuously, chronically hypoxic) (Figure 4.1). The alternative estuarine site, Newport estuary, North Carolina, near the mouth of Core Creek, was selected as a nursery area less impacted by hypoxia, characterized by high tidal flushing and water exchange with the open ocean (Figure 4.1). Tidal creek sites in Bogue Sound were chosen to represent a relatively pristine creek (extensive marsh area, little urbanization; Broad Creek) and an impacted creek (dredged and channelized, much urbanization, boat traffic, two boat marinas; Peletier Creek) (Figure 4.1).

During the summers of 2007-2010 YSI 600XLM data sondes were deployed at the sites in the NRE, ~30 cm off the bottom. The data sondes recorded temperature, salinity, dissolved oxygen and depth every 15 minutes, from early summer (May 25, 2007; May 30, 2008; May 26, 2009; June 14, 2010) to late summer (September 24, 2007; September 25, 2008; August 21, 2009; August 12, 2010). Sondes in the NRE were stationary for the whole season. In 2009 and 2010 sondes were deployed in the two tidal creeks in Bogue Sound in

the same manner as sondes in the NRE; sondes were placed for two periods early in the season (Broad Creek June 1 - 17, 2009, June 15 – July 6, 2010; Peletier Creek June 1 - 17, 2009, June 15 – July 6, 2010) and two periods late in the season (both creeks July 11 – 23, 2009 and August 2 – 10, 2010). The Newport estuary site was monitored from July 16 to August 2, 2010 using the same methods. Sondes were serviced weekly to clean off any accumulated debris, maintain probes and download data. Each location was sampled for fish with an otter trawl (6-m head rope and foot rope with tickler chain attached, 20-mm bar mesh wings, 5-mm bar mesh cod end) to collect spot in 2009 and 2010 from NRE sites and tidal creeks and in 2010 from the Newport Estuary site. NRE sites were sampled for fish approximately weekly from June to August. Fish sampling was conducted twice during each data sonde deployment period at the tidal creek and Newport Estuary sites, one and two weeks after data sonde deployment. From each trawl sample ten randomly selected spot (when available) were measured and weighed, and a white muscle tissue sample and whole liver were collected. Tissue and liver samples were treated the same as in the laboratory experiment.

Laboratory Work

RNA and DNA Nucleic Acid Quantification from Muscle Tissue

RNA and DNA concentrations in white muscle samples were quantified using a one-dye, two-enzyme (ethidium bromide (EB) plus RNase and DNase) fluorometric microplate assay following Caldarone et al. (2001). Briefly: Tissue samples were removed from RNAlater solution, rinsed with deionized water, weighed, and digested with a 2% N-

lauroylsarcosine solution. Once tissues were digested samples were centrifuged to separate cell debris, and supernatants of samples were removed and diluted to a working concentration of 0.1% N-lauroylsarcosine for quantification of nucleic acids. Triplicate aliquots (0.075 ml) of each sample were loaded into 96-well microplates, along with serial-diluted RNA and DNA standards (18S + 28S ribosomal RNA from calf liver, Sigma R-0889; DNA from calf thymus, Sigma D-4764), and stained with 0.075 ml of 2.0 µg ml^{-1} EB. Total fluorescence at 520 nm excitation and 612 nm emission wavelengths was measured using a fluorometer (Fusion Universal Microplate Analyzer, Packard BioScience Company). After initial fluorometric reading, RNA and DNA fluorescence were measured as the decrease in fluorescence after the sequential addition of RNase and DNase, respectively. The slope ratio of DNA to RNA standard curves was 2.41 ± 0.57. Tissue nucleic acids were expressed as the ratio of RNA:DNA (µg RNA µg DNA^{-1}), total RNA concentration per gram wet tissue weight (µg g^{-1}), and total DNA concentration per gram wet tissue weight (µg g^{-1}). Weights of tissue samples from the calibration tank trials inadvertently were not retained, so for these samples we used the average weight of tissue samples collected in the same manner for a similar experiment run in the same time period (mean ± SE, 0.029 ± 0.001 g; Campbell 2012 Chapter 3; estimates of SGR were not related to sample tissue weights ANOVA, n = 55, p = 0.0953); potential effects of this substitution on model predictions are noted in the Results. Recorded individual tissue weights were used for fish from the field.

IGF-I Gene Expression in Liver Tissue

The IGF-I bioindicator was based on measurement of IGF-I gene expression in the liver, the primary source of IGF-I production in fish. Liver tissue total RNA was isolated by TRI reagent extraction (Molecular Research Center, Cincinnati, OH), with an added glycogen removal step using Plant RNA Isolation Aid (Ambion, Austin, TX) (Picha et al. 2008b). For more in-depth description of steps see Campbell (2012, Chapter 3). Briefly: Total RNA was then suspended and checked in triplicate for concentration and purity by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE). Absorbance values ($Abs_{260/280}$) in samples were 1.88 - 1.99. Gel electrophoresis (1% agarose, 0.4 µg ml^{-1} ethidium bromide) confirmed quality of RNA. Samples were then treated with DNase (Turbo DNA-free, Ambion, Austin, TX) and final quantification and integrity was measured by spectrophotometry using a Nanodrop 1000. One microgram of DNase-treated total RNA was reverse transcribed using a High Capacity cDNA Synthesis kit (Applied Biosystems, Foster City, CA).

Liver IGF-I mRNA was measured in triplicate by SYBR Green Real-Time PCR assay (Qiagen, Valencia, CA) using a gene-specific primer pair designed using ABI Primer Express (v 3.0) software (Forward: TGC TGC TTC CAA AGC TGT GA ; Reverse: TCT TGG CAG GTG CAC AGT ACA). For more details see Campbell (2012 Chapter 3). Briefly: Real-time PCR analysis was performed using a 20-ng total RNA sample with Brilliant II SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA) on an ABI 7300 96-well detection system. Real-time measurements were made during the extension step. Cycle

threshold (C_t) values for samples were analyzed by absolute quantification, using standard curves of 10-fold diluted copy number cDNA (dilution ranged from 10^{10} to 10 copies per µl; $R^2 = 0.99$). To normalize for changing liver sizes, liver sample IGF-I gene expression mRNA copy number data were normalized to total RNA concentration, liver size, and body weight (Bustin 2000, Picha et al. 2008b), where expression of IGF-I mRNA in copy number per ng total RNA was used to calculate whole liver IGF-I copy number and then divided by the weight of the fish, resulting in a measure of IGF-I liver expression in units of total liver IGF-I copy number g^{-1} fish body weight). In addition, because normalization to an endogenous reference gene is common in RT-PCR analysis, we also present values of the samples that were normalized to mRNA expression of the housekeeping gene elongation factor 1-α (efl) whose expression was not influenced by treatment. Standard curves for ef1 were calculated like standard curves of IGF-I, and data were normalized using the relative standard curve method as described in the ABI Prism 7700 Sequence Detection System User Bulletin #2, P/N 4303859 (Applied Biosystems, Foster City, CA). Normalized values are expressed as relative mRNA fold change (IGF-I expression fold change) relative to the mean of values from fish sampled on day 7 from the *ad libitum* feeding group.

Growth Modeling

We used results from the laboratory calibration experiment to develop a statistical model to estimate recent growth of juvenile spot that could be applied to fish in the field. Using the observed growth and bioindicator levels from fish in the laboratory, we developed candidate models to estimate growth. The models were created on the basis of literature

review and our own experience of growth dynamics from variables known or suspected to influence growth of juvenile fish. Model variables included: the ratio of RNA:DNA, RNA concentration, DNA concentration, IGF-I liver expression, IGF-I expression fold change, HSI, Fulton's K, initial weight, temperature (as a continuous variable), day in experiment, temperature*RNA concentration, and temperature*RNA:DNA. We chose to include initial weight of the fish to take into account any effect of where a fish was on the size spectrum, but did not use final weight of fish as it would be correlated with calculated SGR. Day in experiment was included to capture response time of variables. Previous studies have found an interaction between temperature and RNA concentration independent of growth rate, so we also included the interaction variables temperature*total RNA and temperature*RNA:DNA. Additionally, since DNA tends not to vary in relation to growth it was not used in any models that did not include RNA (but RNA concentration could be used by itself) nor was the combination of RNA concentration, DNA concentration, and RNA:DNA used in a model as this would be redundant. Given that IGF-I liver expression and IGF-I expression fold change are different ways to quantify gene expression we did not use both in the same model. To meet the assumptions of normality and equality of variance RNA:DNA and IGF-I liver expression data were $\log_{10}$ transformed.

The most appropriate model structure and coefficients were identified using an information theoretic approach (Burnham & Anderson 2002) to compare the relative strengths of the candidate models in explaining growth of spot in the laboratory experiment. Models were fitted with standard least-squares multiple regression in JMP version 9 (SAS

Institute, Inc., Cary, NC). Akaike's information criterion corrected for small sample size (AIC_c) was used to rank candidate models and select the best predictive model (Burnham and Anderson 1998). AIC_c was calculated from residual type I sum of squares of model fits (SS_{resid}) using the least-squares case:

$$AICc = n \cdot \ln \frac{SSresid}{n} + 2K + \frac{2K(K+1)}{n-K-1}$$

where *n* is the number of observations and K is the number of predictors (Burnham and Anderson 1998). Absolute values of AIC_c are arbitrary, but relatively smaller values indicate better models. Model probability weight (w_i) indicates the strength of the evidence for candidate model *i* as the best model of those tested and was calculated for each model (Burnham and Anderson 1998)..

Field Application

After the best predictive growth model was selected, variables from field fish were input to estimate their recent growth rates. For weight we used wet weight at time of capture, and for temperature we used average water temperature for the week prior to capture at the site where the fish was collected. Examination of response times of bioindicator variables (see Campbell 2012 Chapter 3) indicated that most of their response to a change in conditions occurs within a week. Therefore, we set the value of 'day' to seven for field fish and related their estimated growth to DO conditions from the previous week at the site where the fish was captured. Based on these conditions, the week prior to a fish's capture was assigned a DO classification of Good, Moderate or Poor. A classification of Good indicated DO levels above 4.0 mg l^{-1} at least 75% of the time, with only one or two short (< 3 hours)

hypoxic events, if any; Moderate classification denoted DO readings above 4.0 mg l^{-1} less than 75% of the time, but at least 40% between 2 - 4 mg l^{-1}, and few, if any, short to medium duration hypoxic events (< 6 hours); weeks classified as Poor had very little, if any, time above 4.0 mg l^{-1} DO (typically < 15%), more than 25% of the time below 2 mg l^{-1}, many short to medium hypoxic events or prolonged hypoxic events (> 6 hours), or both. Differences in estimated growth were compared among DO classifications, locations sampled, and years, using ANOVA with $\alpha = 0.05$. All statistical tests were performed in JMP version 9 (SAS Institute, Inc., Cary, NC).

To compare the relative effect of differences in DO conditions on growth of juvenile spot in the NRE among years (2007-2010), we first evaluated water quality records from June 15 to August 12 of each year from both the Nearshore and Intermediate site and assigned a DO classification to each day (based on the percent time at various DO levels used in the weekly classification guidelines). Then we simulated growth over this period of a hypothetical spot starting at 6.9 g and 63 mm SL, the average size of spot collected in June 2007 (lengths estimated from the length-weight relationship $W = 0.00006L^{2.82}$, n = 1,388, $R^2 = 0.97$, see Appendix A). Depending on the DO conditions for each day, the fish was grown for that day at the average growth rate estimated for that DO classification (Good, Medium, Poor) in the previously described analysis of NRE field fish. We compared the end weight, length, and overall SGR of the fish simulated under DO conditions from each year and site, and also calculated the percent difference in simulated final weight from each year and site relative to final weight of a fish growing under conditions classified as Good for all 59 days.

RESULTS

Laboratory experiment

Our laboratory calibration experiment successfully produced a range of specific growth rates in spot, from -2.21 to 2.80% body weight per day (% *bw* d^{-1}). No significant differences or marked trends in growth rate by week were found in the *ad libitum* (ANOVA, df = 26, p = 0.28) and high ration groups (ANOVA, df = 25, p = 0.17). In no and low ration groups the highest percentage weight loss occurred during the first week. Fish that were in the starvation group had significantly more negative growth during week 1 (mean ± SE; SGR = -1.30 ± 0.21% *bw* d^{-1}) than estimated for subsequent weeks (week 2 predicted SGR = -0.56 ± 0.12% *bw* d^{-1}; week 3 predicted SGR = -0.32 ± 0.20% *bw* d^{-1}; ANOVA, df = 26, p = 0.0059). A similar, but non-significant, trend also occurred in the low ration group (week 1 SGR = -0.58 ± 0.09% *bw* d^{-1}, week 2 predicted SGR = -0.20 ± 0.27% *bw* d^{-1}; week 3 predicted SGR = -0.01± 0.21% *bw* d^{-1}) (ANOVA, df = 25, p = 0.16).

