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Development and Field Application of a Model Predicting Effects of Episodic Hypoxia on Short-Term Growth of Spot

Lindsay A. Campbell*¹ and James A. Rice

Department of Applied Ecology, North Carolina State University, Campus Box 7617, Raleigh, North Carolina 27695-7617, USA

Abstract

In North Carolina and elsewhere, there is concern that excessive nutrient loading and resulting hypoxic conditions in coastal ecosystems are adversely affecting the native fauna, but quantifying the effects on fish can be difficult. Hypoxia may reduce fish growth via direct exposure or indirectly (e.g., cost of low-oxygen avoidance, reduced food availability, and density-dependent effects in oxygenated refuges). Given the fine spatial and temporal scale of oxygen dynamics in estuarine habitats, evaluating the impacts of hypoxia on fish growth requires short-term growth indicators that integrate the effects of rapidly changing environmental conditions. To address this need, we experimentally determined the sensitivity and response time of a suite of bioindicators of recent growth (RNA:DNA ratio and RNA concentration in muscle tissue; insulin-like growth factor-I messenger RNA expression in the liver; hepatosomatic index; and Fulton's condition factor K) to changes in the specific growth rate of juvenile Spot *Leiostomus xanthurus*. A model based on multiple bioindicators was better at estimating growth rate than models based on single indicators. We used this model to estimate recent growth rates of juvenile Spot collected from the Neuse River estuary and related them to recent dissolved oxygen (DO) conditions. 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 Neuse River estuary in 2007–2010, we estimated that hypoxia dynamics reduced Spot growth over the summer by 6–18% in these years relative to growth under constant good DO conditions. This approach can be used to evaluate impacts of observed or modeled scenarios of water quality dynamics on growth of juvenile Spot and serves as a template for development of predictive growth models for other species.

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,

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*Corresponding author: lindsay_campbell@fws.gov

¹Present address: U.S. Fish and Wildlife Service, San Marcos Aquatic Resources Center, 500 East McCarty Lane, San Marcos, Texas 78666, USA.

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and dissolved oxygen [DO]; Gibson 1994; Beck et al. 2001). Because the productivity and quality of a nursery habitat are 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, 2007b).

In particular, there is growing concern that hypoxia (low DO) due to excessive anthropogenic nutrient loading is having negative impacts on fishes and their habitats in North Carolina (Luettich et al. 2000; McClellan 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, North Carolina: Luettich et al. 2000; Gulf of Mexico “dead zone”: Rabalais et al. 2002), but in the shallower habitats that are more often occupied by juvenile fish, DO levels can change rapidly, creating a more dynamic environment to which fish must respond quickly and continuously (Stierhoff et al. 2009a; Campbell and Rice 2014). The peak of juvenile fish abundance and occupancy in estuaries generally occurs during summer months (Weinstein 1979; Able and Fahay 1998), which correspond with the establishment and spread of hypoxic conditions (Diaz and Rosenberg 2008); as a result, estuary-dependent juvenile fish are particularly susceptible to the effects of hypoxia (Stierhoff et al. 2009a). Given that the frequency of hypoxia is on the rise (Diaz and Rosenberg 2008), quantifying these effects on fish in the field is crucial to understanding the impacts of habitat change.

Laboratory and field enclosure studies have shown that direct exposure to low DO levels can cause reductions in growth or survival for several species of fish (McNatt and Rice 2004; Shimps et al. 2005; Stierhoff et al. 2006). However, 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, National Marine Fisheries Service, Southeast Fisheries Science Center, personal communication) and trawling studies (Eby and Crowder 2002; Bell and Eggleston 2005; Campbell and Rice 2014) 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 underestimates the DO concentrations at which growth is reduced in the field (see Stierhoff et al. 2009a, 2009b). 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 the effects of all the environmental conditions and stressors it experiences and could be considered a direct reflection of habitat suitability, as a 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 from the negative effects of hypoxia on prey resources. As fish crowd into oxygenated refuges, densities can double (Campbell and Rice 2014), leading to density-dependent effects that reduce growth and survival, as shown in cage and pond experiments (Eby et al. 2005; Craig et al. 2007). Even if these individual effects could be teased out, the response of a fish to the suitability of its environment could be better assessed by evaluating how well its recent environmental history has 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 and Rice 2014). 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 the 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; NCDWQ 2009).

Few tools are available to measure short-term growth rates in the field. Because individual fish in the wild cannot readily be recaptured, the traditional approach to calculating individual growth from a 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 and Checkley 1991; Fey 2005; Rakocinski et al. 2006; Searcy et al. 2007a, 2007b) 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 by using morphological, biochemical, physiological, and endocrine indicators that can be measured from a fish captured just once (Ferron and 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. Bioindicators have been used as a proxy for physiological condition (feeding and growth) in larval and small juvenile fishes (Westerman and Holt 1988; Ferron and Leggett 1994; Rooker et al. 1997; Buckley et al. 1999; Glass et al. 2008; Ciotti et al. 2010). In

this study, we estimated the short-term growth of juvenile estuary-dependent fish in the Neuse River estuary (NRE), North Carolina, to directly link changes in DO with impacts on recent growth due to the sublethal effects of hypoxia. To estimate recent growth rate (including both positive [anabolic] and negative [catabolic] changes), 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, the hepatosomatic index (HSI) and Fulton's condition factor (Fulton's K). Variations in muscle RNA relative to DNA reflect protein synthesis demand and can be related to growth (Buckley et al. 1984; Ferron and Leggett 1994). This approach has been successfully applied in a variety of field applications with larval and small juvenile fish to relate recent growth to habitat conditions over the previous 1–3 d to 2 weeks (Malloy and Targett 1994; Rooker and Holt 1996; Stierhoff et al. 2009a, 2009b). The second bioindicator, IGF-I, is a hormone that is responsible for cell differentiation and proliferation; the stimulation of processes related to skeletal elongation; and, ultimately, somatic growth (see Duan 1997; Le Bail et al. 1998 for reviews). Laboratory trials suggest 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 (relative liver weight expressed as a percentage of body weight [bw]), 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 evaluating recent growth (Suthers 1998).

We used laboratory experiments to evaluate the sensitivity and utility of these bioindicators in quantifying recent growth of juvenile Spot *Leiostomus xanthurus*, a representative estuary-dependent fish. Previous studies have assessed how juvenile Spot behaviorally respond to hypoxia (Wannamaker and Rice 2000), how chronic exposure to hypoxia reduces growth (McNatt and Rice 2004), the 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). Spot are found in estuarine nursery areas throughout North Carolina and the southeastern USA and are benthically oriented (where hypoxia is the worst).

Based on these experiments, we developed a set of predictive statistical models relating the indicators (alone and in concert) and environmental variables to Spot growth rate. We then used an information theoretic approach (Burnham

and Anderson 2002) to examine the relative strengths of the candidate models and to guide the selection of the model that best predicted recent Spot growth. We then applied the best predictive growth model from our laboratory study to bioindicator values measured in field-collected Spot 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 reduced 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 of 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 will need when moving forward in understanding the ecosystem-level effects of episodic and chronic hypoxia.

