Development of intensive indoor rearing strategies for largemouth bass (*Micropterus salmoides*) during critical early life history stages

by

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ABSTRACT

Largemouth bass (LMB), *Micropterus salmoides*, is arguably the most sought-after recreational fish in the United States. Recently, there has been rising interest in LMB as a food fish and current production techniques produce marginal results. Thus, this thesis aims to develop intensive indoor RAS techniques to improve hatchery production efficiency. Specific objectives were to: i) identify the temperature (21°C, 24°C, and 27°C) and subspecies (Florida vs. Northern LMB) that maximizes hatchery production efficiency, and ii) optimize first-feeding to fingerling dietary regimes using different live feeds. Results suggest that rearing LMB at 27°C typically improves growth performance during early ontogeny, and Northern LMB can be selected for faster growth when reared in an indoor RAS. Moreover, LMB larvae fed rotifers exhibited a significant increase in morphometric development and yolk characteristics. In conclusion, this thesis furthered our understanding of biological responses, limits, and adaptabilities or preferences to an extrinsic environmental factor (temperature) and different live feeding regimens.

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Chapter 1

Effects of temperature and subspecies during critical early life history stages of largemouth bass (*Micropterus salmoides*)

Abstract

Largemouth bass (LMB), *Micropterus salmoides*, is the most popular sportfish in the United States, with an expanding global food market. Farmers traditionally raise LMB in earthen ponds; however, they are plagued with high mortality during the early life history stages. Replacing initial pond stages with intensive indoor culture would streamline production and minimize losses. Our objectives were to (i) identify an optimal thermal regime for rearing LMB in an indoor recirculation aquaculture system (RAS), (ii) assess the performance of Florida vs. Northern LMB for RAS culture, and (iii) elucidate thermally induced phenotypic changes and inter-linked expression of targeted genes involved in early development. Using RAS technology, Florida and Northern LMB were raised at 21°C, 24°C, and 27°C. Fish were randomly sampled at 2 to 28 days post-hatch (dph) for total length (TL), body area (BA), myotome height (MH), jaw length (JL), eye diameter (ED), oil droplet area (ODA), and yolk area (YA). Condition index, yolk utilization efficiency (YUE), and yolk utilization rate (YUR) were calculated. Wet weight was taken at 29 dph with survival and expression of targeted genes [growth hormone (*gh*), insulin-like growth factor 1 (*igf1*), heat shock protein 70 (*hsp70*)] determined at 8 and 28 dph. There was a temperature effect for all morphometric traits, where both subspecies increased in size over the

temperature gradient, with the largest traits (TL, BA, MH, JL, ED) detected at 27°C. Larvae had smaller remaining ODA and YA reserves as temperatures increased. Larvae reared at 27°C utilized their yolk at faster rates, but were most efficient at converting yolk reserves to body size. Northern LMB had higher YUE than Florida LMB and were typically larger and heavier at 29 dph. LMB reared at 21°C had higher survival than those at 24 or 27°C, while Northern LMB had higher survival than Florida LMB. For Florida LMB, no differences in *gh* and *igf*1 were detected across temperatures at 8 dph. However, at 28 dph, these genes were upregulated at 27°C, while expression of *hsp70* was downregulated at 24 and 27°C. Northern LMB showed similar expression patterns, with no significant differences detected. In conclusion, the present study results suggest that 1) rearing LMB at 27°C typically improves growth performance during early ontogeny, and 2) Northern LMB can be selected for faster growth when reared in an indoor RAS.

1.0. Introduction

Largemouth bass (LMB), *Micropterus salmoides*, is the most widely distributed black bass in the United States, inhabiting an area of 3,297,900 km^2 (MacCrimmon and Robbins, 1975; Lee et al., 1980). The United States currently has ~30 million anglers who target bass, linked to an estimated $60 billion industry (Tidwell et al., 2019). Historically, bass aquaculture has focused on juvenile LMB production for reservoir and pond stockings as a sport fish (Tidwell et al., 2019). However, recently, there has been an increased demand for bass production as a food fish.

Propagation of LMB began over a century ago (Worth, 1895), and since then, this species has been stocked in countries worldwide (Tidwell et al., 2019). Aquaculture of LMB is especially a large industry in Asia (Bai et al., 2009; Bai and Li, 2013). For instance, LMB culture is a thriving food-fish industry in China, where production increased nearly 25% from 125,000 metric tons

(MT) in 2007 to 152,200 MT in 2013 (Zhou and Liu 2019). To put these numbers into perspective, the United States catfish industry, the largest aquaculture industry in the United States, produced 159,421 MT of food size or marketable fish in 2018 (USDA 2019), which accounted for 70% of all United States aquaculture production. Seventy-one commercial farms in the United States recently produced 1,889 tons of market-size LMB with an estimated value of $5.5 million (USDA, 2019). Largemouth bass ranked fifth in food fish production in the United States, behind catfish, trout, tilapia, and hybrid striped bass (USDA, 2019). Given the growing demand and market potential for this species, improving rearing conditions and applying new aquaculture technologies would provide a tremendous opportunity to supply the needs of LMB worldwide (Waite et al., 2014).

LMB production has historically occurred in earthen ponds (Snow, 1968), where farmers have relied on natural reproduction from broodstock (Tidwell et al., 1998). Earthen ponds have been considered economically viable and provide space for large-scale production but are susceptible to natural disturbances (i.e., thermal fluctuations, predation, water quality issues, disease; Park et al., 2015) and require large amounts of land and water (Watts et al., 2016). Another major downfall to this traditional production is the increased handling stress caused by the frequent fish transfer between ponds and indoor facilities. Production of LMB is separated into five phases: the hatching phase, nursery phase, feed-training phase, then first-, and second-year growth (Tidwell et al., 2019). Typically, spawning mats are placed in an earthen pond and broodstock are allowed to spawn naturally. Mats containing eggs are removed and placed into an indoor facility where larvae hatch (Matthews and Stout, 2013). Once the yolk-sac has been absorbed, larvae are transferred to earthen ponds to feed on zooplankton until fingerlings reach ~5 to 8 cm, where they are transferred again to an indoor facility for feed training (Tidwell et al., 2019). Once fingerlings

are conditioned to pelleted feed, they are transferred back to earthen ponds for grow-out (Quintero et al., 2019). These numerous transfers cause stress to the fish and reduced handling would help streamline production. Notably, one of the major advantages of indoor RAS rearing is controlled manipulation of biophysical conditions. Still, despite the promise, a 2013 USDA census found that only 8.7% of the total value of United States aquaculture production was raised in RAS, while 35.8% was produced in earthen ponds (USDA, 2014). Thus, developing RAS techniques for food fish, such as LMB, could increase per unit area production efficiency, while reducing water needs (Timmons et al., 2012).

Fish have shown differential growth and survival during early life stages as a result of differences in biophysical conditions, such as water temperature (Landsman et al., 2011; Myers et al., 2020), photoperiod (Zhang et al., 2019), and salinity (Politis et al., 2018). Temperature is arguably the most important abiotic factor for fish, as it has been shown to influence the time of hatching, developmental rate, somatic growth, metabolic activity, presence of deformities, cellular function, swimming performance, and predator avoidance (Strawn 1961; Leggett et al., 1984; Pepin, 1991; Blaxter, 1991; Somero and Hofmann, 1997; Landsman et al., 2011; Politis et al., 2017). Growth rate of bass has been shown to be temperature dependent and a major factor for cannibalism and predator avoidance (Coutant and DeAngelis, 1983). However, there is a lack of published literature investigating optimal indoor environmental conditions for LMB during early life history stages (i.e., yolk-sac larvae to early juveniles). Understanding these early developmental needs of LMB would benefit the aquaculture industry for food and stocking programs for recreational fisheries.