To evaluate potential indicators of recent growth rate we first ran separate multiple regressions for each bioindicator we evaluated ($\log_{10}$(RNA:DNA), RNA concentration, $\log_{10}$(IGF-I liver expression), IGF-I expression fold change, HSI, and Fulton's K) with SGR as the dependent variable and the bioindicator, temperature, and day in experiment as dependent variables. All of the models relating individual bioindicators to SGR were significant, but the degree of variation they explained varied widely (R^2 0.05-0.75). The least amount of variation explained was by the positive relationship of $\log_{10}$(RNA:DNA) to SGR (R^2 = 0.05) which had no significant interactions with temperature or day in experiment or

other main effects (Table 4.1, Figure 4.2). IGF-I expression fold change had a significant negative relationship with SGR (with no other significant main effects or interactions) that seems contradictory, but is not, since fold change values are relative to the average values of fish fed *ad libitum*, so higher fold change corresponds with lower mRNA expression values at lower SGR. The relationship between SGR and $\log_{10}$(IGF-I liver expression) had low explanatory value ($R^2 = 0.19$), and included an interaction with day in experiment; surprisingly the relationship at 14 days was negative whereas others were positive (ANCOVA, df = 68, p = 0.0185). The relationship of Fulton's K to SGR had low explanatory power ($R^2 = 0.23$) and included an interaction with temperature (ANCOVA, df = 120, p = 0.04) (Figure 4.2, Table 4.1). Significant interaction was found between HSI and temperature (ANCOVA, df = 119, p = 0.015) with day in experiment as a significant main effect covariate resulting in a positive, fairly high explanatory value (R^2=0.59) relationship of HSI with SGR. Temperature was a significant main effect in the relationship between RNA concentration and SGR, and the interaction of RNA*day in experiment was also significant (ANCOVA, df = 106, p = 0.003) (Figure 4.2, Table 4.1). This bioindicator is the only one that could have been affected by the absence of individual tissue sample weights from the lab experiment. Had individual weights been available, RNA concentration may have explained even more variation in SGR, but even with this loss of resolution it was the strongest predictor of SGR ($R^2 = 0.75$) of all the bioindicators we evaluated.

Model Selection

Candidate models to explain specific growth rate using combinations of bioindicators and environmental factors explained up to 80% of the variability in specific growth rate from the lab calibration trials (Table 4.2). Models with combinations of bioindicators performed better than models with single bioindicators and environmental variables, as none of the latter models had any strength of evidence (w_i; Table 4.2). Eleven of our 37 candidate models had some weight (w_i), with all of those models containing HSI, RNA concentration, temperature, and day in experiment. However, the model containing just those four variables had a low model weight ($w_i = 0.06$). The top three models carried the most weight (total 0.51 w_i), with each having similar strength of evidence (w_i of 0.19, 0.17, and 0.15; Table 4.2). Because the top three models all contained similar variables (the top model contained all variables from the second model, the third model contained all variables from the first and second models) and all had nearly identical R^2 values, we decided to use the top ranked model by AIC_c instead of using a model averaging approach (Burnham and Anderson 2002). Models with individual bioindicators did not carry any weight in our model set and were well above a ΔAIC value of 10, denoting essentially no support for the model (Burnham and Anderson 2002). The equation for the top model to estimate SGR based on results of our laboratory calibration experiment is as follows:

$$\text{SGR (\% bw d}^{-1}) = -5.2364 + (1.2119 * \text{HSI}) + (0.0033 * \text{RNA } \mu\text{g g}^{-1}) + (-0.0003 * \text{DNA } \mu\text{g g}^{-1}) + (0.0750 * \text{Temperature } ^{\circ}\text{C}) + (0.5421 * \text{Fulton's K}) + [\text{day 7} = -0.4881, \text{day 14} = 0.1478, \text{day 21} = 0.3403]$$

This model provides a strong, unbiased description of the growth rates observed in our calibration experiment, with the regression of observed growth rates on estimated growth rates explaining 80% of the variability, with a slope of 1 and an intercept virtually indistinguishable from zero (Figure 4.3).

Field Application

Once we had determined the best model for estimating recent growth rate based on the bioindicators we measured in the lab, we applied that model to estimate recent growth of fish collected in the field under various DO conditions. Because the response of growth rate to a change in conditions was most marked in the first week for fish experiencing a shift to negative growth, and did not differ among weeks for fish experiencing positive growth (see above and Campbell 2012 Chapter 3), we set "day" to 7 for model applications to field fish. We then paired the estimated SGR values of individual fish with DO and temperature measurements from the site of capture for the previous week. Good, Moderate, and Poor DO conditions were all found throughout the season in the NRE at both the Nearshore and Intermediate sites. Over 2009-2010 we collected fish samples on a total of 35 occasions.

However, due to budgetary constraints we analyzed fish from a subset of 25 of those occasions, selected based on the previous week's DO conditions to represent all DO categories and sites, and a range of dates throughout the season. Bioindicator values from field fish were within the range of data used to build the model (HSI 100%, Fulton's K 100%, RNA µg g^{-1} 87%, DNA µg g^{-1} 95%).

In the NRE we found that better DO classification was associated with higher estimated growth (ANOVA, df = 2, p = 0.002), and that year sampled also had a significant effect on estimated growth (ANOVA, df = 1, p = 0.001), but there was no interaction between DO classification and year (ANOVA, df = 2, p = 0.064) (overall ANOVA, df = 75, p = 0.0001) (Figure 4.4). Average estimated SGR (both years combined) for fish collected following a week with DO conditions classified as Good (1.39% *bw* d^{-1}) was 14% greater than estimated SGR for fish collected after weeks with DO classified as Moderate (1.19% *bw* d^{-1}), and 42% greater than that of fish collected following a week of Poor DO (0.80% *bw* d^{-1}) (Figure 4.4). Estimates of growth rate for all fish collected in the NRE in 2009 (all DO classifications combined) were significantly higher (1.44% *bw* d^{-1}) than for all fish collected in 2010 (0.95% *bw* d^{-1}) (t-test, df = 75, p = 0.0049), but this difference did not affect the trend of declining estimated growth with declining DO conditions. In both years SGR estimated for fish collected following Good conditions was significantly greater than estimated SGR for fish collected following a week of DO conditions classified as Poor (t-tests, 2009: df = 32, p = 0.0133; 2010: df = 32, p = 0.0043).

The Newport estuary exhibited Good DO ratings for all periods monitored in 2010, with an average estimated growth rate of 1.52% *bw* d^{-1} for all fish collected there (Figure 4.4). This growth rate was slightly higher, but not significantly different from, the estimated SGR of only those fish collected in in the NRE following weeks with a Good DO classification in 2010 (t-test, df = 34, p = 0.268).

Fish from both tidal creeks had variable growth rates by season and year despite Good to Moderate DO ratings, but overall better estimated growth rates than fish from the NRE (Figure 4.5). Peletier creek (2.44% *bw* d^{-1}) and Broad creek (1.94% *bw* d^{-1}) had higher estimated SGRs than the estimated growth rates of fish from the NRE following Good DO weeks (1.46% *bw* d^{-1}) (ANOVA, df = 72, p = 0.0016). There was no difference in growth rates between the two tidal creeks in either year (t-tests, 2009: df = 16, p = 0.0747; 2010: df = 12, p = 0.6882).

Simulated growth rates based on daily DO quality ratings for the NRE across years ranged from a low 6% decrease to a large 18% decrease compared to the simulated growth rate at constant Good DO conditions in the NRE (Table 4.3). As expected, growth was less negatively affected at the Nearshore site than at the Intermediate site, due to generally fewer days of Poor conditions and more days of Good conditions at the Nearshore site. Simulated growth rates for fish at the Nearshore site were similar among years, but still 6-9% lower than growth estimated using a steady state of Good DO conditions (Table 4.3). The simulated growth of a fish at the Newport estuary site (based on estimated growth rate from fish sampled in 2010) was higher than in any of the NRE simulations, and resulted in an end

weight 8% greater than that of a fish grown continuously at the average growth rate corresponding to Good DO conditions in the NRE.

DISCUSSION

The best model we identified used a combination of bioindicators and was able to account for 80% of the variability in recent growth rate of fish in our laboratory trials, giving us a powerful tool we used to estimate recent growth of juvenile spot in the field. The best model using just one bioindicator, RNA concentration (along with temperature and day), explained only 5% less variability in growth rate; thus one might suspect that the advantages of using this simpler model would outweigh this slight loss in predictive power. However, AIC_c analysis indicated that the simpler model had no evidence to support it as being comparable to the top model despite its lower number of parameters, and the additional data requirements for the more complex model are modest; once RNA concentration has been quantified, doing so for DNA entails only one more analytical step, and HSI and Fulton's K require only simple weight and length measurements.

We expected RNA:DNA ratio to be a strong predictor of recent growth since it has been so in numerous other studies (e.g., Buckley et al. 1999, Malloy & Targett 1994, Caldarone et al. 2003, Steirhoff et al. 2009a,b). Its lack of effectiveness in this study may be because we were working with older, relatively large juveniles rather than larvae or smaller juveniles, as studies with other species have also found that RNA:DNA ratio was not a good predictor of recent growth for larger juvenile fishes (Houlihan et al 1993; Buckley et al 1999). In contrast, RNA concentration (RNA:tissue wet weight) was a good predictor of

recent growth rate in our study, and has had better explanatory value than RNA:DNA ratio in other studies as well (Lied & Rosenlund 1984, Mathers et al. 1992, Mathers et al. 1993, Foster et al 1993). The strong relationship between RNA concentration and recent growth both alone and in our best model was somewhat surprising given that we did not have individual tissue sample weights for fish from the calibration experiment; had we had those values RNA concentration might have been an even stronger predictor, but the relationship was remarkably robust despite this loss of resolution. Given the inclusion of RNA concentration in the best model, it was not unexpected that temperature was also a significant component, as many RNA-based indices of growth rate are temperature-dependent (Buckley et al. 1999). Although standardizing RNA to DNA concentration did not prove useful, DNA concentration independent of RNA concentration was a significant variable in our best model. Ciotti et al. (2010) also found that DNA tissue concentration helped explain variation in growth separate from RNA concentration. They suggested that this could be due to weakening of the RNA-growth rate relationship at low growth rates due to biochemical composition reorganization in response to starvation and because RNA-dependent protein synthesis is low. Further, they explain that as fish lose mass, cell number remains constant; because total DNA is proportional to cell number, the increase in DNA concentration from tissues of starved fish (Bulow 1970, Mathers et al. 1993, Malloy & Targett 1994, Fukuda et al. 2001) may be a more informative indicator of growth rate in fish with very low or negative growth rates when the predictive power of RNA concentration is low.

Though HSI is a simple metric, it had surprisingly high explanatory value both alone and as a component of the best model. These results suggest that HSI warrants further consideration as a potentially useful bioindicator of recent growth in other studies. We found it interesting that Fulton's K also was a significant component of the best model, as it is usually associated with long-term rather than short-term growth (Stevenson & Woods 2006, Caldarone et al. 2012). Because this metric gives a general indication of plumpness, or weight relative to length, this might be the reason it added value to our model estimating growth in weight. Many studies measure growth rate via change in length of the fish, however in our study over such short time periods it was more accurate to measure growth rate via change in weight rather than length.

We expected IGF-I to be a better and more sensitive predictor of recent growth rate in our model, based on information in the literature from aquaculture studies and the role of IGF-I in the direct control of somatic growth (LeBail et al. 1998, Beckman et al. 2004, Picha et al. 2008a, Beckman 2011). However, in this study IGF-I mRNA expression accounted for little of the variability in observed specific growth rate of juvenile spot. Based on our previous experiments (Campbell 2012, Chapter 3) and results of the calibration experiment presented here, it appears that IGF-I expression in the liver of juvenile spot may have more of an "On or Off" response reflecting whether fish were feeding or not, as the *ad libitum* group exhibited much higher values than the starved group for $\log_{10}$(IGF liver expression) (t-test, $n = 28$, $p < 0.001$). Thus while IGF-I may be a good indicator of whether or not spot are feeding, it did not provide a graduated response corresponding to different growth rates over

a broad range of feeding rates. It is unclear if IGF-I's lack of discrimination between similar growth rates we observed for spot applies in general to other species, as most other studies of the relationship between IGF-I and growth rate have only compared responses between fed and starved or low ration fish (Beckman 2011). Fukada et al. (2012) found that gene expression of IGF-I in white muscle tissue showed better response to somatic growth and nutrition status in yellowtail *Seriola quinqueradiata* than did IGF-I gene expression from the liver. If this pattern proves to be general for other species, IGF-I gene expression, measured in white muscle rather than liver tissue, may still hold potential as a bioindicator of recent growth in spot.

We originally intended to measure levels of IGF-I circulating in plasma, but many of our blood samples were lost in a freezer malfunction. However, in a related study where we did measure IGF-I plasma levels in a smaller number of spot, this direct measure of IGF-I was no more effective in estimating recent growth rates than was IGF liver expression (Campbell 2012 Chapter 3). While we expected a positive correlation between IGF-I liver expression and IGF-I plasma levels (Beckman 2001), Uchida et al. (2003) did not find a relationship between the two, and information about the relationship between them for spot is currently limited to only one study (Campbell 2012 Chapter 3). Thus it is possible that plasma IGF-I levels may still have potential as a growth indicator for spot even though the relationship was weak for liver expression.

None of the indicators we tested, particularly the RNA-based indicators, responded as quickly to a change in growth conditions as we expected. Others have observed faster

responses in other species (Clemmesen & Doan 1996, Rooker & Holt 1996, Steirhoff 2009a), but based on our laboratory calibration 7 d was about the shortest timeframe we could reliably use to relate recent growth rate to environmental conditions (see also Campbell 2012 Chapter 3). Fish operating in a negative energy budget experienced most of the change in growth and indicators during the first week; furthermore, we would not expect a fish in the wild to experience continuous starvation for three weeks, so any signal would be potentially weakened if evaluated over a longer period.