METHODS

Tank Trials

Juvenile Spot (age 0; mean SL = 80.5 mm, range = 67–102 mm; weight = 11.5 g, range = 6.56–23.2 g) were collected by trawl from the NRE in May and June 2009; fish were transported back to the Fisheries Research Laboratory, located at the University of North Carolina's Institute of Marine Science in Morehead City, and were placed in recirculating holding tanks. Spot were individually marked with visible implant elastomer (Northwest Marine Technology, Shaw Island, Washington), and eight individuals were randomly placed into each of eighteen 75.7-L tanks. Fish were acclimated to two treatment temperatures (24°C and 28°C; nine tanks each) at approximately 25‰ salinity for 2 weeks and were fed ad libitum twice daily with thawed freshwater *Mysis* shrimp (Piscine Energetics, Inc., Vernon, British Columbia). Water was recirculated through a bubble-washed bead biofilter (one filter for each temperature treatment) with a 20% water change daily. Air stones were placed in each tank to ensure good oxygen levels. 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; for each tank group, there was daily monitoring to confirm that the DO concentration was above 4.75 mg/L. Other parameters (e.g., nitrites and pH) were monitored intermittently to ensure that water quality remained good.

For this study, we chose to generate a range of growth rates by manipulating food ration rather than DO levels. Reductions in growth rate due to hypoxia exposure are

well documented for juvenile fishes (Stierhoff et al. 2006). In Spot, these effects occur over a narrow range of DO concentrations just above lethal levels (McNatt and Rice 2004). Evidence indicates that hypoxia reduces growth primarily by reducing feeding rather than by altering the growth process directly (e.g., Stierhoff et al. 2006). For these reasons and because we sought bioindicators that would reflect short-term growth regardless of the factor(s) driving it, we used ration rather than DO level to alter growth rates. 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 spanning the full spectrum of both positive (anabolic) and negative (catabolic) growth (i.e., not to test for differences between the feeding levels). Feeding levels were as follows: no food (0% bw/d total), minimum ration (2–3% bw/d total), intermediate ration (4–5% bw/d total), high ration (8–10% bw/d total), and ad libitum (>10–15% bw/d total). Fish were fed half of their food ration in the morning and half in the late afternoon. Each food level had two randomly assigned tanks per temperature treatment, except the intermediate ration, which had only one tank per temperature due to space limitations. On day 0, fish changed from ad libitum acclimation feeding to their assigned feeding level. 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 ($n = 52$). At the time of sampling, each fish was euthanized with an overdose of tricaine methanesulfonate (MS-222; Argent, Redmond, Washington). 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 on the day it was sampled (day 7, 14, or 21). All weights and measurements were taken in the morning before the first daily feeding (at least 12 h after the last feeding). No mortalities due to ration level occurred during our experiment, although 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, Texas), stored at 4°C for 24–48 h, and then stored at –80°C until analysis. For each fish, we calculated the specific growth rate (SGR, % bw/d; Sutcliffe 1970; Houde and Schekter 1981), HSI, and Fulton's K .

Specific growth rate was calculated as

$$\text{SGR} = 100(e^G - 1),$$

where G is the instantaneous weight-based growth coefficient. This coefficient was calculated as

$$G = (\log_e W_2 - \log_e W_1)/t,$$

where W_1 is the weight (g) of the fish at the beginning of the growth interval, W_2 is weight at the end of the growth interval, and t is the interval (d) between the two measurements. To minimize stress, the fish were only weighed and measured at the start of the experiment and when they were removed for analysis. This allowed us to calculate individual growth rates of fish sampled at the end of week 1, but initial weights were not available for estimating week-specific individual growth rates for weeks 2 and 3. Because growth rate might change over time, we wanted to estimate growth during weeks 2 and 3 as well. To estimate SGR during week 2, we used the measured weight of each fish sampled on day 14 as W_2 , and we estimated W_1 using the measured weight of each fish on day 0 and the average SGR of fish in that feeding group during week 1; SGR estimates for week 3 were calculated similarly (see the Supplement available separately online for details).

HSI was calculated as

$$\text{HSI} = \left[\frac{\text{liver weight (g)}}{\text{body weight (g)}} \right] \times 100.$$

Fulton's K was calculated as

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

where W is final weight (g) and L is final SL (cm).

Multiple linear regression was used to describe the relationship between SGR and each of the single bioindicators (RNA: DNA ratio, RNA concentration, IGF-I liver yield expression, IGF-I expression fold change, HSI, and Fulton's K) along with temperature and day sampled and all potential interactions; nonsignificant terms were dropped from regressions.

Field Collection

Our field sites included both impacted and reference areas in different types of nursery habitat that varied substantially in the severity, frequency, and duration of hypoxia: the mainstem NRE, where hypoxia dynamics are generally large scale and episodic; a similar but smaller estuary that is 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 represented areas that are typically oxygenated, with minimal impacts of hypoxia; and the second site (intermediate) was further from shore and deeper (~2–3 m), representing areas with greater impacts of hypoxic events (usually daily hypoxic episodes but not continuously, chronically hypoxic;

Figure 1). The alternative estuarine site was in the Newport River estuary, North Carolina, near the mouth of Core Creek; this site was selected as a nursery area less impacted by hypoxia and was characterized by high tidal flushing and water exchange with the open ocean (Figure 1). Tidal creek sites in Bogue Sound were chosen to represent a relatively pristine creek (Broad Creek; with extensive marsh area and little urbanization) and an impacted creek (Peletier Creek; dredged and channelized, with much urbanization, boat traffic, two boat marinas; Figure 1).

During summer in 2007–2010, YSI 600XLM data sondes (YSI, Yellow Springs, Ohio) were deployed at the sites in the NRE, approximately 30 cm off the bottom. The data sondes recorded temperature, salinity, DO, and depth every 15 min from early to late summer (May 25–September 24, 2007; May 30–September 25, 2008; May 26–August 21, 2009; June 14–August 12, 2010). Sondes in the NRE were stationary for the entire season. During 2009 and 2010, sondes were deployed within the two tidal creeks in Bogue Sound in the same manner as the NRE sondes; the sondes were placed for two periods early in the season (both creeks: June 1–17, 2009, and 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 River 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 headrope and footrope with tickler chain attached; 20-mm bar-mesh wings; 5-mm bar-mesh cod end) to collect Spot from NRE sites and tidal creeks in 2009 and 2010 and from the Newport River estuary site in 2010. The 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 River estuary sites, 1 and 2 weeks after data sonde deployment. From each trawl sample, 10 randomly selected Spot (when available) were euthanized with an overdose of MS-222, measured, and weighed; a white muscle tissue sample and the whole liver were collected from each selected individual. Tissue and liver samples were treated the same as in the laboratory experiment.