Genetic variants also need to be considered for indoor RAS culture. Northern LMB (*M. salmoides salmoides*) and Florida LMB (*M. salmoides floridanus*) are two LMB subspecies that have been explored for aquaculture production. Fishery biologists have been researching differences (Inman et al., 1977) between Florida and Northern bass subspecies since their identification by Baily and Hubbs (1949). However, previous studies have focused on fishery management needs, utilizing earthen ponds or static water baths (Strawn, 1961; Zolczynski and Davies, 1976; Isely et al., 1987; Kleinsasser et al., 1990). Thus, there are gaps in our understanding on how LMB subspecies perform and develop in intensive indoor RAS units.

Given the present limitations of the LMB industry, the objectives of this study were to improve LMB hatchery production efficiency by: (i) identifying the optimal thermal regime (21°C, 24°C, and 27°C ± 0.2°C) for intensive rearing of LMB in an indoor RAS; (ii) assessing the performance of Florida and Northern LMB in RAS culture; and (iii) determining if one of the subspecies is more suitable for indoor aquaculture production by elucidating thermally induced phenotypic changes and the interlinked expression of targeted genes (*gh, igf, hsp70*) involved in early life development. Together, this research represents a step forward toward improving rearing conditions for an essential aquaculture species of worldwide importance.

1.1. Materials and Methods

1.1.1. Animal care

Protocols for fish experimentation complied with the Animal Care and Use Program of Auburn University (IACUC# 2020-3772).

1.1.2. Broodstock facility

Florida and Northern LMB broodstock were raised at Red Hills Fishery in Boston, Georgia, USA (30.8478ºN, -83.7606ºW). Florida LMB broodstock were fed goldfish and the Northern LMB were fed a combination of goldfish and a broodstock diet (Richloam Bass Fry #14; 64% fish meal, 13% corn gluten meal, 2% fish oils, 1% vitamin supplements) at ~2% body weight per day. All fish were fed to satiation. Each subspecies was raised in a concrete raceway (27 m × 3 m × 1 m) with a flow rate of 340 L/min. To initiate spawning, fish were reared on a 10 h photoperiod the first four weeks, then 8 h photoperiod for 3 to 4 weeks, then back up to 10 h photoperiod for two weeks, and finally 14 h for the last two weeks. Spawning mats (Spawntex, Pentair Aquatic Eco-Systems, Apopka, FL, USA) were evenly distributed along the length of the raceway. The Northern subspecies had 34 males and 40 females, with length and weight ranging from 321 to 584 mm and 0.48 to 3.97 kg, respectively. Meanwhile, the Florida subspecies had 74 males and 56 females, with length and weight ranging from 330 to 481 mm and 0.56 to 1.99 kg, respectively. During the spawning season (1 Sept 2020 to 27 Oct 2020), water temperature and dissolved oxygen (DO) in the raceways ranged from 19.5 to 23.0°C and 8.15 to 10.23 mg/L, respectively for Florida LMB and 19.4 to 22.7°C and 10.17 to 12.25 mg/L, respectively for Northern LMB. Mean nitrite was 0.019 mg/L, mean nitrate was 0.8 mg/L, and mean total ammonia nitrogen (TAN) was 0.07 mg/L at embryo collection.

1.1.3. Embryo collection and rearing

Spawning mats were checked twice daily (08:00 and 17:00). Once eggs/embryos were detected they were transported to the Auburn University E.W. Shell Fisheries Center (32.6526ºN, -85.4859ºW) in 114 L coolers (Coleman, Chicago, IL, USA) with 40 L of raceway water. Florida

LMB embryos (represented by six spawning mats) were transported on 25 Sept. 2020, and Northern LMB embryos (represented by ten spawning mats) were transported on 5 Oct. 2020.

Upon arrival, the eggs/embryos were immediately suspended 15 cm below the water surface in 75 L black aquaria, where they were incubated at ~21°C until hatched. Aquaria were equipped with RAS technology, containing a UV filter (Emperor smart DC2305, Pentair Aquatic Eco-Systems, Apopka, FL, USA), bead filtration system (Bubble Bead Filter XS10000, Aquaculture Systems Technology, Baton Rouge, LA, USA), bag filter (Pall x-100, Pall Corporation, Port Washington, New York, USA), 0.5 hp pump (PerformancePro Cascade, Cascade Pump Company, Santa Fe Springs, CA, USA), 17 × 75 L aquaria, three 795 L circular blue tanks, two 190 L sump tanks, and chiller (AquaLogic Delta Star DS-9, Aqua Logic Inc, San Diego, CA, USA), heat-pump (AquaLogic Delta Star DSHP-9, Aqua Logic Inc, San Diego, CA, USA), or in-line heater (AquaLogic Titanium Evo Z31E, Aqua Logic Inc, San Diego, CA, USA). In addition, each system was equipped with diffused air and a water flow rate of ~7 L/min. In total, there were three separate RAS systems each held at 21°C, 24°C, or 27°C ± 0.2°C. Each RAS was backwashed twice weekly with approximately 40% water removal.

Temperature and DO were checked twice daily (~08:00 and ~16:00; YSI model 58 with 550A probe; YSI, Yellow Springs, OH, USA). Other water quality parameters were tested twice weekly using a spectrophotometer (D/R 2000 Direct Reading, Hach, Colorado, USA) and pH meter (pH30 meter, Oakton Instruments, Vernon Hills, IL, USA). Nitrite and nitrate levels were maintained between 0 to 0.02 mg/L, ammonia 0 to 0.05 mg/L, pH 7.2 to 7.7, alkalinity 95 to 125 mg/L $CaCO_3$, and hardness 80 to 90 mg/L $CaCO_3$. The facility was kept at 23°C ± 0.5°C and rearing of offspring took place under a 12 h light/12 h dark photoperiod at ~250 lux.

1.1.4. Larval and juvenile rearing

Eggs/embryos were monitored every 3 h, starting at 48 h before hatch. Once >50% of larvae hatched, they were gently pipetted into 30 mesh baskets (25 cm × 33 cm × 13 cm); 15 for each subspecies. Each basket was stocked with 2,200 yolk-sac larvae equally represented by the different spawning mats; ~367 larvae per spawning matt for Florida LMB and ~220 larvae for Northern LMB. Once all baskets were stocked, ten baskets per subspecies were gently transferred to two separate RAS systems at 21°C. The water in each RAS was slowly raised to the desired temperature (24°C or 27°C) by adjusting the water flow rate to 0.5 L/min (~1°C/h). The other ten baskets remained in the 21°C RAS. In the end, each RAS had five replicate baskets in a corresponding aquarium for each subspecies. Once >50% of the larvae reached the swim-up stage, they were released from the baskets, and the aquarium water level was reduced to ~20 L.

Starting at 3 days post-hatch (dph), larvae were fed Premium Grade A *Artemia* (Brine Shrimp Direct, Ogden, UT, USA) at 2/mL every 2 h from 06:00 to 24:00. In addition, Otohime B2 micro-diet (Marubeni Nisshin Feed, Tokyo, Japan) was added to each aquarium (~0.5 to 1.0 g/tank) starting at 7 dph. Mortalities, excess feed, and fecal matter were removed daily. The number of survivors in each treatment tank combination were counted at 28 days. Survival was not adjusted for the 5.4% of larvae removed for morphometric and molecular analysis sampling.