When we applied our model to estimate recent growth of juvenile spot collected in the NRE, we were able to detect marked differences associated with DO conditions over the previous week; growth rates associated with Good DO conditions were almost twice those following a week of Poor DO conditions. Because our model was based on data from fish in the laboratory that were not subject to many of the demands experienced by fish in the wild (e.g., avoiding predators and hypoxia, having to search for food), actual growth rates estimated for fish in the field should obviously be interpreted with caution. However, we believe relative differences can be interpreted with confidence. Hypoxic conditions were not site specific and occurred throughout the season in the NRE, providing additional evidence that the growth differences we detected were driven by recent DO conditions. The effects of recent DO conditions on spot growth that we estimated are likely conservative. Given their strong hypoxia avoidance behavior (Wannamaker and Rice 2000, Campbell 2012, Chapter 2) it is highly unlikely that juvenile spot remained in the immediate vicinity of their collection site for the entire previous week if DO conditions were Moderate or Poor. Furthermore,

other environmental variables effect growth and may have masked the effects of DO to some extent. That growth differences were still evident despite these ameliorating effects provides strong evidence that hypoxia negatively affects juvenile spot growth in the field, and that avoidance behavior cannot fully alleviate both the direct and indirect effects of hypoxia on growth.

The negative effects of hypoxia on growth rate that we detected may have to do with the highly dynamic nature of hypoxia in the NRE, in contrast to studies that do not find a negative effect on growth where hypoxic zones are fairly stable (e.g., Roberts et al. 2012). Under stable conditions fish are able to move away from hypoxic zones into well-oxygenated areas and remain there. However, where hypoxia is more dynamic fish are likely to intermittently encounter hypoxia at least briefly, and must adjust to constant changes in their environment.

Simulations of seasonal growth based on records of DO from the NRE in 2007-2010 showed that hypoxia dynamics in recent years have reduced growth on the order of 6% to 18% relative to growth expected under constant Good DO conditions. However, these results may be conservative given that other variables may affect growth regardless of DO. The Intermediate depth site was consistently predicted to support lower growth based on DO qualities, but fish keep returning to this area (see Campbell 2012, Chapter 2), so this area must offer some other benefit that outweighs this cost. Nearshore areas were better for growth based on DO levels, but still were impacted by hypoxia and had lower growth rates than that estimated under constant Good DO conditions. In a previous study we evaluated

the potential magnitude of density-dependent effects on growth of juvenile spot at our Nearshore site due to habitat compression in this "refuge" area during periods of expanded hypoxia. That analysis estimated that density-dependent effects resulting from hypoxia avoidance behavior decreased cumulative seasonal growth in 2007 ~4% (Campbell 2012, Chapter 2). However our simulation presented here for the Nearshore site estimated that the overall effects of hypoxia reduced growth 9% in 2007, demonstrating that density-dependent effects are only one component of the total effect hypoxia dynamics has on fish. This comparison emphasizes the point that while studies estimating single, specific effects on growth due to hypoxia or other environmental factors may be informative, their estimates may be less than the total cumulative effects on fish growth.

In different years different factors may take on the role of the main driver in growth variability, thus the differences in overall growth rates among years, such as higher growth in 2009 than 2010, could be due to other abiotic or biotic factors besides differences in DO conditions. Since growth integrates everything the fish experiences, other factors, such as food quality, food availability or fish density (among others), may have an overriding influence. We did not measure density of fish during our sampling in 2009 and 2010, which could affect overall growth rates (Campbell 2012, Chapter 2). A reduced food base could have been another effect of underlying DO dynamics. In 2010 there were more hypoxic events recorded by our data sondes than in 2009, so the slower growth we estimated in 2010 could be due in part to the cumulative effects of hypoxia exposure on prey availability, not just conditions during the previous week. Despite differences in overall estimated growth

rates between the two years, substantial differences in estimated growth rate were still evident between periods of Good vs. Poor DO classification. Growth rates associated with the Moderate DO classification were much more variable, which could be due to low sample size, or the way we defined the Moderate classification may not have accurately categorized the DO experience.

Overall, spot collected from both tidal creeks had higher estimated growth rates than those from the NRE, and we did not see the expected differences in growth between the two tidal creeks based on human impacts to their watersheds. The variability in estimated growth in the tidal creeks suggests that factors other than DO played a major role in driving growth. The two tidal creeks were selected based on their differences in surrounding habitat characteristics as Broad Creek had a less impacted habitat with marsh areas and minimal anthropogenic alterations, while Peletier Creek had been channelized, with extensive bulkheads and little natural shoreline, and contained two boat marinas. Thus, we expected that Peletier Creek would have poorer DO conditions than Broad Creek, but that was not the case. Broad Creek had Moderate categories of DO mainly due to diel cycling of oxygen that dipped in early morning hours in the shallow creek. Despite all the anthropogenic factors that could potentially negatively impact DO levels in Peletier Creek, DO quality was in the Good category, but other aspects of the impacted habitat may affect the creek's utility as good fish habitat. The variation in estimated growth in Peletier Creek most likely was driven by factors we did not quantify (e.g., other water quality impacts from increased boat traffic and marina operations, or effects of channel dredging on prey availability).

Though the Newport estuary was not sampled as extensively as the NRE, it consistently had good DO conditions during periods when DO conditions varied in the NRE. In addition, estimated growth of spot in the Newport estuary was even better than for fish in the NRE that had recently experienced good DO conditions. All this suggests that the Newport estuary may be a better habitat for growth than the NRE, perhaps at least in part due to better DO conditions.

This study demonstrated the value of incorporating physiological, temporal, and environmental data into predictive growth models to more accurately depict how temporally dynamic hypoxia effects growth in juvenile spot. Many studies have employed growth rate relationships based on single bioindicators, but for juvenile spot an approach incorporating multiple bioindicators coupled with environmental data was the most effective. A similar multi-indicator approach, perhaps including some of the same bioindicators we used, may prove useful in studies evaluating recent growth rate of other fishes. While growth rates of juvenile spot varied both among and within habitats and sites and among years in our study, our results suggest that some habitats promote better growth than others, and that one of the main factors driving these differences is DO levels. We were able to demonstrate that increased frequency and severity of hypoxia reduced estimated growth rates of juvenile spot in an estuarine setting. Despite variation from year to year our results suggest that recent levels of hypoxia are having a negative effect on growth of juvenile spot. High levels of nutrient loading may increase overall system productivity, but can also increase the frequency and duration of hypoxia events (Diaz & Rosenberg 2008, Breitburg et al. 2009).

While enhanced productivity in the NRE may help fish achieve higher growth rates at times, those benefits are offset, at least in part, by the effects of hypoxia. Even though other factors also affect growth, the overall implications suggest that improved DO conditions will lead to improved growth of juvenile fish in the NRE.

REFERENCES

Able KW, Fahay MP (1998) The first year of the life of estuarine fishes in the Middle Atlantic Bight. Rutgers University Press, New Brunswick, New Jersey

Beck MW, Heck KLJ, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern B, Hays CG, Hoshino K, Minello TJ, Orth RJ, Sheridan PF, Weinstein MP (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. BioScience 51(8):633

Beckman BR (2011) Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. Gen Comp Endocrinol 170:233-252

Bell GW, Eggleston DB (2005) Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia. Mar Biol 146:761-770

Breitburg DL (1990) Near-shore hypoxia in the Chesapeake Bay: Patterns and relationships among physical factors. Est Coast Shelf Sci 30:593-609

Breitburg DL, Craig JK, Fulford RS, Rose KW, Boynton WR, Brady DC, Ciotti BJ, Diaz RJ, Friedland KD, Haggy JD III, Hart DR, Hines AH, Houde ED, Kolesar SE, Nixon SW, Rice JA, Secor DH, Targett TE (2009) Nutrient enrichment and fisheries exploitation: interactive effects on estuarine living resources and their management. Hydrobiologia 629:31-47

Breitburg DL (2002) Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. Estuaries 25:767-781

Buckley LJ, Turner SI, Halavik TA, Smigielski AS, Drew SM, Laurence GC (1984) Effects of temperature and food availability on growth, survival and RNA-DNA ratio of larval and sand lance (*Ammodytes americanus*). Mar Ecol Prog Ser 15:91-97

Buckley L, Caldarone E, Ong T (1999) RNA-DNA ratio and other nucleic acid based indicators for growth and condition of marine fishes. Hydrobiologia 401:265–277

Bulow FJ (1970) RNA-DNA ratios as indicators of recent growth rates of a fish. J Fish Res Board Can 27:2343-2349

Burnham KP, Anderson DR (2002) Model selection and multimodel inference (Second Edition). Springer Science, New York

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169-193

Caldaron EM, MacLean SA, Sharak B (2012) Evaluation of bioelectrical impedance analysis and Fulton's condition factor as nonlethal techniques for estimating short-term responses in postsmolt Atlantic salmon (*Salmo salar*). Fish Bull 110:257-270

Caldaron EM, St. Onge-Burns JM, Buckley LJ (2003) Relationship of RNA/DNA ratio and temperature to growth in larvae of Atlantic cod Gadus morhua. Mar Ecol Prog Ser 262:229-240

Caldarone EM, Wagner M, St. Onge-Burns J, Buckley LJ (2001) Protocol and guide for estimating nucleic acids in larval fish using a fluorescence microplate reader. Ref Doc 01-11:1-22, National Marine Fisheries Service, Woods Hole, MA. Also available at www.nefsc.noaa.gov/nefsc/publications/crd/crd0111/0111.htm

Campbell LA (2012) Direct and indirect effects of hypoxia on juvenile fish in the Neuse River Estuary, North Carolina. PhD dissertation, North Carolina State University, Raleigh, NC

Clemmesen C, Doan T (1996) Does otolith structure reflect the nutritional condition of a fish larva? Comparison of otolith structure and biochemical index (RNA/DNA ratio) determined on cod larvae. Mar Ecol Prog Ser 138:33-39

Ciotti BJ, Targett TE, Nash RDM, Batty RS, Burrows MT, Geffen AJ (2010) Development, validation and field application of an RNA-based growth index in juvenile plaice *Pleuronectes platessa*. J Fish Biol 77:2181-2209

Craig JK, Rice JA, Crowder LB, Nadeau DA (2007) Density-dependent growth and mortality in an estuary-dependent fish: An experimental approach with juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 343:251-262

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321:926-929

Duan C (1997) The insulin-like growth factor system and its biological actions in fish. Am Zool 37:491-503

Eby LA, Crowder LB (2002) Hypoxia-based habitat compression in the Neuse river estuary: Context-dependent shifts in behavioral avoidance thresholds. Can J Fish Aquat Sci 59: 952-965

Eby LA, Crowder LB, McClellan CM, Peterson CH, Powers MJ (2005) Habitat degradation from intermittent hypoxia: Impacts on demersal fishes. Mar Ecol Prog Ser 291:249-262

Ferron A, Leggett WC (1994) An appraisal of condition measures for marine fish larvae. Adv Mar Biol 30:217-303

Fey DP (2005) Is the marginal otolith increment width a reliable recent growth index for larval juvenile herring? J Fish Biol 66:1692-1703

Foster AR, Houlihan DF, Hall SJ (1993) Effects of nutritional regime on correlates of growth rate in juvenile Atlantic cod (*Gadus morhua*): comparison of morphological and biochemical measurements. Can J Fish Aquat Sci 50**:**502-512

Fukada H, Murashita K, Furutani T, Masumoto T (2012) Yellowtail insulin-like growth factor 1: molecular cloning and response to various nutritional conditions. Domest Anim Endocrin 42:220-229

Fukuda M, Sako H, Shigeta T, Shibata R (2001) Relationship between growth and biochemical indices in laboratory-reared juvenile Japanese flounder *(Paralichthys olivaceus*), and its application to wild fish. Mar Biol 138:47-55

Gibson RN (1994) Impact of habitat and quality and quantity on the recruitment of juvenile flatfishes. Neth J Sea Res 32:191-206

Houde ED (1987) Fish early life dynamics and recruitment variability. Am Fish Soc Symp 2:17-29

Houlihan DF, Mathers EM, Foster A (1993) Biochemical correlates of growth rate in fish. In: Rankin JC, Jensen FB (eds) Fish Ecophysiology. Chapman & Hall, London p 45-71

JMP, Version 9. SAS Institute Inc., Cary, NC, 1989-2012

Kuropat C, Mercaldo-Allen R, Caldarone E, Goldberg R, Phelan B, Thurberg F (2002) Evaluation of RNA concentration as an indicator of growth in young-of-the-year winter flounder *Pseudopleuronectes americanus* and tautog *Tautoga onitis*. Mar Ecol Progr Ser 230:265-274

Le Bail PY, Gentil V, Noel O, Gomez JM, Carre F, Le Goff P, Weil C (1998) Structure, function, and regulation of insulin-like growth factors in fish. In: Vandry H, Tonon MC, Roubos EW, de Loof A (eds) Trends in Comparative Endocrinology and Neurobiology: From Molecular to Integrative Biology. The New York Academy of Sciences, New York p 157-161