Laboratory Work

Quantification of RNA and DNA from muscle tissue.—The RNA and DNA concentrations in white muscle samples were measured in triplicate by using a one-dye, two-enzyme (ethidium bromide plus RNase and DNase) fluorometric microplate assay in accordance with Caldarone et al. (2001;

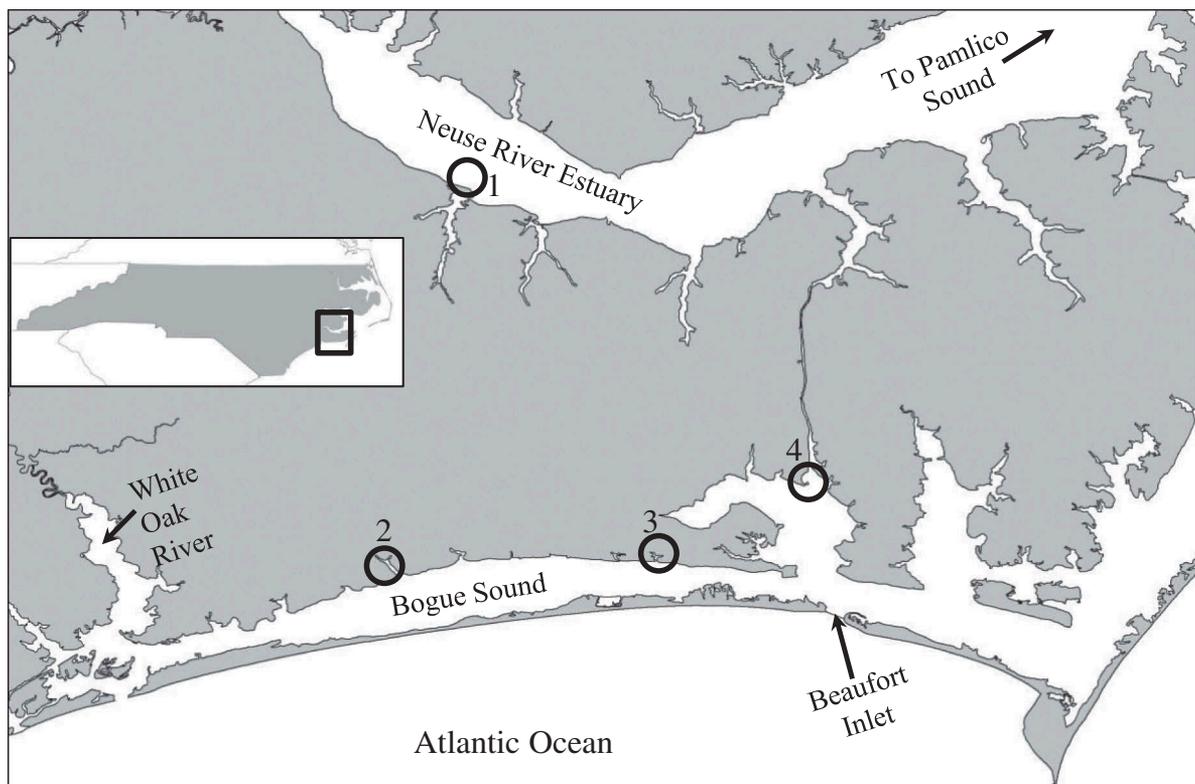


FIGURE 1. Map of study sites along the North Carolina coast; study sites are circled (1 = Neuse River estuary sites; 2 = Broad Creek; 3 = Peletier Creek; 4 = Newport River estuary site). Inset shows North Carolina with the study region outlined.

see the Supplement for a more detailed description). Tissue nucleic acids were expressed as the ratio of RNA:DNA ($\mu\text{g RNA}/\mu\text{g DNA}$), the total RNA concentration per gram of wet tissue weight ($\mu\text{g/g}$), and the total DNA concentration per gram of wet tissue weight ($\mu\text{g/g}$). 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 \pm SE = 0.029 ± 0.001 g; Campbell 2012; 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 sampled from the field.

Expression of the IGF-I gene 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, Ohio), with an added glycogen removal step using Plant RNA Isolation Aid (Ambion; Picha et al. 2008b). For a more in-depth description of steps, see Campbell (2012) and the Supplement.

Liver IGF-I messenger RNA (mRNA) was measured in triplicate by SYBR Green Real-Time PCR (RT-PCR) assay (Qiagen, Valencia, California) using a gene-specific primer pair designed in Applied Biosystems, Inc. (ABI), Primer Express version 3.0 (forward: TGC TGC TTC CAA AGC TGT GA; reverse: TCT TGG CAG GTG CAC AGT ACA; ABI, Foster City, California; for more details, see Campbell 2012 and the Supplement). The RT-PCR analysis was performed using a 20-ng total RNA sample with Brilliant II SYBR Green Quantitative PCR (qPCR) Master Mix (Agilent Technologies, Santa Clara, California) containing 1.5- μM primer concentrations on an ABI 7300 96-Well Detection System. Melting curve analysis denoted a single gene product. Pooled complementary DNA (cDNA) samples were used to account for assays using multiple plates (across-plate normalization), with negative controls run on each plate. Cycle threshold 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/ μL ; $R^2 = 0.99$). To normalize for changing liver sizes, the liver sample IGF-I gene expression mRNA copy number data were normalized to total RNA concentration, liver size, and bw (Bustin 2000; Picha et al. 2008b), where expression of IGF-I mRNA in copy numbers per nanogram of total RNA was used to calculate whole-liver IGF-I copy number and then was 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 per gram of fish bw. 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*), for which expression was not influenced by treatment. Standard curves for *efl* were calculated by a procedure like that used for standard curves of IGF-I, and data were normalized using the relative standard curve method as described for the ABI Prism 7700 Sequence Detection System (User Bulletin Number 2, P/N 4303859). 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 for estimating 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 based on a literature review and based on our own experience of growth dynamics from variables known or suspected to influence the growth of juvenile fish. Model variables included the RNA:DNA ratio, 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 \times RNA concentration, and temperature \times RNA:DNA. We included initial weight of the fish to account for any effect of a fish's place on the size spectrum, but we did not use the final weight of fish, as it would be correlated with the calculated SGR. Day in experiment was included to capture the 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 \times total RNA and temperature \times 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); the combination of RNA concentration, DNA concentration, and RNA:DNA was not 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, the 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 and Anderson 2002) to compare the relative strengths of the candidate models in explaining the growth of Spot in the laboratory experiment. Models were fitted with standard least-squares multiple regression in JMP Pro version 11 (SAS Institute, Inc., Cary, North Carolina). Akaike's information criterion corrected for small sample size (AIC_c) was used to rank candidate models and to select the best predictive model (Burnham and Anderson 1998). The AIC_c was calculated from the residual type I sum of squares of model fits (SS_{resid}) using the least-squares case,

$$AIC_c = n \cdot \log_e \left(\frac{SS_{resid}}{n} \right) + 2K + \frac{2K(K+1)}{n-K-1},$$

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 smaller values indicate better models. Model probability weight (w_i), which indicates the strength of evidence for candidate model i as the best model among those tested, was calculated for each model (Burnham and Anderson 1998).

Field Application

After the best predictive growth model was selected, variables from field-collected fish were input to estimate their recent growth rates. For weight, we used wet weight at the time of capture; 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 and Results) indicated that most of their response to a change in conditions occurred within 1 week. Therefore, we set the value of “day” to 7 for field fish.