1.1.5 Data collection

1.1.6. Morphology

Fish morphological sampling occurred at 0, 2, 4, 8, 12, and 28 dph. For each sampling day, 20 individuals (10 for morphology and 10 for molecular analyses) were randomly selected from each replicate and temperature treatment. For this study, 0 dph was defined as the time when >50% of eggs hatched. Fish were euthanized with 200 ppm MS-222 (tricaine methanesulphonate; Argent Laboratories Inc., Redmond, WA, USA), and digital images were obtained using a Zeiss stereomicroscope (SterREO Discovery V12) equipped with 0.5 to 1.0× objectives and ZEN 2.5 imaging software (blue edition). Measurements were extracted using ImageJ (Version 1.46r) software. Total length (TL, distance from tip of snout to tip of tail), body area (BA, body area excluding fin-fold area and yolk sac), myotome height (MH, body height measured posterior to anus), jaw length (JL), eye diameter (ED), oil droplet area (ODA), and yolk area (YA) were obtained for each individual. The condition index was calculated by dividing MH by TL (following Koslow et al., 1985). Yolk utilization efficiency (YUE) was also calculated by dividing the increase in BA from 2 to 4 dph by the corresponding decrease in YA. In comparison, the yolk utilization rate (YUR) was calculated by the reduction in YA from 4 to 2 dph divided by the corresponding time interval (Hardy and Litvak, 2004; Politis et al., 2017). For each subspecies, 29 dph wet weights (± 0.001 g) were determined for 50 fish per aquaria. Survival was estimated by subtracting the original stocking number by the number of fish left in each tank.

1.1.7. Gene expression analysis

Gene expression was determined for fish from the various temperatures and subspecies at 8 and 28 dph. At each sampling point, ten fish were collected from each aquarium (n = 15 per subspecies), placed into labeled 1.5 mL sterile microcentrifuge tubes, and flash-frozen at -196^{o}C. Fish were then stored at -80^{o}C until used in RNA extractions. For extractions, randomly collected

fish per tank were homogenized using 2.0 mm beads in a 2 mL screw-cap tube in a VWR Bead Mill homogenizer (VWR International; Radnor, PA). RNA was extracted using a *Quick*-RNA Miniprep Plus kit (Zymo Research Corporation; Irvine, CA), according to the manufacturer's directions. Isolated RNA was then quantified on a NanoDrop One (Thermo Fisher Scientific; Waltham, MA), and all samples were diluted to a concentration of 100 ng/μL. RNA (10 μL) was then converted to cDNA in a 20 μL reaction, using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific; Waltham, MA), and the product was stored at -20^{o}C until used in qPCR reactions. For qPCR analyses, previously published primer sets for growth hormone (*gh*), insulin-like growth factor 1 (*igf1*), and heat shock protein 70 (*hsp70*) were used, along with the housekeeping genes beta actin (*actb*) and elongation factor 1α (*ef1α*; Table 1; Eurofins Genomics, Louisville, KY). Primer set efficiencies were assessed using a series of 2- or 10-fold dilutions of pooled cDNA in 10 μL reactions before analyzing the experimental samples. Additionally, all products were evaluated via PCR, and product sizes were verified on a 2% agarose gel.

The 10 μL reaction consisted of 5 μL of SYBER PowerUP 2x Master Mix (ThermoFisher Inc., Waltham, MA), 0.4 μL of each forward and reverse primer (400 nM final), 1.2 μL of molecular grade water, and 3 μL of 3.33 ng/μL cDNA. Each reaction was performed in duplicate (i.e., technical replicates) on a Quantstudio 5 with a 0.2 × 96-well standard block (Applied Biosystems Corp.; Waltham, MA). The reaction conditions were as follows: 50°C for 2 min and 95°C for 2 min for initial denaturation and then 40 cycles of 95°C for 15 s to denature, 60°C for 15 s to anneal, and 72°C for 30 s to extend. A melt curve from 58-95°C was then performed for product verification. Non-template controls (molecular grade water instead of cDNA template) were also included in the qPCR runs. qPCR data were log-transformed and then analyzed using

the comparative Ct method ($2^{-\Delta\Delta Ct}$; Schmittgen and Livak, 2008). With this approach, *actb* and *ef1α* were first used to normalize the genes of interest, and then compared to the respected control group (i.e., either subspecies or time 0 for temperature).

1.1.8. Parentage analysis

Individual fish (2 subspecies × 3 temperatures × 5 aquaria × ~9-10 fish = 296 samples) were weighed (± 0.001 g) and placed directly into labeled 1.5 mL sterile microcentrifuge tubes containing ~1 mL of 96-100% non-denatured ethanol. Tubes were then sealed with parafilm and shipped to the Center for Aquaculture Technologies (CAT, San Diego, CA, USA) to perform parentage assignment analyses, using an established 192-single nucleotide polymorphism (SNP) panel explicitly designed for LMB (based on n = 151 Northern LMB and n = 108 Florida LMB broodstock from Red Hill Fisheries). DNA extractions from ~20 mg of tissue, library preparation, and sequencing were all conducted by CAT following the manufacturer's protocols. The quality control process removed SNPs with less than a 95% call rate. The resulting SNP genotype data were subjected to exclusion and likelihood-based analyses to assign parentage to broodstock pairs. A tank-spawning matrix was not available for candidate broodstock crosses for these samples. Due to the absence of sex-specific markers in the SNP panel, it was impossible to assign specific sex to most broodstock. Thus, all analyses were performed with sex defined as unknown.

1.1.9 Statistical analyses

Data were analyzed using SAS software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were evaluated for normality using Shapiro–Wilk tests and homoscedasticity using plots of residuals vs. predicted values to ensure they met model assumptions. Data were transformed

(log_{10} or arcsine square root) when necessary. Alpha was set at 0.05 for main effects and interactions. Tukey's post-hoc analyses were used to compare least-squares means between treatments.

In our study, Florida LMB broodstock were exclusively fed goldfish, while Northern LMB broodstock were fed a combination of goldfish and a commercial broodstock feed. As such, we need to be cautious when interpreting any Florida vs. Northern LMB results as broodstock dietary regime has been correlated to progeny performance in fish (see Sink et al., 2010 among others). Thus, we independently ran statistical models for each subspecies to identify the optimal thermal regime for intensive indoor RAS culture (see *Section 2.6.1*). Subspecies comparisons were then conducted for specific traits, realizing that dietary regimes may have confounded results (see *Section 2.6.2*). Both approaches are described below.

To examine temperature effects on morphometric traits (TL, BA, MH, JL, ED, condition index, ODA, and YA), we analyzed data using repeated measures mixed-model ANOVAs. Models were run separately for each subspecies and contained the temperature (21, 24, and 27°C) and age (0 to 28 dph) main effects and the temperature × age interaction. If a significant temperature × age interaction was detected, the model was decomposed into a series of reduced ANOVA models to determine the effect of temperature for each age. Reduced models involved only preplanned comparisons and did not reuse data, so alpha level corrections for posteriori comparisons were unnecessary.

The effect of temperature on YUE, YUR, survival, wet weight, and targeted gene expression (*gh, igf1, hsp70* at 8 and 28 dph) was determined using a series of one-way ANOVA models. Moreover, linear regression was used to model the growth of TL and BA between

temperatures for each subspecies. Homogeneity of slopes and t-tests with Bonferroni correction (sequential) were then employed to detect temperature differences.

A series of two-factor ANOVA models were used to compare TL and BA (at 28 dph), as well as YUE, survival, wet weight, and targeted gene expression (*gh*, *igf1*, *hsp70* at 8 and 28 dph) of progeny from Florida and Northern LMB at each rearing temperature. If an interaction between subspecies and temperature was detected, separate t-tests were performed at each temperature to evaluate the effect of subspecies alone.