Lied E, Rosenlund G (1984) The influence of the ratio of protein energy to total energy in the feed on the activity of protein synthesis *in vitro*, the level of ribosomal RNA and the RNA-DNA ratio in white trunk muscle of Atlantic cod (*Gadus morhua*). Comp Biochem Physiol A 77**:**489-494

Luettich RA Jr, McNinch JE, Pinckney JL, Alperin MJ, Martens CS, Paerl HW, Peterson CH, Wells JT (2000) Neuse River Estuary modeling and monitoring project, Final Report: Monitoring Phase. UNC Water Resources Research Institute, Technical Report

Maillet GL, Checkley DM Jr (1991) Storm-related variation in the growth rate of otoliths of larval Atlantic menhaden *Brevoortia tyrannus*: a time series analysis of biological and physical variables and implication for larva growth and mortality. Mar Ecol Progr Ser 79:1-16

Malloy KD, Targett TE (1994) The use of RNA:DNA ratios to predict growth limitation of juvenile summer flounder (*Paralichthys dentatus*) from Delaware and North Carolina estuaries. Mar Biol 118:367-375

Mathers EM, Houlihan DF, Cunningham MJ (1992) Nucleic acid concentrations and enzyme activities as correlates of growth rate of the saithe *Pollachius virens*: growth-rate estimates of open-sea fish. Mar Biol 112:363-369

Mathers EM, Houlihan DF, McCarthy ID, Burren LJ (1993) Rates of growth and protein synthesis correlated with nucleic acid content in fry of rainbow trout, *Oncorhynchus mykiss*: effects of age and temperature. J Fish Biol 43:245-263

McClellen C, Eby L, Crowder L (2002) Neuse River estuary fish monitoring project phase II: Effects of hypoxia on the use and quality of fish habitat. Water Resources Research Institute of the University of North Carolina, Report 343D

McNatt RA, Rice JA (2004) Hypoxia-induced growth rate reduction in two juvenile estuary-dependent fishes. J Exp Mar Biol Ecol 311:147-156

NCDWQ (2009) Neuse river basinwide water quality plan 2009. North Carolina Department of Environmental and Natural Resources, Division of Water Quality

Perez-Jimenez A, Guedes MJ, Morales AE, Oliva-Teles A (2007) Metabolic responses to short starvation and refeeding in *Dicentrarchus labrax* effect of dietary composition. Aquaculture 265:325-335

Picha ME, Silverstein JT, Borski RJ (2006) Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops x Monrone saxatilis*). Gen Comp Endocrinol 147:196-205

Picha ME, Turano MJ, Beckman BR, Borski RJ (2008a) Endocrine biomarkers of growth and applications to aquaculture: a mini-review of growth hormone, Insulin-like Growth Factor-I and IGF binding proteins as growth indicators in fishes. N Am J Aquacult 70:196-211

Picha ME, Turano MJ, Tipsmark CK, Borski RJ (2008b) Regulation of endocrine and paracrine sources of Igfs and Gh receptor during compensatory growth in hybrid striped bass (*Morone chrysops* X *Morone saxatilis*). J Endorin 199:81-94

Rabalais NN, Turner RE, Scavia D (2002) Beyond science into policy: Gulf of Mexico hypoxia and the Mississippi River. BioScience 52:129-142

Rakocinski CF, Peterson MS, Comyns BH, Zapfe GA, Fulling GL (2006) Do abiotic factors drive the early growth of juvenile spot (*Leiostomus xanthurus*)? Fish Res 82:186-193

Roberts JJ, Brandt SB, Fanslow D, Ludsin SA, Pothoven SA, Scavia D, Höök TO (2012) Effects of hypoxia on consumption, growth, and RNA:DNA ratios of young yellow perch. Trans Am Fish Soc 140:1574-1586

Rooker JR, Holt GJ (1996) Application of RNA:DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). Mar Freshw Res 47:283-290

Rooker JR, Holt GJ, Holt SA (1997) Condition of larval and juvenile red drum (*Sciaenops ocellatus*) from estuarine nursery habitats. Mar Biol 127:387-394

Rose KA (2000) Why are quantitative relationships between environmental quality and fish populations so elusive? Ecol Appl 10:367-385

Scharf FS (2000) Patterns in abundance, growth, and mortality of juvenile red drum across estuaries on the Texas coast with implications for recruitment and stock enhancement. Trans Am Fish Soc 129:1207-1222

Searcy SP, Eggleston DB, Hare JA (2007a) Is growth a reliable indicator of habitat quality and essential fish habitat for a juvenile estuarine fish? Can J Fish Aquat Sci 64:681-691

Searcy SP, Eggleston DB, Hare JA (2007b) Environmental influences on the relationship between juvenile and larval growth for Atlantic croaker, *Micropogonias undulatus*. Mar Ecol Prog Ser 349:81-88

Shimps EL, Rice JA, Osborne JA (2005) Hypoxia tolerance in two juvenile estuary-dependent fishes. J Exp Mar Biol Ecol 325:146-162

Sogard S (1997) Size-selective mortality in the juvenile stage of teleost fishes: A review. Bull Mar Sci 60:1129-1157

Stevenson RD, Woods WA Jr (2006) Condition indices for conservation: new uses for evolving tools. Integr Comp Biol 46:1169-1190

Stierhoff KL, Targett TE, Miller K (2006) Ecophysiological responses of juvenile summer and winter flounder to hypoxia: Experimental and modeling analyses of effects on estuarine nursery quality. Mar Ecol Prog Ser 325:255-266

Stierhoff KL, Targett TE, Power JH (2009a) Hypoxia-induced growth limitation of juvenile fishes in an estuarine nursery: assessment of small-scale temporal dynamics using RNA:DNA. Can J Fish Aquat Sci 66:1033-1047

Stierhoff KL, Tyler RM, Targett TE (2009b) Hypoxia tolerance of juvenile weakfish (*Cynoscion regalis*): Laboratory assessment of growth and behavioral avoidance responses. J Exp Mar Biol Ecol 381:S173-S179

Suthers IM (1998) Bigger? Fatter? Or is faster growth better? Considerations on condition in larval and juvenile coral-reef fish. Aust J Ecol 23:265-273

Tyler RM, Targett TE (2007) Juvenile weakfish Cynoscion regalis distribution in relation to diel-cycling dissolved oxygen in an estuarine tributary. Mar Ecol Progr Ser 333:257-269

Uchida K, Kajimura S, Riely LG, Hirano K, Aida K, Grau EG (2003) Effects of fasting on growth hormone/insulin-like growth factor 1 axis in the tilapia, *Oreochromis mossambicus*. Comp Biochem Physiol A 134:429-439

Wannamaker CM, Rice JA (2000) Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern united states. J Exp Mar Biol Ecol 249:145-163

Weinstein MP (1979) Shallow marsh habitats as primary nurseries for fishes and shellfish, Cape Fear River, North Carolina. Fish Bull US Dep Comm 77:339-357

Weinstein MP (1983) Population dynamics of an estuarine-dependent fish, the spot (*Leiostomus xanthurus*), along a tidal creak-seagrass meadow coenocline. Can J Fish Aquat Sci 40:1633-1638

Weinstein MP, Scott L, O'Neil SP, Siegfried RC, Szedlmayer ST (1984) Population dynamics of spot, *Leiostomus xanthurus*, in polyhaline tidal creeks of the York River estuary, Virginia. Estuaries 7:444-450

Table 4.1 Equations for relationships between SGR and bioindicators. In cases where there were significant interactions the full model equation is shown, along with simple individual relationship equations corresponding to relationships shown in Figure 4.1 for illustrative purposes only. Sample sizes vary among indicators due to damaged samples, lost measurements, or exclusion of some samples due to excessive variability among replicates.

	Equation	**R^2**	**n**	**P value**
RNA:DNA ratio	SGR = 0.73($\log_{10}$(RNA:DNA ratio)) - 0.03	0.05	105	0.0257
RNA concentration (full model)	SGR = -5.3 + 0.002*(RNA conc) + 0.11*(Temp) + [day 7 = -0.65, day 14 = 0.26, day 21 = 0.39] + (Temp - 26.09)*((RNA conc - 994.3)*0.000005) + (RNA conc - 994.3)*[day 7 = 0.00016, day 14 = 0.00044, day 21 = -0.00060]	0.75	107	<0.0001
	[1]$SGR_{7\ day}$ = 0.0047*RNA conc - 226.77	*0.68*	*29*	
	[1]$SGR_{14\ day}$ = 0.0054*RNA conc - 2.35	*0.81*	*31*	
	[1]$SGR_{21\ day}$ = 0.0034*RNA conc - 1.22	*0.62*	*47*	
IGF-I liver expression (full model)	SGR = -1.15 + [day 7 = -0.14, day 14 = 0.27, day 21 = -0.12] + 0.28*($\log_{10}$(IGF-I liver expression) + [day 7 = 0.71, day 14 = -1.20, day 21 = 0.48] *($\log_{10}$(IGF-I liver expression) – 6.92)	0.19	69	0.0185
	[1]$SGR_{7\ day}$ = 0.98* $\log_{10}$(IGF-I liver expression) -6.23	0.18	15	
	[1]$SGR_{14\ day}$ = 0.93* $\log_{10}$(IGF-I liver expression) + 7.39	0.09	20	
	[1]$SGR_{21\ day}$ = 0.75* $\log_{10}$(IGF-I liver expression) - 4.61	0.21	34	
IGF-I expression fold change	SGR = -0.72(IGF-I expression fold change) + 1.11	0.06	78	0.0298
HSI (full model)	SGR = -0.81 -0.04*Temp + [day 7 = -0.28, day 14 = 0.15, day 21= 0.13] + 3.09*HSI + (Temp - 25.95)*((HSI - 0.63)*0.36))	0.59	119	<0.0001
	[2]$SGR_{24°C}$ = 2.29(HSI at 24°C) - 1.16	0.47	61	
	[2]$SGR_{28°C}$ = 3.69(HSI at 28°C) – 2.20	0.65	58	
Fulton's K (full model)	SGR = -5.57 - 0.01*Temp + 2.89*Fulton's K + (Temp-25.98)*((Fulton's K-2.04)*-0.50)	0.23	121	<0.0001
	[2]$SGR_{24°C}$ = 3.88(Fulton's K at 24°C) – 7.71	0.30	61	
	[2]$SGR_{28°C}$ = 1.89(Fulton's K at 24°C) - 3.69	*0.16*	*60*	

Table 4.1 Continued

[1]Equation for total RNA concentration or $\log_{10}$(IGF-I liver expression) grouped by day sampled (both temperatures combined) and no interactions for illustrative purposes (see Figure 4.1)

[2]Equation for just HSI or Fulton's K grouped by temperature (all days sampled combined) and no interactions for illustrative purposes (see Figure 4.1)

Table 4.2 Least-squares multivariate regression candidate models developed to predict SGR based on bioindicators and environmental variables (see Methods), ordered by Akaike's information criterion (AIC_c); K = number of parameters in the model, ΔAIC_c = distance of each model from the best AIC_c model, w_i = model probability weight. The top three models are indicated in bold. Individual bioindicator models shown in Table 4.1 are italicized.

Model	R^2	n	K	AIC_C	ΔAIC_C	W_i
HSI, RNA, DNA, temp, Fulton's K, day	**0.80**	**103**	**8**	**-144.72**	**0.00**	**0.19**
HSI, RNA, temp, Fulton's K, day	**0.80**	**105**	**7**	**-144.50**	**0.21**	**0.17**
HSI, RNA, DNA, temp, Fulton's K, day, int. wgt	**0.81**	**103**	**9**	**-144.23**	**0.48**	**0.15**
HSI, RNA, DNA, temp, Fulton's K, day, RNA*temp	0.80	103	9	-142.85	1.87	0.08
HSI, RNA, logRNA:DNA, temp, Fulton's K, day	0.80	102	8	-142.84	1.88	0.07
HSI, RNA, DNA, temp, int. wgt, day	0.80	103	8	-142.81	1.90	0.07
HSI, RNA, temp, Fulton's K, day, RNA*temp	0.80	105	8	-142.73	1.99	0.07
HSI, RNA, temp, day	0.79	105	6	-142.34	2.38	0.06
HSI, RNA, temp, int. wgt, day	0.80	105	7	-142.19	2.52	0.05
HSI, RNA, DNA, temp, Fulton's K, day, int. wgt, RNA*temp	0.81	103	10	-142.04	2.67	0.05
HSI, RNA, logRNA:DNA, temp, int. wgt, day	0.80	102	8	-141.08	3.63	0.03
RNA, DNA, temp, Fulton's K, day	0.75	105	7	-125.60	19.11	0.00
RNA, DNA, temp, int. wgt, day, Fulton's K	0.75	105	8	-124.41	20.31	0.00
RNA, temp, Fulton's K, day	0.75	107	6	-123.61	21.11	0.00
RNA, temp, Fulton's K, day, int. wgt	0.75	107	7	-123.58	21.14	0.00
*RNA, temp, day, RNA*day*	0.75	107	7	-121.40	23.31	0.00
RNA, DNA, temp, int. wgt, day	0.73	105	7	-117.14	27.57	0.00
RNA, temp, int. wgt, day	0.72	107	6	-112.58	32.14	0.00
*HSI, temp, day, HSI*temp*	0.59	119	6	-84.27	60.45	0.00
HSI, logRNA:DNA, temp, Fulton's K, day	0.63	103	7	-82.63	62.09	0.00