Once individual growth rates were estimated, we tested for differences in recent growth in relation to DO conditions from the previous week at the site where the fish was captured. Based on the severity, frequency, and duration of hypoxic events at a fish’s collection site during the week prior to its capture, the DO conditions associated with that fish’s growth rate estimate were assigned a DO classification of “good,” “moderate,” or “poor.” A classification of good indicated DO levels that were above 4.0 mg/L at least 75% of the time, with only one or two short (<3-h) hypoxic events, if any. The moderate classification denoted DO readings above 4.0 mg/L less than 75% of the time but at least 40% between 2 and 4 mg/L and few, if any, short- to medium-duration (<6-h) hypoxic events. Weeks classified as poor had very little, if any, time above 4.0 mg/L DO (typically < 15%); more than 25% of the time below 2 mg/L; and many short to medium hypoxic events, prolonged hypoxic events (>6 h), or both. Differences in estimated growth were compared among DO classifications, locations sampled, and years by using ANOVA ($\alpha = 0.05$). All statistical tests were performed in JMP Pro version 11.

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 for June 15–August 12 of each year from both the nearshore site and the intermediate site, and we assigned a DO classification to each day (based on the percentage of time at various DO levels used in the weekly classification guidelines). We then simulated growth over this period for 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 [L]–weight [W] relationship: $W = 0.00006L^{2.82}$, $n = 1,388$,

$R^2 = 0.97$; see Campbell 2012). 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, moderate, or 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 we also calculated the percent difference in simulated final weight from each year and site relative to the final weight of a fish growing under conditions classified as “good” for all 59 d.

RESULTS

Laboratory Experiment

Our laboratory calibration experiment successfully produced a range of SGRs in Spot, from -2.19% to 2.87% bw/d. A full-factorial ANOVA model was run with SGR as the dependent variable and with temperature, ration group, days in experiment, and their interactions as dependent variables; the only significant factor affecting SGR was ration group (ANOVA, overall: $df = 121$, $P < 0.0001$; ration group: $df = 4$, $P < 0.0001$). As expected, Tukey’s honestly significant difference test identified the SGR in each ration group (all temperatures and days in experiment combined) as being significantly different from the others.

Separate SGR estimates for each week of the experiment showed that in the no-food and low-ration groups, the highest percentage weight loss occurred during the first week. Fish that were in the no-food group had significantly more negative growth during week 1 (measured SGR [mean \pm SE] = $-1.30 \pm 0.21\%$ bw/d) than estimated for subsequent weeks (week 2: estimated SGR = $-0.56 \pm 0.12\%$ bw/d; week 3: estimated SGR = $-0.32 \pm 0.20\%$ bw/d; ANOVA, $df = 26$, $P = 0.0059$). A similar but nonsignificant trend also occurred in the low-ration group (week 1: measured SGR = $-0.58 \pm 0.09\%$ bw/d; week 2: estimated SGR = $-0.20 \pm 0.27\%$ bw/d; week 3: estimated SGR = $-0.01 \pm 0.21\%$ bw/d; ANOVA: $df = 25$, $P = 0.16$). No significant differences or marked trends in growth rate by week were found in groups with positive growth (ANOVA, ad libitum ration: $df = 26$, $P = 0.28$; high ration: $df = 25$, $P = 0.17$; Supplementary Table S.1).

To evaluate potential indicators of recent growth rate, we first ran separate multiple regressions for each bioindicator, with SGR as the dependent variable and with the bioindicator, temperature, and day in experiment as independent variables (see Table S.2 for a summary of trial results). All of the models relating single bioindicators to SGR were significant, but the degree of variation explained varied widely among bioindicators ($R^2 = 0.05$ – 0.81 ; Table 1). The least amount of variation was explained by the positive relationship of $\log_{10}(\text{RNA}:\text{DNA})$ to SGR ($R^2 = 0.05$), which had no other significant main effects or interactions with temperature or day in experiment ($F_{1, 104} = 5.14$, $P = 0.0254$; Table 1; Figure 2). Temperature was a significant positive main effect in the relationship between RNA

TABLE 1. Equations for relationships between Spot specific growth rate (SGR) and single bioindicators (IGF-I = insulin-like growth factor I; Fulton's K = Fulton's condition factor; HSI = hepatosomatic index). In the full model equations, only main effect and interaction terms that were significant are shown. In cases where there were significant interactions, simple individual relationship equations corresponding to the relationships shown in Figure 2 are also presented (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.

Bioindicator	Equation	R^2	n	P -value
RNA:DNA ratio (full model)	$SGR = 0.74 \cdot [\log_{10}(\text{RNA:DNA ratio})] - 0.03$	0.05	105	0.0254
RNA concentration (full model)	$SGR = -5.3 + 0.005 \cdot (\text{RNA concentration}) + 0.11 \cdot (\text{Temperature}) + [\text{day 7} = -0.65, \text{day 14} = 0.26, \text{day 21} = 0.39] + (\text{RNA concentration} - 497.15) \cdot [\text{day 7} = 0.0003, \text{day 14} = 0.0009, \text{day 21} = -0.0012]$ $SGR_{7d}^a = 0.0047 \cdot (\text{RNA concentration}) - 2.78$ $SGR_{14d}^a = 0.0054 \cdot (\text{RNA concentration}) - 2.36$ $SGR_{21d}^a = 0.0034 \cdot (\text{RNA concentration}) - 1.22$	0.75	107	<0.0001
IGF-I liver expression (full model)	$SGR = -1.16 + [\text{day 7} = -0.14, \text{day 14} = 0.27, \text{day 21} = -0.12] + 0.28 \cdot [\log_{10}(\text{IGF-I liver expression})] + [\text{day 7} = 0.72, \text{day 14} = -1.20, \text{day 21} = 0.48] \cdot \{[\log_{10}(\text{IGF-I liver expression})] - 6.92\}$ $SGR_{7d}^a = 0.99 \cdot \log_{10}(\text{IGF-I liver expression}) - 6.31$ $SGR_{14d}^a = -0.93 \cdot \log_{10}(\text{IGF-I liver expression}) + 7.45$ $SGR_{21d}^a = 0.75 \cdot \log_{10}(\text{IGF-I liver expression}) - 4.61$	0.19	69	0.0185
IGF-I expression fold change (full model)	$SGR = -0.73 \cdot (\text{IGF-I expression fold change}) + 1.12$	0.06	78	0.0298
Fulton's K (full model)	$SGR = -5.58 - 0.005 \cdot (\text{Temperature}) + 2.899 \cdot (\text{Fulton's } K) + (\text{Temperature} - 25.98) \cdot [(\text{Fulton's } K - 2.04) \cdot (-0.50)]$ $SGR_{24^\circ C}^b = 3.89(\text{Fulton's } K \text{ at } 24^\circ C) - 7.74$ $SGR_{28^\circ C}^b = 1.89(\text{Fulton's } K \text{ at } 24^\circ C) - 3.67$	0.23	121	<0.0001
HSI (full model)	$SGR = -0.81 - 0.04 \cdot (\text{Temperature}) + [\text{day 7} = -0.28, \text{day 14} = 0.16, \text{day 21} = 0.13] + 3.10 \cdot (\text{HSI}) + (\text{Temperature} - 25.95) \cdot [(\text{HSI} - 0.63) \cdot 0.36]$ $SGR_{24^\circ C}^b = 2.299 \cdot (\text{HSI at } 24^\circ C) - 1.16$ $SGR_{28^\circ C}^b = 3.699 \cdot (\text{HSI at } 28^\circ C) - 2.20$	0.59	119	<0.0001

^a Equations for total RNA concentration or \log_{10} (IGF-I liver expression) grouped by day sampled (both temperatures combined) with no interactions are presented for illustrative purposes only (see Figure 2).