Linear regression was used to compare TL and BA growth dynamics for both subspecies at each temperature. Homogeneity of slopes and t-tests with Bonferroni correction (sequential) were then employed to detect differences between the subspecies.

Finally, a series of t-tests were run to compare the expression of targeted genes (*gh*, *igf1*, *hsp70* at 8 and 28 dph) between Florida and Northern LMB at each rearing temperature.

1.2. Results

1.2.1. Effect of rearing temperature

Florida LMB morphometrics and growth

Repeated measures ANOVA models indicated significant temperature × age interactions for all Florida LMB morphometric traits (Table 1.2.). Therefore, the models were decomposed into a series of one-way ANOVA models at 2, 4, 8, 12, 20, and 28 dph for each morphometric trait. There was a significant temperature effect for all morphometric traits at each dph ($p < 0.001$; Table 1.3.), where fish increased in size over the temperature gradient, and the largest morphometric

traits (TL, BA, MH, JL, and ED) were detected at 27°C from 2 to 28 dph (Fig. 1.1.A-E). Additionally, temperature significantly impacted condition index, where fish demonstrated differential changes in condition index based on age; but typically increased with increasing temperature (Fig. 1.1.F).

The linear regressions used to model growth over time at each temperature showed highly significant ($r^2 \geq 0.94$, $p < 0.0001$) growth trajectories for both TL and BA (Table 1.4.). The regressions showed significant differences among slopes with temperature, where fish grew faster at 24°C and 27°C for TL (Fig. 1.2.A) and at 27°C for BA (Fig. 1.2.B).

Florida LMB yolk-sac *characteristics*

The temperature × age interaction was significant for both ODA ($F_{2,12} = 80.87$, $p = < 0.0001$) and YA ($F_{2,12} = 957.81$, $p = < 0.0001$). Therefore, the two saturated models were decomposed into separate one-way ANOVAs at 2 and 4 dph. ODA ($F_{2,12} \geq 2199.03$, $p = < 0.0001$; Fig. 1.4.A) and YA ($F_{2,12} \geq 9830.87$, $p = < 0.0001$; Fig. 1.4.B) both decreased with increasing temperatures. Similarly, temperature significantly influenced YUE ($F_{2,12} = 106.09$, $p = < 0.0001$; Fig. 1.5.A) and YUR ($F_{2,12} = 4176.04$, $p = < 0.0001$; Fig. 1.5.B), where both traits increased with increasing temperature.

Florida LMB final wet weight and survival

Temperature had a significant effect on the survival of Florida LMB ($F_{2,12} = 23.49$, $p = < 0.0001$), such that survival decreased with increasing rearing temperature (Fig. 6A). Temperature also impacted wet weights ($F_{2,12} = 18.03$, $p = 0.0002$, Fig. 1.6.B), where the 27°C treatment produced heavier fish after 29 days of rearing.

Northern LMB morphometrics and growth

For Northern LMB, all morphometric traits were significantly influenced by the temperature × age interaction (Table 1.5.), and temperature (Table 1.6.). In addition, Northern LMB showed similar morphometric size trends observed to the Florida LMB, where TL, BA, MH, JL, and ED increased with temperature for each dph, and fluctuations in condition factor were detected as the fish aged (Fig. 1.3.).

Highly significant ($r^2 \geq 0.94$, $p < 0.0001$; Table 1.4.) linear growth trajectories were detected for Northern LMB. These regressions showed significant differences among slopes across temperatures, where fish grew faster at 27°C for TL (Fig. 1.2.C) and at 24 and 27°C for BA (Fig. 1.2.D).

Northern LMB yolk-sac characteristics

In Northern LMB, the temperature × age interaction was also significant for ODA ($F_{2,12} = 593.32$, $p = < 0.0001$) and YA ($F_{2,12} = 542.08$, $p = < 0.0001$), thus the models were decomposed to determine the effect of temperature at 2 and 4 dph (Fig. 1.4.CD). During these early life stages the larvae had smaller remaining yolk reserves (YA and ODA) as temperatures increased ($F_{2,12} \geq 5709.15$, $p = < 0.0001$). At the same time, YUE and YUR were impacted by rearing temperature, such that larvae reared at 27°C were most efficient at converting their yolk reserves to body size ($F_{2,12} = 56.14$, $p = < 0.0001$, Fig. 1.5.C), while larvae reared at 27°C also utilized their yolk at the fastest rate ($F_{2,12} = 1365.60$, $p = < 0.0001$, Fig. 1.5.D).

Northern LMB final wet weight and survival

Temperature did not significantly impact the survival ($F_{2,12} = 1.11$, $p = 0.360$) of Northern LMB (Fig. 1.6.C). However, temperature was found to have a significant effect on final

weights ($F_{2,12}$ = 16.74, p = 0.0003), such that, like the Florida LMB, final weights increased with increasing rearing temperature (Fig. 1.6.D).

1.2.2. Gene expression analysis

Florida LMB

Expression patterns of selected genes were compared across temperatures at 8 and 28 dph for Florida LMB. No differences in *gh* and *igf*1 expression were detected across the temperature gradient at 8 dph; however, at 28 dph these growth-related genes were upregulated in fish reared at 27°C (Fig. 1.7.AB). On the contrary, expression of stress/repair-related *hsp70* was downregulated on 8 dph when fish were reared at 27°C and on 28 dph when fish were reared at 24 and 27°C (Fig. 1.7.C).

Northern LMB

Northern LMB showed similar expression patterns as Florida LMB across the temperature gradient, but with no significant differences in *gh*, *ifg*1, and *hsp70* detected (Fig. 1.7.D-F).

1.2.3. Effect of subspecies

Morphometric traits and growth dynamics

The temperature × subspecies interaction was significant for both TL ($F_{2,24}$ = 26.25, p < 0.0001; Fig. 1.8.A) and BA ($F_{2,24}$ = 481.29, p < 0.0001; Fig. 1.8.B); thus, these models were decomposed to determine the effect of subspecies for each temperature. Concerning TL and BA, Northern LMB were larger across the entire temperature gradient (all p < 0.0001).

Linear regressions were used to model differences in growth trajectories over time between the two subspecies at each temperature (Table 1.4.). Growth trajectories for TL were not significant between Florida and Northern LMB at 21, 24, or 27°C (all $p \geq 0.158$). Growth trajectories for BA

were also not significant at 21 (p = 0.112) and 27°C (p = 0.310), while Northern LMB grew faster at 24°C than their Florida LMB counterparts (p = 0.011).

Yolk-sac characteristics

Both temperature ($F_{2,24}$ = 144.92, p < 0.0001; Fig 1.9.B) and subspecies ($F_{2,24}$ = 495.89, p < 0.0001; Fig. 1.9.C) impacted YUE, such that larvae increased their efficiency at utilizing yolk reserves as rearing temperature increased and Northern LMB had higher YUE than Florida LMB.

Final wet weight and survival

The temperature × subspecies interaction was not significant for survival ($F_{2,24}$ = 1.47, p = 0.250; Fig. 1.9.D), as such main effects were interpreted. Both temperature ($F_{2,24}$ = 9.40, p = 0.001) and subspecies ($F_{1,24}$ = 6.50, p = 0.018) had a significant impact on survival, such that LMB reared at 21°C had higher survival than those reared at 24 or 27°C (Fig. 1.9.E), and Northern LMB had higher survival than Florida LMB (Fig. 1.9.F).

The interaction between temperature × subspecies did not significantly impact the wet weight of LMB at 29 dph ($F_{2,24}$ = 1.08, p = 0.356) (Fig. 1.9.G). On the contrary, an increase in weight was detected as rearing temperature increased ($F_{2,24}$ = 69.79, p < 0.0001; Fig. 1.9.H) and Northern LMB were heavier than Florida LMB ($F_{1,24}$ = 15.02, p = 0.0007; Fig. 1.9.I).