Table 4.2 Continued

HSI, RNA, DNA, fold change, temp, int. wgt, day	0.76	67	9	-80.24	64.48	0.00
HSI, logRNA:DNA, temp, int. wgt, day	0.62	103	7	-79.90	64.82	0.00
HSI, temp, Fulton's K, day	0.58	119	6	-79.19	65.53	0.00
HSI, RNA, fold change, temp, int. wgt, day	0.76	69	8	-78.27	66.45	0.00
HSI, temp, int. wgt, day	0.57	119	6	-76.57	68.15	0.00
RNA, DNA, fold change, temp, Fulton's K, day	0.71	68	8	-74.24	70.48	0.00
HSI, RNA, log IGF-I liver exp , temp, int. wgt, day	0.72	61	8	-71.41	73.31	0.00
HSI, RNA, DNA, temp, log IGF-I liver exp , Fulton's K, day	0.73	61	9	-70.03	74.69	0.00
HSI, RNA, DNA, logIGF-I liver exp , temp, int. wgt, day	0.72	61	9	-68.99	75.73	0.00
RNA, DNA, logIGF-I liver exp , temp, Fulton's K, day	0.68	62	8	-65.55	79.17	0.00
HSI, logIGF-I liver exp , temp, int. wgt, day	0.51	68	7	-45.39	99.33	0.00
HSI, fold change, temp, int. wgt, day	0.56	77	7	-42.13	102.58	0.00
HSI, logRNA:DNA, temp, logIGF-I liver exp, Fulton's K, int. wgt, day	0.55	62	9	-41.76	102.96	0.00
HSI, logRNA:DNA, temp, fold change, Fulton's K, int. wgt, day	0.56	68	9	-41.42	103.30	0.00
logIGF-I liver exp, temp, Fulton's K, day	0.23	69	6	-18.20	126.51	0.00
logRNA:DNA, temp, Fulton's K, day	0.27	105	6	-15.31	129.41	0.00
*logIGF-I liver exp, day, logIGF-I liver exp*day*	0.19	69	6	-15.07	129.65	0.00
*Fulton's K, temp, Fulton's K*temp*	0.23	121	4	-13.33	131.39	0.00
logIGF-I liver exp , temp, int. wgt, day	0.15	69	6	-11.92	132.79	0.00
fold change, temp, Fulton's K, day	0.23	78	6	-2.75	141.97	0.00
fold change, temp, int. wgt, day	0.17	78	6	3.65	148.36	0.00
logRNA:DNA	0.05	105	2	4.14	148.85	0.00

Table 4.2 Continued

fold change	0.06	78	2	4.24	148.96	0.00
logRNA:DNA, temp, int. wgt, day	0.06	105	6	11.98	156.69	0.00

Table 4.3. Simulated growth values for a juvenile spot starting at 6.9 g and 63 mm SL, over the period of June 15 to August 12, based on recorded DO values for the two NRE sites (Nearshore and Intermediate) 2007-2010, and the Newport estuary in 2010. For each site-year combination the number of days categorized as Good, Moderate, and Poor DO conditions are given, along with the end weight and length of the simulated fish, its average specific growth rate (SGR), and average growth rate in length. For comparison, values are given for growth at constant Good, Moderate and Poor DO conditions, based on average growth rates for fish collected in the NRE in 2009-2010. Values for the simulation of growth at constant Good DO conditions are in bold; estimated fish end weights from each of the other simulations are compared to the estimated end weight from this simulation.

Year	Site	Number of Days at Each DO Rating			Predicted		Weight difference from constant Good DO conditions	Average SGR (% *bw* day^{-1})	Average Growth Rate in length (mm d^{-1})
		Good	Moderate	Poor	End Weight (g)	End SL (mm)			
Simulations based on actual DO conditions in the NRE									
2007	Nearshore	35	12	12	14.2	81	-9%	1.22	0.31
	Intermediate	29	9	21	13.5	80	-13%	1.14	0.29
2008	Nearshore	32	25	2	14.7	82	-6%	1.28	0.33
	Intermediate	14	29	16	13.4	79	-14%	1.12	0.28
2009	Nearshore	27	25	7	14.2	81	-9%	1.23	0.31
	Intermediate	19	23	17	13.5	79	-14%	1.13	0.28
2010	Nearshore	39	12	8	14.5	82	-7%	1.26	0.32
	Intermediate	12	19	28	12.7	78	-18%	1.04	0.26
Simulations based on assumed constant DO conditions									
	Good	**59**	**-**	**-**	**15.6**	**84**	**-**	**1.39**	**0.36**
	Moderate	-	59	-	13.8	80	-11%	1.19	0.30
	Poor	-	-	59	11.0	74	-29%	0.80	0.19
	Newport Estuary 2010	-	-	-	16.8	86	+8%	1.52	0.39

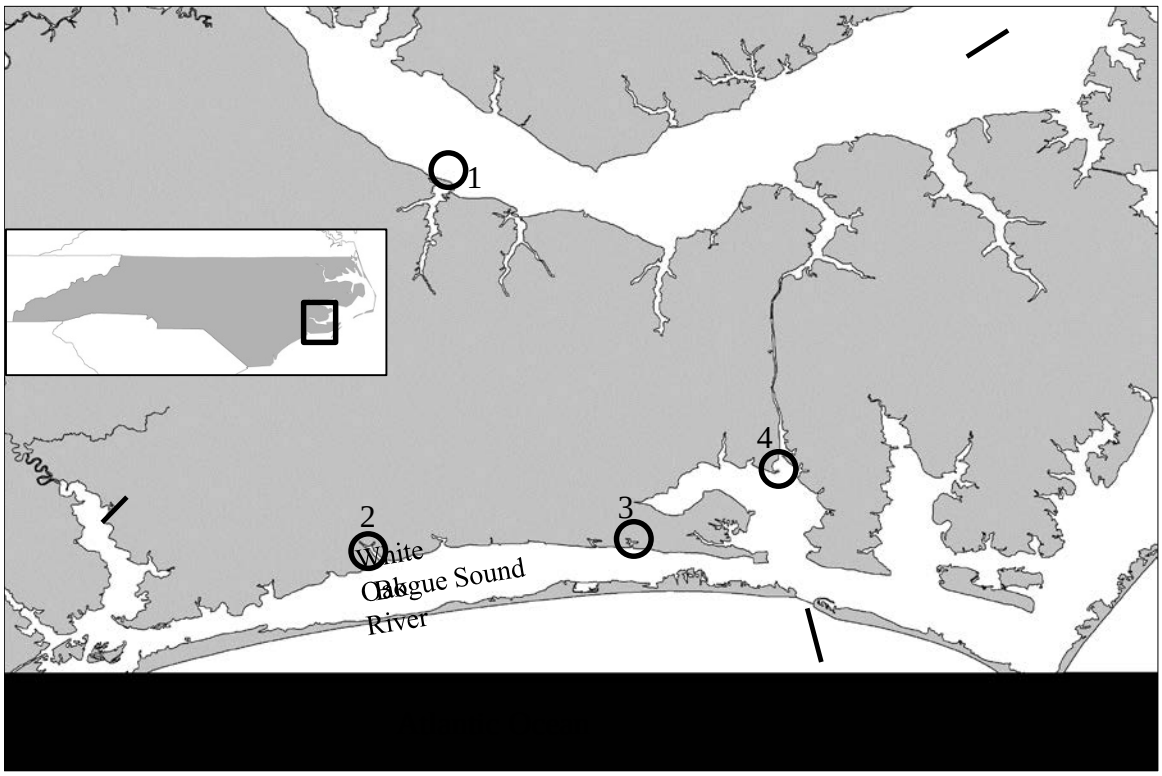


Figure 4.1 Map of study sites along North Carolina coast, inset is state with area of study boxed. Circles are around study areas: (1) Neuse River Estuary sites, (2) Broad Creek, (3) Peletier Creek, (4) Newport estuary site.

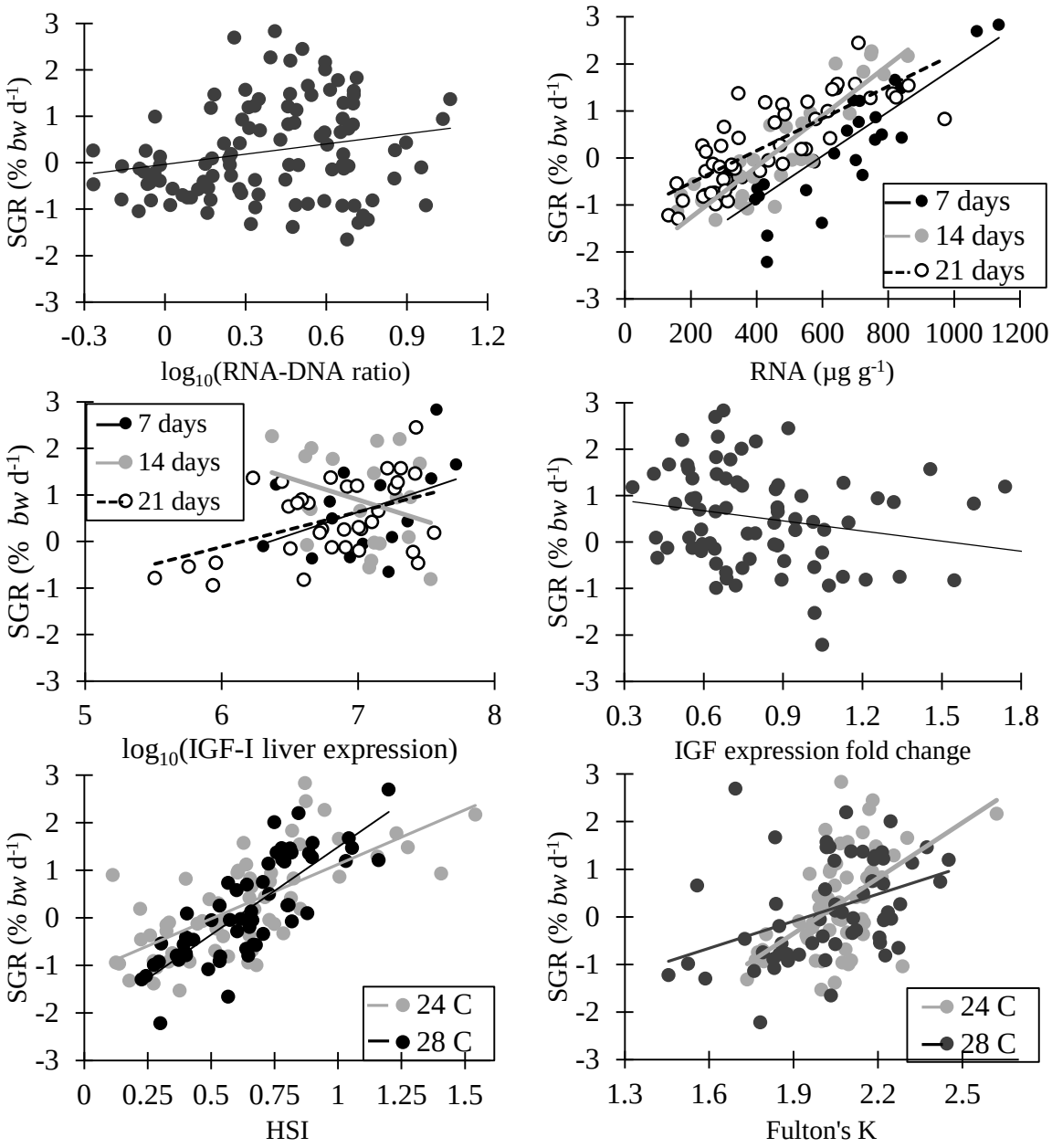


Figure 4.2 Relationships between each bioindicator and SGR from the laboratory calibration experiment. In cases where there were significant interactions (RNA*day in experiment, $\log_{10}$(IGF-I liver expression)*day in experiment, HSI*temperature, Fulton's K*temperature) individual relationship lines are shown for illustrative purposes only. See Table 4.1 for regression equations and R^2 values.

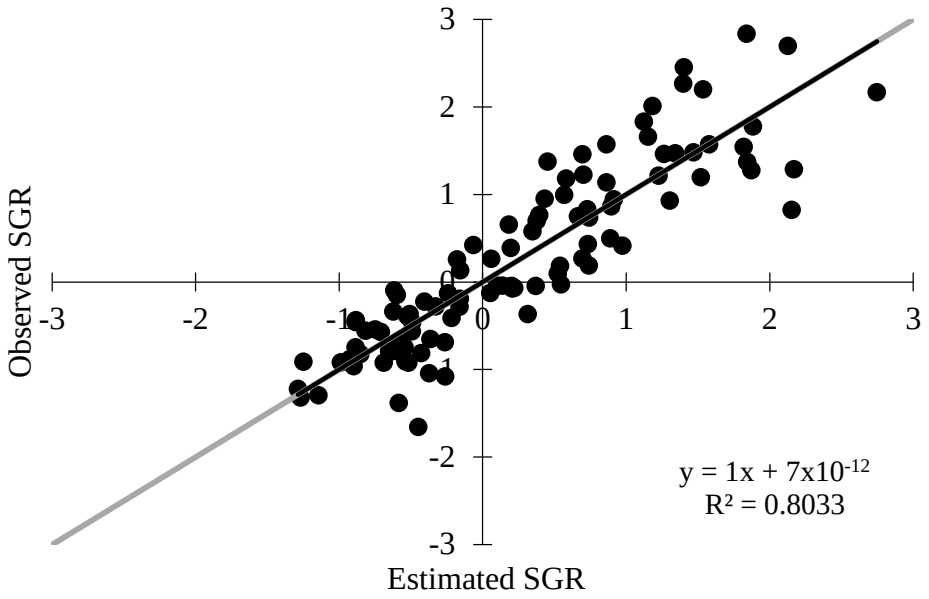


Figure 4.3 Relationship between observed growth rates of fish in the calibration experiment and corresponding growth rates estimated by the best model identified using AIC_c. One to one line in grey. Fitted line in black.