^b Equations for Fulton's K and HSI grouped by temperature (all days sampled combined) with no interactions are presented for illustrative purposes only (see Figure 2).

concentration and SGR, and the interaction of RNA \times day in experiment was also significant (interaction: $F_{2, 106} = 6.15$, $P = 0.003$; Table 1; Figure 2). This bioindicator is the only one that could have been affected by the absence of individual tissue sample weights from the laboratory experiment. Had individual weights been available, the RNA concentration might have explained even more variation in SGR, but even with this loss of resolution it was the strongest predictor of SGR ($R^2 = 0.75$) among the bioindicators we evaluated. The positive relationship between SGR and \log_{10} (IGF-I liver expression) had low explanatory value ($R^2 = 0.19$; Table 1; Figure 2) and included an interaction with day in experiment (interaction: $F_{2, 68} = 3.72$, $P = 0.0298$); surprisingly, the relationship at 14 d was negative, whereas the relationships at 7 and 21 d were positive. The IGF-I expression fold change had a significant negative relationship with SGR (with no other significant main effects or interactions; Table 1; Figure 2) that seemed contradictory, but it is not,

because fold change values are relative to the average values of fish fed ad libitum, so a higher fold change corresponds with lower mRNA expression values at lower SGR. The positive relationship between Fulton's K and SGR had low explanatory power ($R^2 = 0.23$) and included an interaction with temperature (interaction: $F_{1, 120} = 4.17$, $P = 0.0434$; Table 1; Figure 2). A significant interaction was found between HSI and temperature (interaction: $F_{1, 118} = 8.85$, $P = 0.0036$) with day in experiment as a significant main effect covariate, resulting in a positive relationship between HSI and SGR that generated a fairly high explanatory value ($R^2 = 0.59$).

Model Selection

Candidate models to explain SGR using combinations of bioindicators and environmental factors explained up to 80% of the variability in SGR from the laboratory calibration trials (Table 2). Models with combinations of bioindicators performed better than

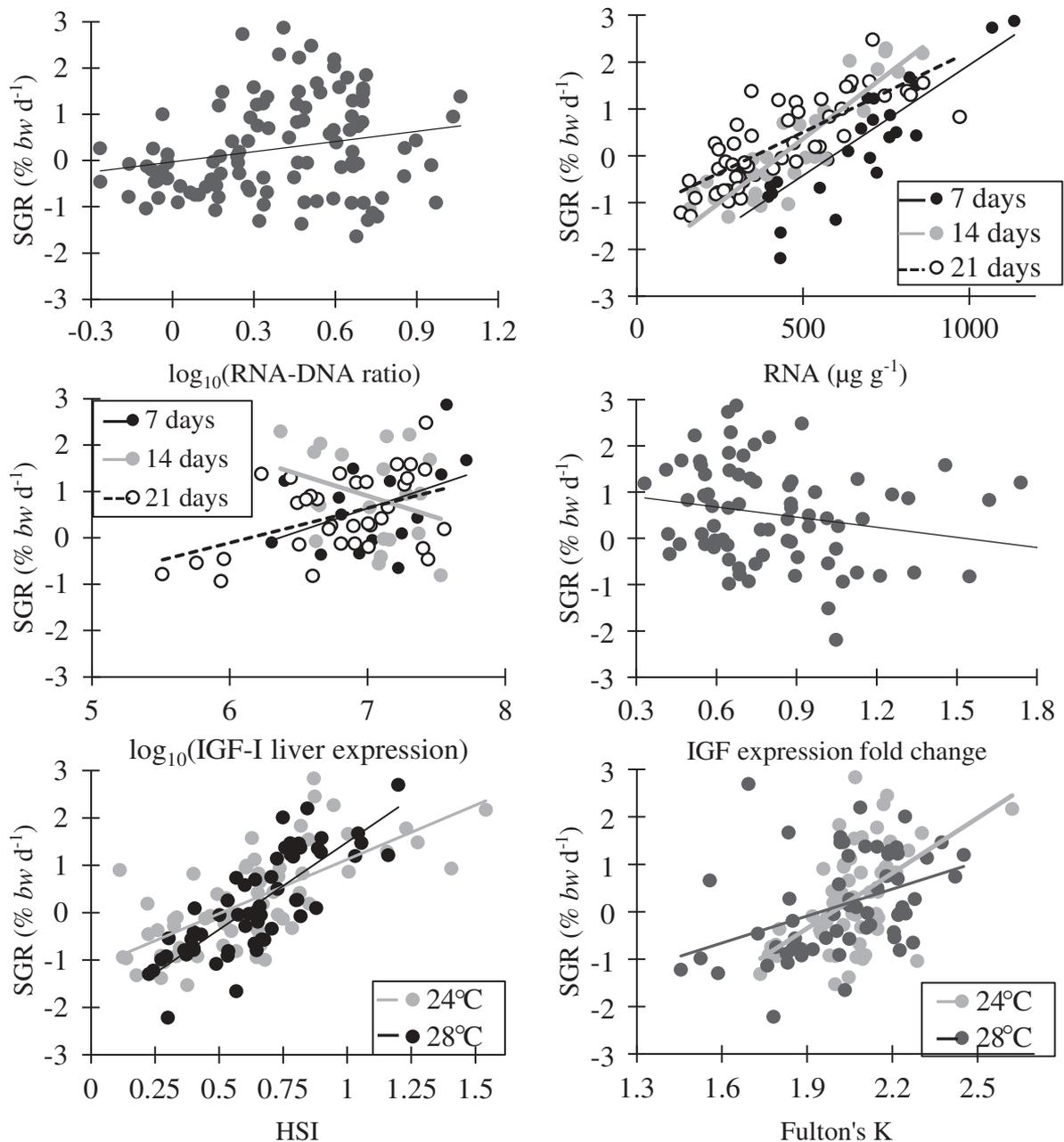


FIGURE 2. Relationships between each bioindicator and specific growth rate (SGR; % body weight [bw]/d) of Spot from the laboratory calibration experiment (RNA = RNA concentration; IGF-I = insulin-like growth factor I; HSI = hepatosomatic index; Fulton's K = Fulton's condition factor). In cases of significant interactions (RNA \times day in experiment; \log_{10} [IGF-I liver expression] \times day in experiment; HSI \times temperature; or Fulton's K \times temperature), relationship lines are shown for illustrative purposes only. See Table 1 for regression equations and R^2 values.

models with single bioindicators and environmental variables, as none of the latter models had any strength of evidence (based on w_i ; Table 2). Eleven of our 37 candidate models had some weight (w_i), with all of those models including HSI, RNA concentration, temperature, and day in experiment. The top-three models together accounted for half of the total weight ($0.501w_i$), with each having similar strengths of evidence ($w_i = 0.182, 0.176, \text{ and } 0.142$;

Table 2). Because there were only minor differences among them and all had nearly identical R^2 values, we decided to use the top-ranked model selected by AIC_c instead of using a model-averaging approach (Burnham and Anderson 2002). Models with single bioindicators did not carry any weight in our model set and were well above an AIC_c difference (ΔAIC_c) value of 10 (including the model based on RNA concentration, despite its high R^2 value),

TABLE 2. Least-squares multivariate regression candidate models developed to predict the specific growth rate (SGR) of Spot based on bioindicators and environmental variables (see Methods) and their R^2 values, ordered by Akaike's information criterion (AIC_c ; K = number of parameters in the model; ΔAIC_c = difference in AIC_c between each model and the best-performing model; w_i = model probability weight; HSI = hepatosomatic index; Fulton's K = Fulton's condition factor; RNA:DNA = ratio of RNA to DNA). The top three models are indicated in bold italics. Only models that had any model probability weight are listed in the table.