Gene expression

The expression of selected genes was compared between Florida and Northern LMB at each temperature (Table 1.7.). At 8 dph, Northern LMB larvae had increased expression of *gh* at 27°C and increased expression of *igf*1 at 21 and 27°C. Conversely, at both points in ontogeny, Northern LMB had decreased expression of *hsp70* across the temperature gradient (21-27°C) compared to their Florida LMB counterparts (Table 1.7.).

1.2.4. Parental assignment

A 192 SNP panel designed exclusively for LMB successfully assigned 271 of 297 fish (progeny) to single parent pairs. Parent-pair contributions to progeny were quantified for each temperature regime and subspecies. Florida LMB were represented by ten parental combinations (Families 1-10, Fig. 1.10.A), while Northern LMB were represented by 16 parental combinations (Families 11-26, Fig. 1.10.B). More specifically, progeny from Florida LMB were represented by six families (1, 2, 3, 7, 8, 10) across all temperatures, with a more significant proportion of progeny sampled from families 3, 7, and 10 (Fig. 1.10.A). In some cases, specific family contributions (n = 4) were only represented at 1 or 2 rearing temperatures. Northern LMB progeny were also represented by six families across rearing temperatures (14, 19, 22, 23, 25, 26), with many other families having a less prominent parental contribution (Fig. 1.10.B).

1.3. Discussion

Hatchery production is one of the highest costs and constraints for aquaculture production for food fish and stocking programs (Migaud et al., 2013). In this study, several key findings to improve hatchery function and efficiency for LMB are reported: 1) temperature had a significant impact on a series of key morphological traits during early life history; 2) there is a difference in performance between the two subspecies of LMB, where Northern typically outperformed the Florida subspecies; and 3) performance during these trials suggests promising potential for indoor RAS culture for this socially and economically important food and recreational fish in the United States.

RAS technology provides greater control of the thermal environment than ponds and, therefore, finer production control. Our study utilized RAS technology to investigate indoor options for the culture of early life history stages of Florida and Northern LMB reared at different temperatures within their thermal threshold tolerance limits. Here, there was a significant temperature effect for all morphological traits. Both subspecies increased in size over the temperature gradient, with the largest traits (TL, BA, MH, JL, ED) typically detected at 27^{o}C. This is important as the thermal environment has been shown to influence the formation and development of structures and organs responsible for early life development, including prey capture and foraging efficiency after the switch to exogenous feeding (Helvik et al., 2001; Hall et al., 2004; Sala et al., 2005). For instance, formation of fin structures aid in coordinated locomotive ability, which is essential for all fishes (Tanaka et al., 2002). Moreover, the development of larger muscle myotomes allows for greater propulsion potential (Fisher et al., 2000; Fisher and Hogan, 2007; Nanami, 2007). Furthermore, the development of larger mouthparts provides the ability for larvae and early juveniles to ingest a greater size distribution of feeds due to larger gape sizes, while larger eyes improve visual acuity (Shirota, 1970; Kerrigan, 1997; Sabatés and Saiz, 2000; Hunt von Herbing, 2001; Rideout et al., 2004). Therefore, optimizing temperature regimes during early ontogeny, as demonstrated in this study, can significantly improve LMB production.

Larvae development, YUE, and YUR were accelerated with the increase in rearing temperature. This was also reflected in the YA and ODA, as both decreased faster with the increase in temperature. Similar results were found with Guadalupe bass (*Micropterus treculii*), where yolk and oil globule depletion rates increased across a temperature gradient between 22 and 24°C (Prangnell and Matthews, 2019). However, it is worth mentioning that this phenomenon occurs within the favorable thermal threshold limits, as unfavorable thermal conditions close to or beyond

species-specific tolerance limits are known to cause less efficient yolk utilization, resulting in reduced growth (Rombough, 1996; Politis et al., 2014). In our study, larvae experiencing colder temperatures utilized their yolk reserves less rapidly and grew slower (leading to increased stage duration) than those reared at higher temperatures.

In accordance with our findings, previous studies suggest a thermal window of 26 to 30°C for increased growth of LMB, however with diminishing growth benefits at temperatures above 30°C (Strawn, 1961; Tidwell et al., 2003; Fantini et al., 2021). This raises concerns about the current rearing procedures in outdoor ponds, due to fluctuating ambient temperatures, especially considering that recent climate models predict increasing temperatures by 2.2°C to 5.5°C in the Southeastern United States over the next 30 years (National Fish and Wildlife Foundation, 2010). Therefore, developing indoor rearing techniques and utilizing RAS technology by not relying on fluctuating climatic conditions but instead enabling stable biophysical conditions and water quality, will benefit early life performance and increase LMB production.

While survival in the lower treatment was highest, it does not consider the developmental stage achieved in each treatment. Suppose we use total length as a proxy for the development state. In that case, we can use our regression data to determine at what age the fish reared in the higher temperature treatments reached the average size attained in the low-temperature treatment. For the Florida and Northern subspecies reared at 21°C, the average size at 28 DPH was 12.04 and 12.56 mm, respectively. Percent mortality per day for the duration of the experiment can then be used to estimate the proportion surviving in the 24 and 27°C treatments for each subspecies when they reached the size of those reared at 21°C. It took 18 and 14 days for both subspecies reared in the 24 and 27°C treatments, respectively, to reach the size attained in their 21°C treatments. Northern LMB survival for both the 24 and 27°C treatments were 43% and 55%, respectively. Similar results

were observed for the Florida subspecies with survival to this point for both the 24 and 27^{o}C treatments being 40% and 52%, respectively. This is more than two times higher survival than the 21^{o}C treatment for both subspecies. Clearly, care must be taken when interpreting survival results from fish grown in temperature treatments. This suggests, as was seen in our work, that we should examine survival to an equivalent developmental or size stage when comparing survival.

This study also examined the molecular attributes of two subspecies reared at different temperatures. At 27°C, Florida LMB showed upregulated expression of growth-related *gh* and *igf*, providing molecular evidence for higher ontogenetic growth potential. Under suitable rearing temperatures, both hormones tightly control growth homeostasis as critical drivers of the hypothalamic-pituitary-somatotropic axis (Picha et al., 2006; Reinecke et al., 2005; Picha et al., 2008). As such, choosing a rearing temperature regimen that promotes the most efficient growth potential and reduces unnecessary rearing duration is beneficial.

Interestingly, Florida LMB reared at 21°C had a higher expression of *hsp70* than Northern, which indicates a potential stress response, as this gene is known to encode heat shock protein chaperones, activated to facilitate re-folding of miss-folded proteins to ensure normal development (Roberts et al., 2010; Politis et al., 2017). This does make sense on two levels. First, it agrees with our finding that survival to their terminal size at 28 dph in the 21^{o}C treatment was stressful as it took much less time in the higher temperatures to grow to that size. Second, the Florida subspecies are from a lower latitude and do not experience as low temperatures as the Northern subspecies. The Northern subspecie's greater adaptability to lower temperatures and growth rates (Garvey and Marschall, 2003) suggests it's a better candidate for indoor RAS culture.