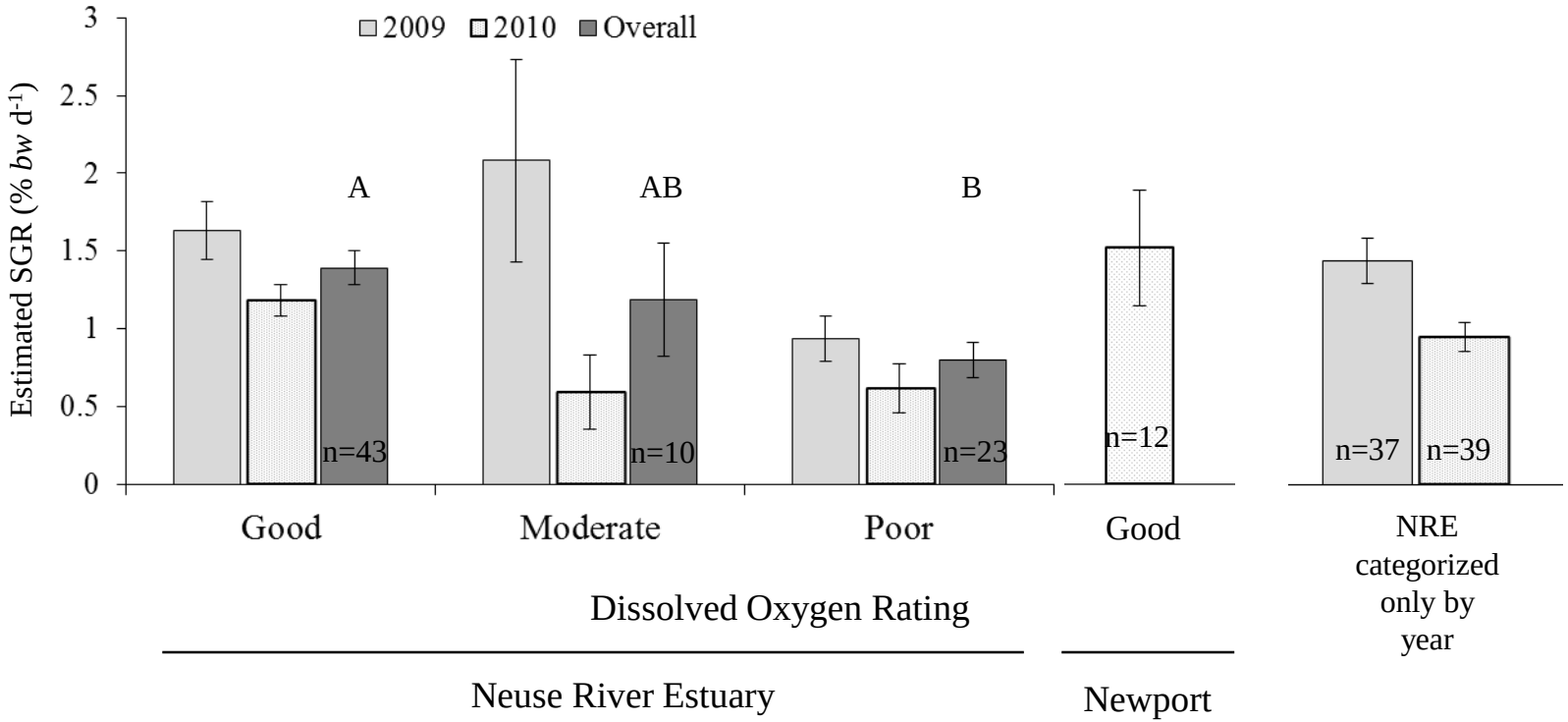


Figure 4.4. Mean ± SE estimated SGR for field fish by DO classification for the Neuse River estuary (NRE) and the Newport estuary 2010 and mean ± SE estimated SGR by year for NRE. ANOVA tests found significant main effects of DO classification and year, but not an interaction. Estimated SGR for NRE by year are presented for illustrative purposes. For the NRE overall Tukey's HSD levels are denoted by letters.

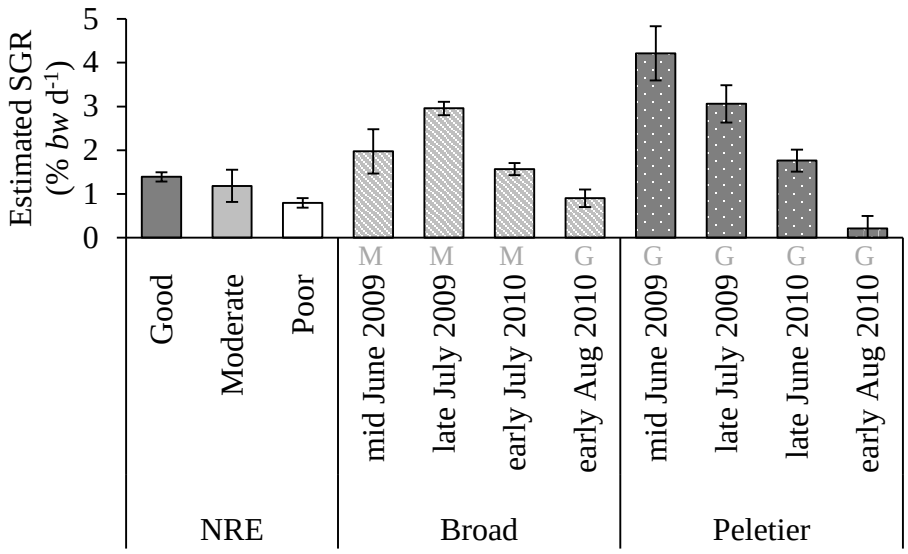


Figure 4.5 Mean ± SE estimated SGR for fish collected from Broad and Peletier tidal creeks in comparison to estimated SGR of fish from the NRE. Values shown for the NRE are for fish collected in 2009 and 2010 following weeks of Good, Moderate, and Poor DO conditions. For each creek sample, DO classification for the week preceding fish collection is indicated in grey letters below the bar (G = Good rating, M = Moderate rating).

Chapter 5

Conclusions

Understanding the effects of hypoxia dynamics on fish is becoming critically important as the frequency and magnitude of hypoxia incidents are on the rise (Diaz & Rosenberg 2008) and insight into these habitat changes will provide much-needed information to guide water quality and natural resource management. Yet, despite extensive work to understand and model processes governing nutrient loading and subsequent effects on algal production, ecosystem function, and spatiotemporal patterns of DO concentrations (e.g., Borsuk et al. 2003, Bowen 2003, Wool et al. 2003), none of these models can translate such changes into direct estimates of impacts on growth and production of living resources; negative effects are generally assumed rather than demonstrated. Managers and policy makers urgently need this direct linkage to know how changes in water quality are likely to affect living resources.

Indirect effects (e.g., habitat compression, reduced food availability, density-dependent processes in oxygenated refuge habitats) are likely the primary mechanisms by which hypoxia may impact juvenile fish cohorts (Eby et al. 2005, Craig et al. 2007), because fish are highly mobile and exhibit strong hypoxia avoidance behavior (Wannamaker & Rice 2000, Eby & Crowder 2002, Bell & Eggleston 2005). Therefore, if we are to understand the effects of hypoxia on estuarine fishes we have to evaluate the processes occurring in oxygenated refuges that potentially impact growth and mortality. To do so, we monitored

how hypoxia within season (weekly to monthly) and on an event scale (hourly to daily) altered fish densities, feeding, and exposure to potential predators in oxygenated refuges.

Our study documented substantial hypoxia dynamics over a wide range of spatial and temporal scales in the Neuse River Estuary (NRE); yet despite these dynamics, fish were able to detect and avoid hypoxia and rapidly redistribute once hypoxic waters retreated. Interestingly, throughout the summer fish continued to return to locations that had recently been hypoxic, instead of permanently migrating to habitats less frequently impacted by hypoxia. Densities of fish in nearshore oxygenated refuges increased nearly two-fold when the habitat was compressed by hypoxic waters. Moreover, we found evidence of feeding reduction in compressed refuges that can lead to negative impacts on fish growth and condition. In 2007, compression of the oxygenated refuge had the potential to reduce average growth rate over the season by a minimum of 4%, due to density-dependent effects on growth (Craig et al. 2007). Increases in duration and expansion of hypoxia will only intensify reductions in growth due to density-dependent processes. Our results facilitate understanding the magnitude of the indirect effects of hypoxia on juvenile fish in order to understand its impact on fish production.

While indirect effects of hypoxia on growth are important factors in regards to fish production, recent research results implied that direct effects of hypoxia on fish in the wild are likely more substantial than laboratory work has previously suggested (Stierhoff et al. 2009). Stierhoff et al.'s study showed that relationships between growth and direct exposure to hypoxia measured in the laboratory underestimate DO concentrations at which growth is

reduced in the field. Even if direct effects on survival are minimal, the cumulative effects of even relatively modest growth reductions may have significant consequences for cohort production and may increase mortality from size-dependent mechanisms such as predation or over-winter survival. Given the fine spatial and temporal scale of oxygen dynamics in the NRE, the costs associated with hypoxia are likely greater than anticipated, and laboratory studies alone are not sufficient to quantify them.

Our next step in evaluating the impacts of hypoxia on fish growth required the use of short-term growth indicators that could measure the integrated effects of rapidly changing environmental conditions. Specifically, we were interested in assessing changes in short-term (e.g., one to several days) growth rates of individual fish, and linking them to observed changes in dissolved oxygen concentrations to quantify the sublethal effects of hypoxia on growth of juvenile estuary-dependent fishes in nursery habitats. To do this we determined the sensitivity and response time of a suite of biological indicators (RNA:DNA ratio, RNA concentration, hepatic IGF-I mRNA expression, IGF-I plasma levels, HSI, and Fulton's K) to changes in environmental conditions and assessed their feasibility and utility for evaluating effects on growth in the field. This study was the first to examine IGF-I production (via both circulating plasma levels and mRNA expression) in a wild-caught estuarine fish that is not used in aquaculture. In addition, we evaluated RNA and DNA concentrations, which had not previously been done with spot. We found that, in juvenile spot, the bioindicators we tested did not fully respond to changes in feeding level until after a week. Despite fairly strong patterns associated with feeding levels, individual indicators did not consistently explain a

high proportion of the variation in specific growth rate. In spite of this, when multiple indicators were combined we were able to produce a predictive model of specific growth rate that accounted for 80% of the variability in growth rates of laboratory fish, thus providing a predictive growth equation we could use to evaluate recent growth of juvenile spot collected in the field.

We applied the model using the appropriate indicators and environmental data to spot collected from the field to relate changes in spot growth rates to the temporal patterns of hypoxia in sites in the NRE, Newport estuary, and in two marsh creeks. These comparisons allowed us to test the hypothesis that increased severity, frequency and duration of hypoxia will reduce spot growth. We were able to detect differences in estimated growth rates in relation to DO conditions, with growth rates associated with good DO conditions the previous week almost twice those of growth rates associated with Poor DO conditions the previous week. These results suggest that recent levels of hypoxia are having a negative effect on juvenile fish using the NRE as nursery habitat. Despite the tendency of fish to evade hypoxia, fish were still experiencing declines in growth when the previous week had less than optimum DO levels and increased hypoxia, suggesting that avoidance cannot mitigate both the direct and indirect effects of hypoxia on growth.

This research provided a quantitative estimate of the combined direct and indirect effects of hypoxia dynamics in North Carolina nursery habitats on growth of a representative estuary-dependent fish. We demonstrated the value of incorporating multiple physiological and morphological into predictive growth models, whereas most studies using biological

indicators to estimate growth only use one indicator per model. Our model provides researchers and managers with a tool to evaluate impacts of observed or modeled scenarios of water quality dynamics on fish growth rates and a means to begin comparing the relative impacts on fishery resources of water quality degradation, harvest, and habitat loss. It also serves as a template for development of predictive growth models for other species. The processes governing recruitment and transport of larval estuary-dependent fishes are largely beyond our control, but we show that hypoxia has important consequences for juveniles during their stay in the estuary, suggesting that enhanced yield per recruit may be achieved by improving habitat and water quality. These benefits will be applicable to coastal systems regionally, nationally, and globally.

This research adds to the limited number of studies that have attempted to directly relate patterns of hypoxia dynamics to growth of juvenile fish in the wild (e.g., Stierhoff et al 2009, Roberts et al. 2012). We suggest that researchers conduct more detailed monitoring of hypoxia dynamics in estuaries, and further explore the use of multi-indicator models to estimate effects of those dynamics on fishes. While IGF-I was not highly correlated with recent growth rates in our study, it has shown high explanatory value in aquaculture studies (e.g., Beckman et al. 2004, Beckman 2011), so we believe there is still value in further investigating this hormone for use as a growth indicator in field studies, whether based on circulating levels in plasma, or measures of IGF-I gene expression. We did not expect the simple metrics HSI and Fulton's K to be very useful in estimating recent growth, but both of

them (especially HSI) were surprisingly informative; therefore, they too warrant further consideration for inclusion in multi-indicator models.