Model	R^2	N	K	AIC_c	ΔAIC_c	w_i
<i>HSI, RNA concentration, Temperature, Fulton's K, Day</i>	0.80	105	7	-143.52	0.00	0.182
<i>HSI, RNA concentration, DNA concentration, Temperature, Fulton's K, Day</i>	0.80	103	8	-143.46	0.07	0.176
<i>HSI, RNA concentration, DNA concentration, Temperature, Fulton's K, Day, Initial weight</i>	0.81	103	9	-143.03	0.50	0.142
HSI, RNA concentration, DNA concentration, Temperature, Initial weight, Day	0.80	103	8	-141.76	1.77	0.075
HSI, RNA concentration, Temperature, Fulton's K , Day, RNA concentration \times Temperature	0.80	105	8	-141.74	1.79	0.075
HSI, RNA concentration, DNA concentration, Temperature, Fulton's K , Day, RNA concentration \times Temperature	0.80	103	9	-141.58	1.94	0.069
HSI, RNA concentration, \log_{10} (RNA:DNA), Temperature, Fulton's K , Day	0.80	102	8	-141.56	1.96	0.068
HSI, RNA concentration, Temperature, Day	0.79	105	6	-141.55	1.98	0.068
HSI, RNA concentration, Temperature, Initial weight, Day	0.80	105	7	-141.46	2.06	0.065
HSI, RNA concentration, DNA concentration, Temperature, Fulton's K , Day, Initial weight, RNA concentration \times Temperature	0.81	103	10	-140.83	2.70	0.047
HSI, RNA concentration, \log_{10} (RNA:DNA), Temperature, Initial weight, Day	0.80	102	8	-140.01	3.51	0.031

denoting essentially no support for these models (Burnham and Anderson 2002). The equation for the top model to estimate SGR based on the results of our laboratory calibration experiment is as follows:

$$\begin{aligned} \text{SGR}(\% \text{bw/d}) = & -5.2469 + (1.2681 \times \text{HSI}) + \\ & (0.0032 \times \text{RNA, } \mu\text{g/g}) \\ & + (0.0661 \times \text{Temperature, } ^\circ\text{C}) \\ & + (0.6177 \times \text{Fulton's } K) \\ & + [\text{day 7} = -0.5160, \text{day 14} = 0.1657, \\ & \text{day 21} = 0.3503]. \end{aligned}$$

This model provides a strong, unbiased description of the growth rates observed in our calibration experiment; the regression of observed growth rates on estimated growth rates explained 80% of the variability, with a slope of 1.0 and an intercept virtually indistinguishable from zero (Figure 3).

Field Application

Once we had determined the best model for estimating recent growth rate based on the bioindicators we measured in the laboratory, we applied that model to estimate recent growth of Spot collected in the field under various DO conditions. Because the response of growth rate to a change in conditions was most marked during the first week for fish experiencing a shift to negative growth and because the

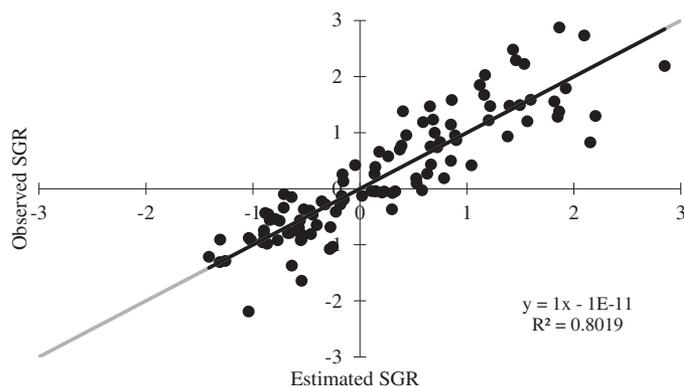


FIGURE 3. Relationship between observed specific growth rates (SGRs; % body weight/d) of Spot in the calibration experiment and corresponding SGRs estimated by the best model identified using Akaike's information criterion corrected for small sample size. The 1:1 line is shown in gray; the fitted line is shown in black.

response did not differ among weeks for fish experiencing positive growth (see above and Campbell 2012), we set "day" to 7 for model applications to field-sampled fish. We then paired the estimated SGR values of individual fish with the DO classification and average temperature 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. In 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, which were selected based on the previous week's DO conditions to represent all DO categories and sites as well as a range of dates throughout the season. Bioindicator values from field fish were within the range of data used to build the model (HSI was 100% within the range, Fulton's K was 100% within the range, and RNA concentration [$\mu\text{g/g}$] was 87% within the range; see Table S.3 for a summary of field results).

In the NRE, we found that higher estimated growth was associated with increasingly better DO conditions (ANOVA: $F_{2, 75} = 6.4669$, $P = 0.0026$; Figure 4). Year sampled also had a significant effect on estimated growth (ANOVA: $F_{1, 75} = 16.7040$, $P = 0.001$), but there was no interaction between DO classification and year (ANOVA: $F_{2, 75} = 2.7702$, $P = 0.0695$; overall ANOVA: $df = 75$, $P = 0.0001$; Figure 4). Average estimated SGR (both years combined) for fish collected after a week with DO conditions classified as good (1.38% bw/d) was 18% greater than that of fish collected after a week with DO classified as moderate (1.17% bw/d) and was 75% greater than that of fish collected after a week of poor DO conditions (0.79% bw/d; Figure 4). Estimates of growth rate for all fish collected in the NRE during 2009 (all DO classifications combined) were significantly higher (1.43% bw/d) than for all fish collected during 2010 (0.93% bw/d; t -test: $df = 75$, $P = 0.0051$), but this difference did not affect the trend of declining estimated growth with declining DO conditions. In both years, the SGR estimated for fish collected after good DO conditions was significantly greater than that estimated for fish collected after a week of DO conditions classified as

poor (t -test, 2009: $df = 32$, $P = 0.0156$; 2010: $df = 32$, $P = 0.0052$).

The Newport River estuary exhibited good DO ratings for all periods monitored in 2010, with an average estimated growth rate of 1.49% bw/d for all fish collected there (Figure 4). This growth rate was slightly higher than—but not significantly different from—the estimated SGR of only those fish collected in the NRE after weeks with a good DO classification in 2010 (t -test: $df = 33$, $P = 0.2968$).

Spot from both tidal creeks had variable growth rates by season and year despite good to moderate DO ratings, but they exhibited overall better estimated growth rates than fish from the NRE (Figure 5). The estimated SGR for Peletier Creek Spot (2.43% bw/d) was higher than that of NRE fish after good DO weeks (1.46% bw/d; ANOVA, $df = 72$, $P = 0.0113$), whereas the SGR for fish in Broad Creek was similar to both (1.92% bw/d; post hoc Tukey's honestly significant difference test). There was no difference in growth rate between the two tidal creeks in either year (t -test, 2009: $df = 15$, $P = 0.0684$; 2010: $df = 11$, $P = 0.7243$).