Before this study, the role of genetic background and subspecies-specific phenotypic plasticity in LMB reared in RAS was unclear, especially regarding the growth potential of

genetically verified Florida and Northern subspecies (Fewell et al., 2020). An early study compared fingerlings (52 - 58 mm) of those subspecies raised for one year in a Florida pond system, where Northern LMB demonstrated larger final weights compared to their Florida counterparts (Clugston, 1964). However, Florida LMB used in that study were not genetically verified. Similarly, Zolczynski and Davies (1976) found Northern LMB to exhibit larger weight gain (225.0 g) compared to the Florida (174.7 g) and F1 hybrid (158.2 g) counterparts when reared in an Alabama pond for six months. However, it is important to note that the Northern LMB used in that study were sourced from Lake Martin (Alabama), where Gowan (2015) found an allele frequency of 0.53, suggesting that Northern LMB populations have been hybridizing with Florida LMB, which further complicates interpretation of these results.

Moreover, most of the published studies available on the genetic potential of Florida and Northern LMB were conducted in outdoor systems, with very few comparative studies occurring in indoor facilities using RAS technology. One of the first indoor experiments addressing the temperature effects of these subspecies was conducted by Fields et al. (1987), where Florida and Northern LMB juveniles (50 – 60 mm) were reared in 600 L aquaria and exposed to various temperature treatments (8, 16, 24, 32°C), exploring critical and chronic thermal thresholds. Interestingly, Florida LMB were shown to have the highest thermal tolerance limits. These findings stress the importance of controlled biophysical parameters (such as temperature) during early development, and the importance of genetics regarding phenotypic characteristics and performance traits in response to key factors such as temperature.

The results of the current study indicate that the Northern LMB subspecies performed better in morphometric development, survival, and final weight than the Florida LMB subspecies. Here, the literature is conflicting regarding stock performance at various temperatures, but

inconsistencies probably reflect the lack of genetic verification (Philipp and Whitt, 1991). Notably, the fish used in the present study were genetically verified using previous broodstock data and an SNP panel designed explicitly for LMB. Interestingly, for both subspecies, only a few parent-pair contributions represented the majority of progeny. Furthermore, specific family contributions were only developed at one or two rearing temperatures in some cases, demonstrating the importance of genetic (in)compatibility and the associated parental combination-specific thermal sensitivity. Consequently, these results illustrate that applying genetic tools in future selective breeding programs and most importantly utilizing RAS technology to control environmental parameters according to genetically preprogrammed environmental thresholds and preferences, will significantly improve performance, leading to successful and efficient culture of LMB species.

In conclusion, this study furthered our understanding of biological responses, limits, and adaptabilities or preferences of LMB early life stages to an extrinsic environmental factor (temperature). This is especially important in relation to their genetic background, which is key for optimizing rearing techniques for this socially and economically important fish species. As such, the overall knowledge gained provides a promising step towards utilizing RAS technology for controlled and optimized indoor rearing conditions of LMB.

References

Bai, J.J., Li, S.J., Zheng, G., Xie, J., 2009. The culturing status and technology of Largemouth bass in China. Chinese Fisheries Economics 7, 15-17.

Bai, J.J., Li, S., 2013. Current status and development trend on China largemouth bass industry. Chinese Fisheries Economics 5, 104-108.

Bailey, R.M., Hubbs, C.L., 1949. The black basses (*Micropterus*) of Florida, with description of a new species. Papers of the Museum of Zoology, University of Michigan 516, 1-43.

Blaxter, J.H.S.,1991. The effect of temperature on larval fishes. Netherlands Journal of Zoology 42, 336-357.

Clugston, J.P., 1964. Growth of the Florida Largemouth Bass, Micropterus salmoides floridanus (LeSueur), and the Northern Largemouth Bass, M.S. salmoides (Lacépède), in Subtropical Florida. Transactions of the American Fisheries Society 93, 146-154.

Coutant, C.C., DeAngelis, D.L., 1983. Comparative Temperature-Dependent Growth Rates of Largemouth and Smallmouth Bass Fry. Transactions of the American Fisheries Society 112, 416-423.

Fantini, L.E., Smith, M.A., Jones, M., Roy, L.A., Lochmann, R., Kelly, A.M., 2021. Growth parameters in northern largemouth bass *Micropterus salmoides salmoides* raised near their upper thermal tolerance for 28 days. *Aquaculture Reports* 21(2021), 100845.

Fewell, A.K., Beck, B., McNulty, S., Davis, A., Peatman, E., 2020. Examining early growth genetic potential of two distinct localities of Florida Bass and an F1 hybrid bass (Micropterus floridanus x Micropterus salmoides). Auburn University thesis.

Fields, R., Lowe, S.S., Kaminski, C., Whitt, G.S., Philipp, D.P., 1987. Critical and Chronic Thermal Maxima of Northern and Florida Largemouth Bass and Their Reciprocal F_1 and F_2 Hybrids. Transactions of the American Fisheries Society 116, 856-863.

Fisher, R., Bellwood, D.R., Job, S.D., 2000. Development of swimming abilities in reef fish larvae. Marine Ecology Progress Series 202, 163-173.

Fisher, R., Hogan, J.D., 2007. Morphological predictors of swimming speed: a case study of pre-settlement juvenile coral reef fishes. Journal of Experimental Biology 210, 2436-2443.

Garvey, J.E., Marschall, E.A., 2003. Understanding latitudinal trends in fish body size through models of optimal seasonal energy allocation. Canadian Journal of Fish Aquatic Sciences 60, 938-948.

Gowan, S.W., 2015. Development and application of diagnostic SNP marker resources for Northern (*Micropterus salmoides salmoides*) and Florida (*Micropterus salmoides floridanus*) largemouth bass. (Master's thesis). Auburn University, Auburn, AL.

Hall, T.E., Smith, P., Johnston, I.A., 2004. Stages of the embryonic development in the Atlantic cod *Gadus morhua*. Journal of Morphology. 259, 255–270.

Hardy, R.S., Litvak, M.K., 2004. Effects of Temperature on the Early Development, Growth, and Survival of Shortnose Sturgeon, *Acipenser brevirostrum*, and Atlantic Sturgeon, *Acipenser oxyrhynchus*, Yolk-Sac Larvae. Environmental Biology of Fishes 70, 145–154.

Helvik, J.V., Drivenes, O., Naess, T.H., Fjose, A., Seo, H.C., 2001. Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (Hippoglossus hippoglossus). Visual neuroscience 18, 767-780.

Hunt von herbing, I., 2001. Development of feeding structures in larval fish with different life histories: Winter flounder and Atlantic cod. Journal of Fish Biology 59, 767-782.

Inman, C.R., Dewey, R.C., Durocher, P.P.,1977. Growth comparisons and catchability of three largemouth bass strains. Fisheries 2, 20-25.

Isely, J.J., Noble, R.L., Koppelman, T.B., Philipp, D.P., 1987. Spawning period and first-year growth of Northern, Florida, and intergrade stocks of largemouth bass. Transactions of the American Fisheries Society 116, 757-762.

Kamler, E., 2002. Ontogeny of yolk-feeding fish: an ecological perspective. Revision of Fishery Biology 12, 79-103.

Kerrigan, B.A., 1997. Variability in larval development of the tropical reef fish *Pomacentru amboinensis* (Pomacentridae): The paternal legacy. Marine Biology 127, 395-402.

Kleinsasser, L.J., Williamson, J.H., Whiteside, B.G., 1990. Growth and Catchability of Northern, Florida, and F1 Hybrid Largemouth Bass in Texas Ponds. North American Journal of Fisheries Management 10, 462-468.

Koslow, J.A., Brault, S., Dugas, J., Fournier, R.O., Hughes, P., 1985. Condition of larval cod (*Gadus morhua*) off southwest Nova Scotia in 1983 in relation to plankton abundance and temperature. Marine Biology 86, 113–121.

Kovác, V., 2002. Synchrony and herochrony in ontogeny (of fish). Journal of Theoretical Biology 217, 499-507.