Comparisons among estuaries of hypoxia impacts on fish growth rates would provide valuable insight regarding the extent to which hypoxia is impairing the nursery function of estuaries, and help identify those that would benefit most from water quality improvements. When estimated growth is lower than would be expected due to effects of hypoxia alone, as in the marsh creeks we studied, efforts can be focused on other water quality or habitat conditions that may be responsible. The findings presented here could only be achieved by combining biotechnology techniques with traditional field ecology methods. Fisheries ecologists can benefit greatly by establishing collaborations with scientists who regularly employ these techniques, but typically work in controlled laboratory settings. Such interdisciplinary approaches are becoming essential to address many of the increasingly complex questions and issues facing researchers, managers and policy makers.

REFERENCES

Beckman BR (2011) Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. Gen Comp Endocrinol 170:233-252

Beckman BR, Fairgrieve W, Cooper KA, Mahnken CVW, Beamish RJ (2004) Evaluation of endocrine indices of growth in individual postsmolt Coho salmon. Trans Am Fish Soc 133:1057-1067

Bell GW, Eggleston DB (2005) Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia. Mar Biol 146:761-770

Borsuk ME, Stow CA, Reckhow KH (2003) An integrated approach to TMDL development for the Neuse river estuary using a Bayesian probability network model (Neu-BERN). J Water Resour Plann Manage 129:271-282

Bowen JD, Asce AM, Hieronymus JW (2003) A CE-QUAL-W 2 model of Neuse estuary for total maximum daily load development. J Water Resour Plann Manage 129:283-294

Craig JK, Rice JA, Crowder LB, Nadeau DA (2007) Density-dependent growth and mortality in an estuary-dependent fish: An experimental approach with juvenile spot *Leiostomus xanthurus*. Mar Ecol Prog Ser 343:251-262

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321: 926-929

Eby LA, Crowder LB (2002) Hypoxia-based habitat compression in the Neuse river estuary: Context-dependent shifts in behavioral avoidance thresholds. Can J Fish Aquat Sci 59: 952-965

Eby LA, Crowder LB, McClellan CM, Peterson CH, Powers MJ (2005) Habitat degradation from intermittent hypoxia: Impacts on demersal fishes. Mar Ecol Prog Ser 291:249-262

Roberts JJ, Brandt SB, Fanslow D, Ludsin SA, Pothoven SA, Scavia D, Höök TO (2012) Effects of hypoxia on consumption, growth, and RNA:DNA ratios of young yellow perch. Trans Am Fish Soc 140:1574-1586

Stierhoff KL, Targett TE, Power JH (2009) Hypoxia-induced growth limitation of juvenile fishes in an estuarine nursery: assessment of small-scale temporal dynamics using RNA:DNA. Can J Fish Aquat Sci 66:1033-1047

Wannamaker CM, Rice JA (2000) Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern united states. J Exp Mar Biol Ecol 249:145-163

Wool TA, Davie SR, Rodriquez HN (2003) Development of Three-Dimensional Hydrodynamic and Water Quality Models to Support Total Maximum Daily Load Decision Process for the Neuse River Estuary, North Carolina. J Water Resour Plann Manag 129:295-306

APPENDIX

Appendix A

Juvenile Spot (*Leiostomus xanthurus*) Length-Weight Relationship

I estimated the Length-Weight relationship of juvenile spot *Leiostomus xanthurus* based on 1,388 individuals collected from June-September 2007 in the Neuse River estuary. The analysis resulted in the equation:

$$W = 0.00006L^{2.82}$$

where W is the weight of the fish in grams and L is the Standard Length (SL) of the fish in mm, with an R^2 of 0.97 (Figure A.1). Weight of fish ranged from 0.99 – 70.49 grams, with a mean of 9.62 ± 0.21 (mean ± SE). Lengths ranged from 32 – 136 mm, with a mean of 67 ± 0.21 (Table A.1).

Table A.1 Standard Lengths (SL, to the nearest mm) and weight (to the nearest 0.01 g) of juvenile spot collected in the Neuse River estuary June – September 2007.

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
32	0.99	54	4.51	59	6.26	67	8.23	83	13.18
34	1.04	54	4.12	59	5.93	67	8.52	83	15.06
35	1.23	54	4.74	59	5.27	67	8.07	83	15.54
35	1.11	54	4.68	59	5.73	67	8.45	83	15.83
36	1.35	54	4.99	59	5.79	67	7.75	83	17.36
37	1.62	54	4.53	59	6.33	67	9.72	83	15.99
38	1.70	54	4.04	59	5.78	67	7.87	83	17.04
39	1.56	54	4.91	59	6.06	67	8.59	83	13.90
39	1.63	54	4.17	59	4.90	67	9.55	83	13.71
39	1.92	54	4.71	59	5.37	67	8.60	83	16.24
39	1.81	54	4.07	59	6.19	67	8.22	83	15.98
40	1.44	54	4.88	59	5.32	67	8.63	83	15.44
40	1.90	54	4.53	59	5.27	67	9.78	83	15.77
40	1.71	54	5.15	59	5.33	67	8.68	83	14.77
40	2.10	54	4.90	59	6.14	67	9.81	83	18.61
40	1.85	54	4.48	59	5.67	67	7.16	83	13.81
40	2.03	54	4.52	59	5.64	67	8.24	83	13.97
42	2.06	54	4.42	59	2.98	67	6.79	83	13.09
42	2.15	54	4.55	59	5.11	67	9.05	83	14.47
43	2.25	54	5.15	59	5.19	67	8.34	84	15.73
43	2.27	54	4.43	59	5.36	67	8.95	84	17.27
43	2.43	54	4.87	59	2.90	67	8.09	84	15.28
43	2.34	54	4.25	59	5.36	67	8.20	84	16.06
43	2.25	54	4.47	59	6.04	68	7.99	84	16.66
44	1.92	54	4.72	59	6.20	68	9.42	84	14.90
44	2.62	54	4.48	59	5.98	68	9.23	84	14.97
44	2.58	54	4.29	60	5.24	68	7.74	84	14.86
44	2.30	54	4.86	60	6.08	68	9.43	84	13.98
44	2.47	54	4.32	60	5.34	68	9.64	84	15.71
44	2.53	54	3.96	60	5.24	68	10.01	84	15.59

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
45	2.22	54	4.19	60	5.52	68	10.89	85	19.19
45	2.46	54	3.97	60	5.94	68	9.10	85	18.75
45	2.09	54	4.47	60	6.13	68	9.47	85	18.08
45	2.80	54	5.22	60	6.29	68	8.76	85	16.68
45	2.87	54	4.61	60	6.36	68	8.92	85	16.39
45	2.71	54	4.61	60	6.01	68	8.71	85	14.60
45	2.44	54	4.11	60	5.15	68	8.95	85	15.76
45	2.59	54	4.54	60	5.80	68	7.57	85	15.27
45	2.53	54	4.89	60	5.82	68	8.09	85	18.12
45	2.56	54	4.61	60	5.90	68	9.48	85	17.51
45	2.32	54	4.14	60	5.91	68	8.46	85	15.92
45	2.70	54	4.75	60	6.03	68	7.24	85	15.00
45	2.47	54	4.32	60	5.64	68	8.33	85	15.32
45	2.46	55	3.94	60	5.95	68	9.30	85	14.04
45	2.29	55	4.37	60	5.23	68	9.69	85	15.68
46	2.44	55	4.44	60	5.81	68	9.15	85	15.31
46	2.02	55	4.97	60	5.84	68	8.10	85	13.87
46	2.70	55	3.40	60	6.63	68	8.61	85	13.50
46	2.76	55	5.57	60	6.56	68	9.12	86	18.04
46	2.71	55	4.44	60	6.33	69	8.21	86	20.42
46	2.91	55	4.04	60	6.28	69	9.38	86	16.62
46	2.68	55	4.81	60	5.95	69	7.15	86	16.20
46	3.00	55	4.62	60	6.80	69	9.94	86	14.69
46	2.83	55	4.03	60	6.33	69	7.34	86	15.23
46	2.78	55	3.88	60	5.50	69	9.75	86	16.10
46	12.19	55	4.28	60	5.60	69	8.87	86	16.01
47	3.38	55	4.51	60	5.10	69	10.14	86	14.96
47	3.56	55	4.88	60	4.97	69	10.56	86	16.39
47	2.49	55	4.15	60	5.77	69	9.10	86	10.19
47	3.37	55	4.62	60	6.08	69	8.94	86	15.24
47	3.05	55	4.93	60	6.91	69	10.53	86	15.46
47	2.93	55	4.52	60	5.92	69	9.55	86	14.83

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
47	3.54	55	4.56	60	6.09	69	9.33	87	16.19
47	3.06	55	4.65	60	6.34	69	9.34	87	19.30
47	2.96	55	4.60	60	5.51	69	10.56	87	20.65
47	2.74	55	4.00	60	4.66	69	9.32	87	16.09
47	2.91	55	4.73	60	5.23	70	8.41	87	17.10
47	3.10	55	5.21	60	6.28	70	7.62	87	15.04
47	2.80	55	4.48	60	6.82	70	12.00	87	17.67
47	3.24	55	4.31	60	6.14	70	9.43	87	15.94
48	2.72	55	3.87	60	5.58	70	8.97	87	16.08
48	3.04	55	4.82	60	6.42	70	8.86	87	15.38
48	1.68	55	4.77	60	5.30	70	7.86	87	16.68
48	2.75	55	4.38	60	6.53	70	8.10	87	17.32
48	3.38	55	4.24	60	6.21	70	10.38	87	16.10
48	3.28	55	4.60	60	6.41	70	8.61	87	16.47
48	3.19	55	4.68	60	5.43	70	9.49	87	18.45
48	3.17	55	4.60	60	6.00	70	8.81	87	17.82
48	3.40	55	4.47	60	5.56	70	9.43	87	17.14
48	3.34	55	3.96	60	6.55	70	10.10	88	19.52
48	2.61	55	4.40	60	5.84	70	10.06	88	17.03
48	2.99	55	5.83	60	5.32	70	9.23	88	16.39
48	2.99	55	5.04	60	5.70	70	9.69	88	23.55
48	3.06	55	5.29	60	7.97	70	12.01	88	17.58
48	3.07	55	4.61	60	7.19	70	7.95	88	19.22
48	3.41	55	4.63	60	6.53	70	9.06	88	15.64
48	3.75	55	4.78	60	7.78	70	8.46	88	16.87
48	2.77	55	4.97	60	7.15	70	10.71	88	19.68
48	2.83	55	5.21	60	6.38	70	7.56	88	17.05
48	3.02	55	4.59	60	5.83	70	8.51	88	15.68
49	3.71	55	5.93	60	6.31	70	10.45	88	16.14
49	2.60	55	5.21	60	6.47	70	9.25	88	18.30
49	2.93	55	4.24	60	6.39	70	9.21	88	17.08
49	2.92	55	4.48	60	6.27	70	8.93	88	16.73

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
49	3.10	56	4.01	60	5.69	70	10.10	88	16.42
49	3.32	56	5.11	60	5.63	70	9.80	88	16.49
49	3.23	56	6.01	61	5.34	70	10.15	88	16.11
49	2.67	56	3.36	61	6.54	70	8.51	88	16.76
49	3.51	56	2.98	61	6.75	70	8.45	88	16.04
49	3.40	56	4.58	61	6.67	70	9.04	88	17.01
49	3.16	56	5.65	61	6.20	71	10.73	88	16.27
49	3.37	56	5.09	61	6.28	71	8.84	88	17.51
49	2.89	56	4.97	61	6.44	71	12.08	88	16.87
49	3.84	56	5.00	61	6.37	71	10.81	88	17.02
49	3.59	56	5.43	61	6.04	71	10.74	88	15.00
49	3.75	56	4.50	61	5.90	71	10.18	89	22.22
49	3.70	56	4.74	61	6.85	71	10.58	89	22.98
49	2.50	56	5.06	61	6.39	71	9.57	89	21.98
49	3.68	56	5.04	61	5.84	71	10.05	89	20.15
49	3.58	56	5.02	61	6.74	71	11.04	89	21.93
49	3.31	56	4.67	61	6.02	71	11.54	89	17.30
49	3.71	56	5.02	61	5.08	71	10.55	89	19.03
49	3.40	56	5.43	61	5.80	71	9.38	89	17.06
49	3.50	56	5.14	61	7.39	71	9.26	89	17.71
50	3.82	56	4.96	62	7.20	71	9.48	89	16.09
50	3.49	56	5.08	62	5.95	71	10.02	89	15.81
50	3.12	56	4.25	62	6.15	71	11.10	89	16.52
50	3.36	56	4.96	62	5.87	71	10.63	89	17.39
50	3.57	56	4.11	62	6.22	72	11.69	90	19.72
50	3.39	56	4.40	62	6.33	72	9.13	90	23.62
50	3.18	56	5.29	62	6.55	72	9.52	90	21.09
50	3.43	56	4.86	62	6.35	72	10.65	90	19.34
50	3.57	56	4.94	62	7.11	72	9.86	90	18.75
50	2.92	56	6.00	62	5.76	72	9.90	90	17.57
50	2.90	56	5.80	62	7.63	72	10.46	90	18.51
50	3.15	56	5.32	62	7.08	72	9.30	90	17.64