Simulated growth rates based on daily DO quality ratings for the NRE across years ranged from 6% to 18% less than the simulated growth rate assuming constant good DO conditions in the NRE (Table 3). As expected, growth was less negatively affected at the NRE 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 Spot at the nearshore site were similar among years but were still 6–9% lower than growth estimated using a steady state of good DO conditions (Table 3). The simulated

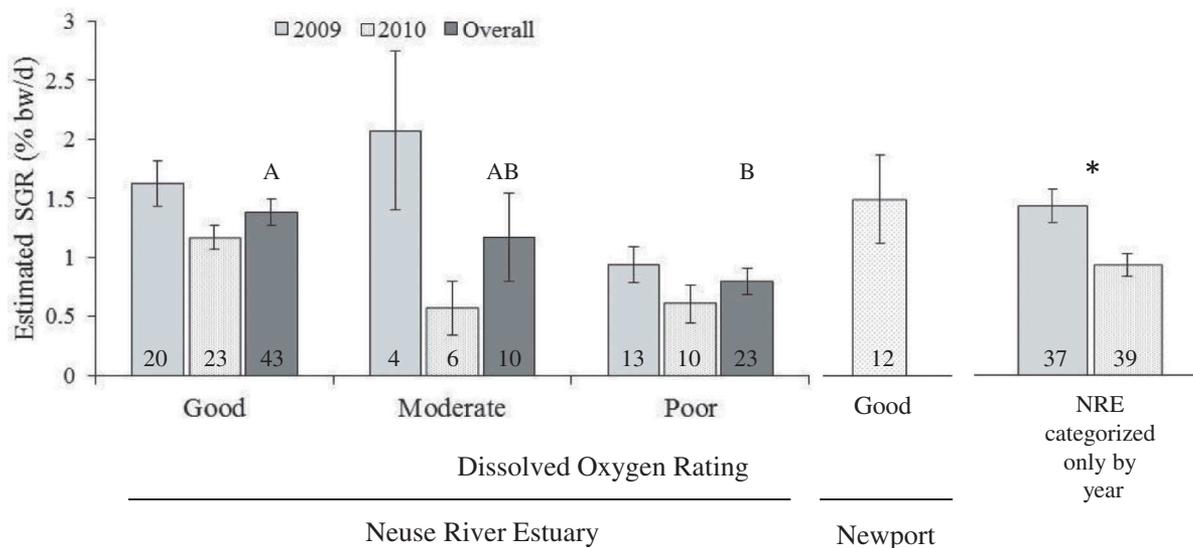


FIGURE 4. Mean (\pm SE) estimated specific growth rate (SGR; % body weight [bw]/d) of field-caught Spot presented in relation to dissolved oxygen (DO) classification for the Neuse River estuary (NRE) in 2009 and 2010 and the Newport River estuary in 2010. Estimated SGRs for NRE by year only (denoted by the asterisk) are presented for illustrative purposes. Numbers in bars are the n -values. The ANOVA tests identified significant main effects of DO classification and year but not an interaction. Letters denote results of Tukey's honestly significant difference test for comparisons among DO classifications in the NRE for both years combined.

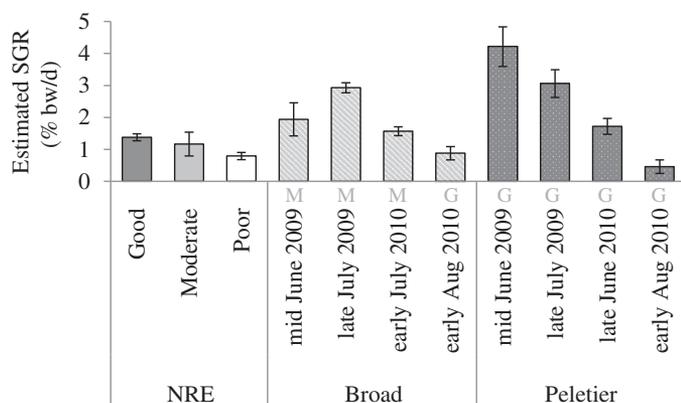


FIGURE 5. Mean (\pm SE) estimated specific growth rate (SGR; % body weight [bw]/d) for Spot collected from two tidal creeks (Broad and Peletier creeks) in comparison to the estimated SGR of fish from the Neuse River estuary (NRE). Values shown for the NRE are for fish collected in 2009 and 2010 (combined) after weeks of good, moderate, and poor dissolved oxygen (DO) conditions. For each creek sample, the DO classification for the week preceding fish collection is indicated in gray letters below the bar (G = good DO rating; M = moderate DO rating).

growth of a fish at the Newport River estuary site (based on the estimated growth rate from fish sampled in 2010) was higher than in any of the NRE simulations and resulted in an end weight that was 7% 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 (HSI, RNA concentration, and Fulton's K , along with temperature and day) and accounted for 80% of the variability in recent growth rate of fish in our laboratory trials, giving us a powerful tool for estimating the recent growth of juvenile Spot in the field. The best model using just one bioindicator (RNA concentration, along with temperature, day, and the RNA concentration \times day interaction) explained only 5% less of the variability in growth rate; thus, one might suspect that the advantages of using this simpler model would outweigh the 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; HSI and Fulton's K require only simple weight and length measurements.

We expected the RNA:DNA ratio to be a strong predictor of recent growth since it has been so in numerous other studies (e.g., Malloy and Targett 1994; Buckley et al. 1999; Caldarone et al. 2003; Stierhoff et al. 2009a, 2009b). 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 the 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 the RNA:DNA ratio in other studies as well (Lied and Rosenlund 1984; Mathers et al. 1992, 1993; Foster et al. 1993). The strong relationship between RNA concentration and recent growth, both alone and in our next-best models, was somewhat surprising given that we did not have individual tissue sample weights for fish from the calibration experiment; if those values had been available, the 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 surprising that temperature was also a significant component, since many RNA-based indices of growth rate are temperature dependent (Buckley et al. 1999).

Although 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. Interestingly, Fulton's K also was a significant component of the best model, despite the fact that it is usually associated with long-term rather than short-term growth (Stevenson and Woods 2006; Caldarone et al. 2012). This metric gives a general indication of plumpness, or weight relative to length, which might be the reason it added value to our model estimating growth in weight. Many studies measure growth rates via change in the length of the fish; however, over short time periods like those examined in this study, it is more accurate to measure growth rate via the change in weight rather than the change in 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 from studies on the role of IGF-I in the direct control of somatic growth (Le Bail et al. 1998; Beckman et al. 2004; Picha et al. 2008a; Beckman 2011). 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 than IGF-I liver expression in estimating recent growth rates (Campbell 2012). Although we expected a positive correlation between IGF-I liver expression and IGF-I plasma levels (Beckman 2011), Uchida et al. (2003) did not find a relationship between the two, and limited information available from the one study with juvenile Spot also did not suggest a clear relationship between them (Campbell 2012). In the findings presented here, IGF-I mRNA expression accounted for little of the

TABLE 3. Simulated growth values for a juvenile Spot (starting at 6.9 g and 63 mm SL) over the period June 15–August 12 based on recorded dissolved oxygen (DO) values for the two Neuse River estuary (NRE) sites (nearshore and intermediate) during 2007–2010 and for the Newport River estuary during 2010. For each site × year combination, the number of days categorized as having good, moderate, or poor DO conditions is given, along with the end weight and length of the simulated fish, its average specific growth rate (SGR; % body weight [bw]/d), 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 during 2009–2010. Values for the simulation of growth at constant good DO conditions are in bold italics; estimated fish end weights from each of the other simulations are compared with the estimated end weight from this simulation.