Landsman, S.J., Gingerich, A.J., Philipp, D.P., Suski, C.D., 2011. The effects of temperature change on the hatching success and larval survival of largemouth bass *Micropterus salmoides* and smallmouth bass *Micropterus dolomieu*. Journal of Fish Biology 78, 1200-1212.

Lee, D.S., Gilbert, C.R., Hocutt, C.H., Jenkins, R.E., McAllister, D.E., Stauffer, J.R., 1980. Atlas of the North American Freshwater Fishes. North Carolina State Museum of Natural History, Raleigh, NC.

Leggett, W.C., Frank, K.T., Carscadden, J.E., 1984. Meteorological and hydrographic regulation of year-class strength in capelin (*Mallotus villosus*). Canadian Journal of Fisheries and Aquatic Sciences 41, 1193-1201.

MacCrimmon, H.R., Robbins, W.N., 1975. Distribution of black basses in North America. Black Bass Biology and Management 56-66.

Matthews, M.D., Stout, R.B., 2013. Out-of-Season Spawning Methods for Florida Largemouth Bass to Produce Advanced-Sized Fingerlings by Early Spring. North American Journal of Aquaculture 75, 524-531.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture 5, 194-223.

Myers, J.N., Chatakondi, N.G., Dunham, R.A., Butts, I.A.E., 2020. Genetic architecture of early life history traits for channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂ hybrid production. Aquaculture 514, 734436.

Nanami, A., 2007. Juvenile swimming performance of three fish species on an exposed sandy beach in Japan. Journal of Experimental Marine Biology and Ecology 348, 1-10.

National Fish and Wildlife Foundation, 2010. A Business Plan for the Conservation of Native Black Bass Species in the Southeastern US: A Ten-Year Plan.

Park, J., Renukdas, N., Luna, T., Roy, L.A., 2015. The effects of biomass density on size variability and growth performance of juvenile largemouth bass, *Micropterus salmoides*, in a semi-closed recirculating system. Journal of World Aquaculture 46, 283-291.

Pepin, P., 1991. Effect of temperature and size on development, mortality and survival rates of the pelagic early life stages of marine fish. Canadian Journal of Fisheries and Aquatic Sciences 48, 503-518.

Philipp, D.P., Whitt, G.S., 1991. Survival and growth of northern, Florida, and reciprocal F_1 hybrid largemouth bass in central Illinois. Transactions of the American Fisheries Society 120, 58-64.

Picha, M.E., Silverstein, J.T., Borski, R.J., 2006. Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). General and Comparative Endocrinology 147, 196-205.

Picha, M.E., Turano, M.J., Beckman, B.R., Borski, R.J., 2008. Endocrine Biomarkers of Growth and Applications to Aquaculture: A Minireview of Growth Hormone, Insulin-Like Growth Factor (IGF)-I, and IGF-Binding Proteins as Potential Growth Indicators in Fish. North American Journal of Aquaculture 70, 196-211.

Politis, S.N., Dahlke, F.T., Butts, I.A.E., Peck, M.A., Trippel, E.A., 2014. Temperature, paternity and asynchronous hatching influence early developmental characteristics of larval Atlantic cod, *Gadus morhua*. Journal of Experimental Marine Biology and Ecology 459, 70–79.

Politis, S.N., Mazurais, D., Servili, A., Zambonino-Infante J..L, Miest, J.J., Sørensen, S.R., Tomkiewicz, J., Butts, I.A.E., 2017. Temperature impacts gene expression and morphological development of European eel, *Anguilla anguilla* larvae. PLos One 12(8).

Politis, S.N., Servili, A., Mazurais, D., Zambonino-Infante, J.L., Miest, J.J., Tomkiewicz, J., Butts, I.A.E., 2018. Salinity reduction benefits European eel larvae: Insights at the morphological and molecular level. PLos One 13(6).

Prangnell, D.I., Matthews, M.D., 2019. The Early Life History of the Guadalupe Bass: Lessons for Culturing a Threatened Species. North American Journal of Aquaculture 81, 296-325.

Quintero, H.E., Roy, L.A., Kelly, A.M., Park, J., Heikes, D., 2019. Evaluation of alternative pond production systems for raising largemouth bass, *Micropterus salmoides.* Journal of the World Aquaculture Society 50, 622-632.

Reinecke M., Björnsson, T.B., Dickhoff, W.W., McCormick, S.D., Navarro, I., Power, D.M., 2005. Growth hormone and insulin-like growth factors in fish: Where we are and where to go. General and Comparative Endocrinology 142, 20–24.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. Paternal effects on haddock early life history traits. Journal of Fish Biology 64, 695–701.

Roberts, R.J., Agius, C., Saliba, C., Bossier, P., Sung, Y.Y., 2010. Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. Journal of Fish Diseases 33, 789–801.

Rombough, P.J., 1996. The effects of temperature on embryonic and larval development. In: Wood, C.M., McDonald, D.G. (Eds.), Global Warming: Implications for Freshwater and Marine Fish. Soc. Exp. Biol. Sem. Ser. 61, pp. 177–223.Romano, N., Fischer, H., Rossi, W., Quintero, H., Limbaugh, N. and Sinha, A.K., 2021. Effects of bioprocessed soybean

meal and nucleotide supplementation on growth, physiology and histomorphology in largemouth bass, *Micropterus salmoides*, juveniles. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 260, 111038.

Sabatés, A., Saiz, E., 2000. Intra-and interspecific variability in prey size and niche breadth of myctophiform fish larvae. Marine Ecology Progress Series 201, 261-271.

Sala, O.E., van Vuuren, D., Pereira, H.M., 2005. Biodiversity across scenarios. Pages 375– 408 in S.R. Carpenter, L.P. Prabhu, E.M. Bennet, M.B. Zurek, editors. Ecosystem and human well-being: scenarios. Findings of the Scenarios Working Group, millennium ecosystem assessment . Island Press, Washington , DC .

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3, 1101-1108.

Shirota, A., 1970. Studies on the mouth size of fish larvae. Journal of Japanese Society of Fisheries Science 36, 353-368.

Sink, T. D., Lochmann, R.T., Pohlenz, C., Buentello, A., Gatlin, D., 2010. Effects of dietary protein source and protein-lipid source interaction on channel catfish (*Ictalurus punctatus*) egg biochemical composition, egg production and quality, and fry hatching percentage and performance. Aquaculture 298, 251-259.

Snow, J.R., 1968. Some progress in the controlled culture of the Largemouth Bass, *Micropterus salmoides*, (Lac.). Southern Division of the American Fisheries Society Annual Meeting.

Somero, G.N., Hofmann, G.E., 1997. Temperature thresholds for protein adaptation: when does temperature change start to 'hurt'? In *Global Warming: Implications for Freshwater and Marine Fish* (C.M. Wood & D.G. McDonald, eds). Cambridge University Press 1-24.

Strawn, K., 1961. Growth of Largemouth Bass Fry at Various Temperatures. Transactions of the American Fisheries Society 90, 334-335.

Tanaka, M., Münsterberg, A., Anderson, G.W., Prescott, A.R., Hazon, N., Tickle, C., 2002. Fin development in a cartilaginous fish and the origin of vertebrate limbs. Nature 416, 527-531.

Tidwell, J.H., Webster, C.D., Coyle, S.D., Schulmiester, G., 1998. Effect of Stocking Density on Growth and Water Quality for Largemouth Bass, *Micropterus salmoides*, Growout in Ponds. Journal of the World Aquaculture Society 29, 37.