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
50	3.32	56	5.00	62	6.58	72	8.85	90	18.54
50	3.54	56	5.28	62	6.89	72	9.46	90	17.03
50	3.64	56	4.76	62	6.86	72	10.77	90	18.24
50	3.24	56	5.14	62	5.40	72	10.24	90	19.29
50	3.25	56	5.74	62	5.33	72	9.82	90	17.67
50	3.63	56	4.51	62	5.85	72	9.49	90	16.86
50	3.35	56	5.38	62	6.40	72	11.61	90	17.68
50	3.26	56	4.82	62	6.83	72	11.04	90	19.16
50	3.38	56	5.17	62	6.04	72	9.49	90	20.47
50	3.24	56	4.98	62	5.68	72	10.18	90	17.30
50	4.17	56	4.38	62	6.29	73	10.85	90	16.75
50	3.85	56	5.00	62	6.24	73	10.49	91	19.61
50	3.78	56	4.24	62	6.28	73	10.24	91	25.94
50	3.29	56	5.40	62	7.21	73	10.54	91	18.99
50	3.62	56	5.72	62	6.59	73	12.11	91	18.98
50	3.74	56	4.57	62	7.34	73	9.84	91	16.99
50	3.25	56	4.64	63	8.67	73	10.44	92	21.46
50	3.32	56	5.04	63	6.73	73	10.37	92	21.87
50	4.14	56	4.08	63	5.95	73	10.60	92	22.14
50	4.34	56	4.53	63	6.15	73	10.06	92	19.75
50	3.38	56	4.40	63	6.63	73	10.62	92	17.68
51	3.86	56	4.43	63	6.75	73	11.72	92	19.41
51	3.28	56	5.89	63	7.25	73	10.59	92	18.56
51	3.82	56	5.25	63	6.93	73	11.48	92	19.61
51	4.26	56	5.19	63	7.49	73	10.02	92	17.72
51	3.74	56	4.54	63	7.02	73	11.01	93	23.08
51	3.30	56	4.53	63	7.69	73	9.55	93	22.50
51	3.68	56	5.24	63	7.76	74	11.02	93	19.43
51	3.49	56	4.80	63	6.77	74	10.27	93	18.36
51	4.04	57	6.14	63	7.29	74	12.06	93	16.87
51	4.01	57	5.42	63	7.16	74	11.13	93	22.09
51	3.75	57	5.18	63	6.77	74	11.73	93	20.66

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
51	3.50	57	5.83	63	5.97	74	11.70	93	20.96
51	3.56	57	4.91	63	7.06	74	10.52	93	19.55
51	4.01	57	5.20	63	6.57	74	11.35	93	18.29
51	4.94	57	4.94	63	6.24	74	12.38	93	20.60
51	3.71	57	6.24	63	7.24	74	11.94	93	21.13
51	3.76	57	5.20	63	4.45	74	9.25	93	21.91
51	3.74	57	5.00	63	7.11	75	8.07	94	18.96
51	4.37	57	4.82	63	5.69	75	10.96	94	21.49
51	3.41	57	4.94	63	6.42	75	10.50	94	17.87
51	4.04	57	5.65	63	6.90	75	12.34	94	21.21
51	4.89	57	5.24	63	6.19	75	13.37	95	19.24
51	4.13	57	5.21	63	6.18	75	11.87	95	21.84
51	3.48	57	5.16	63	6.54	75	10.81	95	15.37
51	4.23	57	5.31	63	6.60	75	10.04	95	21.35
51	3.48	57	4.79	63	7.24	75	11.21	95	20.45
51	3.53	57	5.14	63	7.14	75	11.16	95	19.16
51	4.32	57	3.71	63	7.54	75	10.92	95	22.88
52	3.90	57	4.58	64	6.61	75	9.87	95	21.38
52	3.76	57	5.37	64	7.28	75	12.27	95	26.17
52	3.50	57	5.03	64	7.44	75	9.84	96	20.03
52	4.13	57	5.11	64	7.02	75	9.14	97	26.91
52	3.92	57	4.62	64	7.69	75	10.19	97	25.17
52	4.22	57	5.26	64	6.14	75	11.97	97	21.36
52	3.70	57	4.49	64	6.96	76	12.73	97	22.76
52	3.61	57	5.24	64	7.64	76	12.96	97	22.29
52	3.69	57	5.59	64	7.51	76	13.02	97	22.13
52	4.46	57	4.10	64	7.96	76	13.82	97	21.98
52	3.82	57	5.00	64	7.98	76	11.08	97	24.64
52	3.84	57	5.25	64	7.56	76	10.78	97	21.38
52	3.54	57	5.35	64	6.81	76	9.21	97	22.64
52	3.43	57	8.39	64	8.17	76	10.25	98	28.43
52	4.20	57	4.52	64	7.38	76	11.19	98	24.74

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
52	3.86	57	4.61	64	7.58	76	10.40	98	22.79
52	3.48	57	5.46	64	6.57	76	10.10	98	20.97
52	4.37	57	5.48	64	7.21	76	10.15	98	20.82
52	4.05	57	5.34	64	7.95	76	11.63	98	22.44
52	4.11	57	5.68	64	6.96	77	12.40	98	24.09
52	4.03	57	5.31	64	8.06	77	12.29	98	18.95
52	3.89	57	4.45	64	8.07	77	10.67	98	23.52
52	3.83	57	4.70	64	7.62	77	8.72	98	24.47
52	3.75	57	16.21	64	7.51	77	12.96	98	27.14
52	4.62	57	5.48	64	6.38	77	11.11	98	23.73
52	3.46	57	5.42	64	7.08	77	11.60	98	25.03
52	3.86	57	5.56	64	6.25	78	12.48	99	25.87
52	3.76	57	5.66	64	7.68	78	13.54	99	23.38
52	3.82	57	5.63	64	7.87	78	12.78	99	25.56
52	3.96	58	6.39	64	7.56	78	11.70	100	22.05
52	4.15	58	5.23	64	7.08	78	13.47	100	21.91
52	3.72	58	7.04	64	7.59	78	16.87	100	24.49
52	3.90	58	6.46	64	7.42	78	14.34	100	24.92
52	4.06	58	4.94	64	7.24	78	12.25	100	24.08
52	4.37	58	5.10	64	7.42	78	13.75	100	22.50
52	4.43	58	4.98	64	7.42	78	12.11	100	22.63
52	4.27	58	5.32	64	8.30	78	11.61	100	21.83
52	4.53	58	5.83	64	7.70	78	11.73	100	24.53
53	4.01	58	6.35	64	8.01	78	16.30	101	25.52
53	3.55	58	5.43	65	6.56	78	16.15	101	24.18
53	4.41	58	5.76	65	7.18	78	11.26	102	30.20
53	4.06	58	4.57	65	7.47	78	11.63	102	29.25
53	3.67	58	4.43	65	7.21	78	12.06	102	26.18
53	4.72	58	5.14	65	8.08	79	13.36	102	25.32
53	3.64	58	4.96	65	7.78	79	13.30	102	24.78
53	3.94	58	4.95	65	8.22	79	11.81	102	25.64
53	3.60	58	5.85	65	6.99	79	13.73	102	24.68

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
53	4.02	58	5.57	65	8.95	79	11.77	102	24.86
53	4.83	58	6.11	65	7.79	79	14.95	103	23.53
53	4.36	58	4.96	65	8.13	79	12.04	103	26.40
53	4.62	58	5.37	65	8.10	80	14.39	103	27.74
53	4.73	58	6.01	65	7.42	80	15.61	104	32.66
53	4.19	58	5.65	65	7.16	80	13.31	104	25.70
53	3.51	58	5.55	65	6.06	80	13.61	104	24.92
53	3.94	58	5.78	65	7.80	80	14.59	104	24.93
53	4.65	58	5.88	65	7.88	80	11.10	104	24.49
53	4.26	58	5.28	65	6.80	80	11.16	104	26.95
53	4.63	58	5.71	65	7.57	80	15.47	105	27.63
53	4.16	58	5.51	65	7.02	80	14.78	105	27.56
53	4.30	58	5.80	65	7.13	80	14.84	105	29.34
53	4.28	58	5.42	65	7.63	80	13.57	106	30.74
53	4.49	58	5.06	65	7.55	80	11.97	107	29.34
53	4.05	58	4.34	65	7.11	80	13.46	108	32.91
53	3.61	58	5.06	65	7.27	80	12.58	109	31.09
53	14.40	58	5.36	65	6.75	80	14.61	110	31.89
53	4.25	58	4.89	65	7.98	80	16.09	110	32.07
53	4.15	58	5.61	65	7.76	80	11.81	110	32.98
53	4.43	58	4.83	65	8.28	80	14.41	111	33.11
53	4.19	58	5.00	65	7.38	80	12.02	111	32.35
53	4.05	58	4.67	65	7.75	80	12.04	112	36.04
53	3.84	58	4.61	65	6.99	80	13.19	113	39.72
53	4.02	58	5.95	66	5.54	80	11.92	113	37.68
53	4.54	58	6.08	66	7.38	80	12.34	113	35.12
53	4.54	58	5.37	66	7.74	80	11.90	114	26.59
53	4.43	58	5.78	66	7.53	80	12.47	115	23.07
53	4.10	58	5.58	66	6.65	80	11.22	115	37.05
53	4.80	58	6.27	66	8.11	80	12.25	115	31.14
53	4.22	58	5.88	66	7.59	80	14.56	116	39.39
53	4.27	58	5.26	66	8.08	81	14.06	116	39.54

Table A.1 Continued

SL	Weight	SL	Weight	SL	Weight	SL	Weight	SL	Weight
53	4.24	58	6.27	66	9.13	81	14.03	116	42.34
53	4.41	58	5.86	66	8.89	81	15.03	117	39.24
53	4.56	58	5.21	66	7.97	81	13.22	117	41.26
53	4.43	58	5.14	66	7.17	81	15.38	117	40.98
53	3.74	59	5.70	66	9.48	81	18.62	117	28.17
53	4.63	59	5.56	66	7.87	81	14.24	119	43.01
53	4.75	59	5.21	66	8.85	81	13.17	120	21.87
54	3.92	59	5.33	66	8.39	81	16.01	120	50.33
54	3.90	59	5.34	66	8.57	81	15.54	125	54.71
54	4.76	59	5.80	66	6.87	81	12.57	125	49.16
54	4.75	59	4.21	66	7.34	81	12.58	128	55.54
54	4.29	59	5.24	66	8.42	81	14.01	128	51.81
54	4.37	59	5.76	66	8.54	81	14.38	130	51.00
54	4.00	59	6.22	66	8.19	82	14.08	131	55.51
54	4.61	59	5.64	66	7.45	82	14.58	134	50.62
54	4.89	59	5.47	66	6.74	82	15.52	134	62.92
54	4.32	59	5.59	66	6.44	82	14.81	135	61.12
54	4.37	59	5.49	66	8.16	82	16.79	136	70.49
54	3.84	59	6.48	66	8.44	82	16.37		
54	3.88	59	5.42	66	8.48	82	14.78		
54	3.99	59	4.99	66	7.50	82	15.54		
54	4.68	59	5.70	67	7.31	82	13.85		
54	4.39	59	5.44	67	7.93	82	13.11		
54	4.52	59	6.08	67	8.36	82	14.37		
54	3.97	59	5.57	67	8.68	82	12.56		

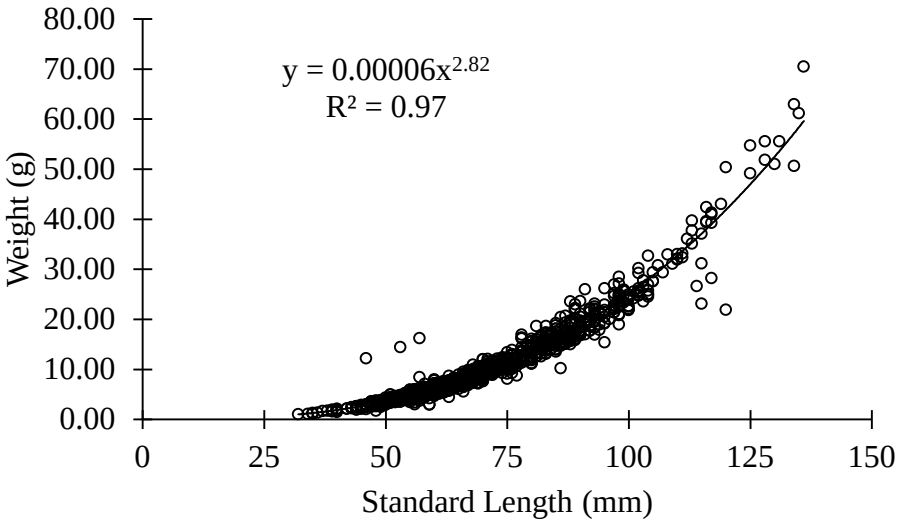


Figure A.1 Length-Weight relationship for 1,388 juvenile spot collected from June-September 2007 in the Neuse River estuary.