Year	DO condition or site	Number of days at each DO rating			Simulated			Average SGR (% bw/d)	Average growth rate in length (mm/d)
		Good	Moderate	Poor	End weight (g)	End SL (mm)	Weight difference from constant good DO conditions		
Simulations based on assumed constant DO conditions									
	<i>Good</i>	<i>59</i>			<i>15.5</i>	<i>84</i>		<i>1.38</i>	<i>0.35</i>
	Moderate		59		13.7	80	–12%	1.17	0.29
	Poor			59	11.0	74	–29%	0.79	0.19
Simulations based on actual DO conditions in the NRE									
2007	Nearshore	35	12	12	14.1	81	–9%	1.21	0.31
	Intermediate	29	9	21	13.5	79	–13%	1.13	0.28
2008	Nearshore	32	25	2	14.5	82	–6%	1.26	0.32
	Intermediate	14	29	16	13.3	79	–14%	1.11	0.28
2009	Nearshore	27	25	7	14.1	81	–9%	1.21	0.31
	Intermediate	19	23	17	13.4	79	–14%	1.12	0.28
2010	Nearshore	39	12	8	14.4	81	–7%	1.25	0.32
	Intermediate	12	19	28	12.7	78	–18%	1.03	0.26
Simulation based on Newport River estuary conditions									
2010	Newport River estuary				16.5	85	+7%	1.49	0.39

variability in observed SGR of juvenile Spot. The difference between this study and previous research may be that the prior studies mainly compared IGF-I levels between fish that were starved or fed ad libitum. A similar comparison of fish starved and fed ad libitum in our study also revealed a significant difference in IGF-I mRNA expression (t -test: $df = 27$, $P < 0.001$), but the relationship across the full spectrum of growth rates was weak. It is unclear whether IGF-I's inability to discriminate smaller differences in growth rates 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 fish that were fed high rations and fish that were starved or fed low rations (Beckman 2011). Fukada et al. (2012) found that IGF-I gene expression in white muscle tissue showed a better response to somatic growth and nutrition status in the 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.

None of the indicators we tested, particularly the RNA-based indicators (RNA:DNA and RNA concentration), responded as quickly as expected to a change in growth conditions. Other authors have observed faster responses in other species (Clemmesen and Doan 1996; Rooker and Holt 1996; Stierhoff et al. 2009a), but based on our laboratory calibration, 7 d constituted the shortest time frame that we could reliably use to relate recent growth rate to environmental conditions (see also Campbell 2012). 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 3 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 detected marked differences associated with DO conditions over the previous week; growth rates associated with good DO conditions were 75% greater than 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 that 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 estimated effects of recent DO conditions on Spot growth in the present study are likely conservative. Given their strong hypoxia avoidance behavior (Wannamaker and Rice 2000; Campbell and Rice 2014), 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. However, although Spot will move away from hypoxia, they are likely to stay in the general area rather than emigrate. Weinstein (1983) and Weinstein et al. (1984) found that Spot belonging to a size range (45.3–97.3 mm SL) similar to that of 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 d (from each study, respectively), which suggests that the responses of Spot in our study could be linked to recent environmental conditions where they were collected. Furthermore, other environmental variables affect 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 the growth of juvenile Spot in the field and that avoidance behavior cannot fully alleviate both the direct and indirect effects of hypoxia on growth.

The detected negative effects of hypoxia on growth rate may be associated with the highly dynamic nature of hypoxia in the NRE, in contrast to studies that have not found a negative effect on growth in areas 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. Avoidance behavior may increase activity costs and interfere with foraging, and crowding in oxygenated refuges can result in density-dependent reductions in growth and survival (Eby et al. 2005; Craig et al. 2007; Campbell and Rice 2014).

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 by 6–18% relative to growth expected under constant good DO conditions. However, these results may be conservative given that other variables could affect growth regardless of DO. The NRE intermediate-depth site was consistently predicted to support lower growth based on DO dynamics, but fish keep returning to this area (see Campbell and Rice 2014), so the site must offer some other

benefit that outweighs the cost. Nearshore areas were better for growth based on DO levels, but they still were impacted by hypoxia and had lower growth rates than those 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 (Campbell and Rice 2014). That analysis estimated that density-dependent effects resulting from hypoxia avoidance behavior decreased the cumulative seasonal growth in 2007 by approximately 4% (Campbell and Rice 2014). However, our simulation presented here for the nearshore site estimated that the overall effects of hypoxia reduced growth by 9% in 2007, demonstrating that density-dependent effects constitute only one component of the total effect of hypoxia dynamics on fish. This comparison emphasizes that although 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 assume 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 in 2010, could be due to abiotic or biotic factors in addition to DO conditions. Since growth integrates everything the fish experiences, other factors (e.g., food quality, food availability, or fish density, among others) may have an overriding influence. We did not measure the density of fish during our sampling in 2009 and 2010, but density could affect overall growth rates (Campbell and Rice 2014). A reduced food base could have been another effect of underlying DO dynamics. There were more hypoxic events recorded by our data sondes in 2010 than in 2009, so the slower growth we estimated in 2010 could be partly attributable to the cumulative effects of hypoxia exposure on prey availability and not just to conditions that were present 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 DO versus poor DO classification. Growth rates associated with the moderate DO classification were much more variable; this could be due to low sample size or to the way we defined the moderate classification, which 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 differences in anthropogenic impacts on 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 differences in their surrounding habitat characteristics: Broad Creek had a less-impacted habitat with marsh areas and minimal anthropogenic alterations, whereas Peletier Creek

had been channelized, was characterized by extensive bulkheads and little natural shoreline, and contained two boat marinas. Thus, we expected Peletier Creek to have poorer DO conditions than Broad Creek, but that was not the case. Broad Creek had moderate DO, mainly due to the diel cycling of oxygen that dipped during early morning hours in the shallow creek. Despite all the anthropogenic factors with a potential to 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 within Peletier Creek most likely was driven by factors that 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).

Although the Newport River 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 River estuary was even better than growth of fish in the NRE that had recently experienced good DO conditions. Collectively, this suggests that the Newport River estuary provides a better habitat for growth than the NRE, perhaps at least in part due to better DO conditions.

The present study demonstrated the value of incorporating physiological, temporal, and environmental data into predictive growth models to more accurately depict how temporally dynamic hypoxia affects growth in juvenile Spot, a representative estuary-dependent species. 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.

Although 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 level. We were able to demonstrate that an increased frequency and severity of hypoxia reduced the estimated growth rates of juvenile Spot in their estuarine nursery habitat. Despite variation from year to year, our results suggest that recent levels of hypoxia are having a negative effect on the 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 and Rosenberg 2008; Breitburg et al. 2009). While enhanced productivity in the NRE may help fish to 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.

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