Tidwell, J.H., Coyle, S.D., Bright, L.A., VanArnum, A., Yasharian, D., 2003. Effect of Water Temperature on Growth, Survival, and Biochemical Composition of Largemouth Bass *Micropterus salmoides*. Journal of the World Aquaculture Society 32, 175-183.

Tidwell, J.H., Coyle, S.D., Bright, L.A., 2019. Largemouth Bass Aquaculture. 5M Publishing, United Kingdom, pp. 91-96.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2012. Recirculating Aquaculture Systems. Cayuga Aqua Ventures, New York.

USDA (United States Department of Agriculture), 2014. 2013 Census of Agriculture 3(3), 1-49.

USDA (United States Department of Agriculture), 2019. 2018 Census of Aquaculture 3(2), 1-55.

Waite, R., Beveridge, M., Brummett, R., Castine, S., Chaiyawannakarn, N., Kaushik, S., Mungkung, R., Nawapakpilai, S., Michael, P., 2014. Improving productivity and environmental performance of aquaculture. Washington, DC: World Resource Institute.

Wang, Y., Ni, J., Nie, Z., Gao, J., Sun, Y., Shao, N., Li, Q., Hu, J., Xu, P. and Xu, G., 2020. Effects of stocking density on growth, serum parameters, antioxidant status, liver and

intestine histology and gene expression of largemouth bass (*Micropterus salmoides*) farmed in the in-pond raceway system. Aquaculture Research 51, 5228-5240.

Watts, C., Bright, L.A., Coyle, S., Tidwell, J.H., 2016. Evaluation of Stocking Density during Second-Year Growth of Largemouth Bass, *Micropterus salmoides*, Raised Indoors in a Recirculating Aquaculture System. Journal of World Aquaculture Society 47, 538-543.

Worth, S., 1895. Report on the Propagation and Distribution of Foodfishes. US Commission of Fisheries, Washington D.C. Government Printing Office, Washington, DC, USA.

Yang, P., Wang, W., Chi, S., Mai, K., Song, F. and Wang, L., 2020. Effects of dietary lysine on regulating GH-IGF system, intermediate metabolism and immune response in largemouth bass (*Micropterus salmoides*). Aquaculture Reports 17, 100323.

Yu, H.H., Liang, X.F., Chen, P., Wu, X.F., Zheng, Y.H., Luo, L., Qin, Y.C., Long, X.C. and Xue, M., 2019. Dietary supplementation of Grobiotic®-A increases short-term inflammatory responses and improves long-term growth performance and liver health in largemouth bass (*Micropterus salmoides*). Aquaculture 500, 327-337.

Zhang, Y., Huang, J., Zhao, J., Bao, E., Zhu, S., Liu, D., Shao, Y., Ye, Z., 2019. Effects of different light spectra on the growth performance and survival of largemouth bass (*Micropterus salmoides*) larvae in recirculating aquaculture systems. Transactions of the American Society of Agricultural and Biological Engineers 62, 1811-1818.

Zolczynski, S.J., Davies, W.D., 1976. Growth characteristics of the Northern and Florida subspecies of Largemouth bass and their hybrid, and a comparison of catchability between the subspecies. Transactions of the American Fisheries Society 105, 240-243.

Table 1.1. Primer pairs utilized in qPCR analysis of gene expression for growth hormone (*gh*), insulin-like growth factor 1 (*igf1*), and heat shock protein 70 (*hsp70*), along with the reference genes beta actin (*actb*) and elongation factor 1α (*ef1α*). Primer efficiency was computed using a series of 2 or 10-fold dilutions.

Primer	**Forward (5'-3')**	**Reverse (5'-3')**	**Product size (bp)**	**Efficiency (%)**	**Reference**
actb	ATCGCCGCACTGGTTGTTGAC	CCTGTTGGCTTTGGGGTTC	187	92.3	Wang et al., 2020
ef1a	TGCTGCTGGTGTTGGTGAGTT	TTCTGGCTGTAAGGGGGCTC	147	97.3	Yu et al., 2019
gh	CCCCCAAACTGTCAGAACT	ACATTTCGCTACCGTCAGG	224	86.7	Yang et al., 2020
igf1	GATCACGTGGCATTGTGGAC	AGCAGGCTTGCTAGTCTTGG	95	98.6	Romano et al., 2021
hsp70	CAGTGATGAAGACAAGCAGAAGA	GCCACCAGCACTCTGATACA	163	91.3	Wang et al., 2020

Table 1.2. Summary of temperature × age interaction statistics (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, $f = f$ value, $p = p$ value) for Florida largemouth bass (*Micropterus salmoides floridanus*) morphometric traits obtained from repeated measures mixed-model ANOVAs.

Morphometric trait	**DFN**	**DFD**	***f***	***p***
Total length	10	36.5	2655.88	<0.0001
Body area	10	44.8	951.49	<0.0001
Myotome height	10	41.6	1250.13	<0.0001
Eye diameter	10	37.4	80.66	<0.0001
Jaw length	10	49.1	114.62	<0.0001
Yolk area	2	12	957.81	<0.0001
Oil droplet area	2	12	80.87	<0.0001
Condition index	10	39.8	450.35	<0.0001

Table 1.3. Summary of temperature effect statistics (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, *f* = *f* value) for Florida largemouth bass (*Micropterus salmoides floridanus*) morphometric traits (TL = total length, BA = body area, MH = myotome height, ED = eye diameter, JL = jaw length, YA = yolk area, ODA = oil droplet area, CF = condition index) obtained from decomposed ANOVA models run at each age.

	Day 2			**Day 4**			**Day 8**			**Day 12**			**Day 20**			**Day 28**		
Trait	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****
TL	2	12	253.1	2	12	1284	2	12	2206	2	12	8697	2	12	14699	2	12	11657
BA	2	12	3946	2	12	8252	2	12	3106	2	12	10578	2	12	2672	2	12	4418
MH	2	12	119.6	2	12	252	2	12	166.7	2	12	7207	2	12	3605	2	12	12546
ED	2	12	326.3	2	12	1397	2	12	1314	2	12	4541	2	12	2203	2	12	1289
JL	2	12	583.3	2	12	35.3	2	12	345.8	2	12	21813	2	12	2790	2	12	5902
YA	2	12	957.8	2	12	9831												
ODA	2	12	2278	2	12	2199												
CF	2	12	22.1	2	12	14.8	2	12	8.8	2	12	4818	2	12	558	2	12	832.4

* $p < 0.001$

Table 1.4. Linear relationships between total length (mm) and body area (mm^2) over time for Florida largemouth bass (*Micropterus salmoides floridanus*) and Northern largemouth bass (*M. salmoides salmoides*) at each temperature.

Morphometric trait	**Temperature**	**Slope (±SEM)**	**Intercept (±SEM)**	**r^2**	***p***
Florida					
	21°C	0.24 (±0.005)	5.82 (±0.07)	0.98	<0.0001
Total length	24°C	0.37 (±0.006)	5.31 (±0.09)	0.99	<0.0001
	27°C	0.47 (±0.01)	5.26 (±0.18)	0.97	<0.0001
	21°C	0.84 (±0.03)	0.22 (±0.41)	0.96	<0.0001
Body area	24°C	1.01 (±0.03)	0.40 (±0.39)	0.98	<0.0001
	27°C	1.49 (±0.07)	-1.25 (±0.94)	0.94	<0.0001
Northern					
	21°C	0.25 (±0.005)	5.62 (±0.07)	0.99	<0.0001
Total length	24°C	0.41 (±0.008)	5.12 (±0.12)	0.99	<0.0001
	27°C	0.54 (±0.02)	4.80 (±0.23)	0.97	<0.0001
	21°C	0.96 (±0.03)	-0.05 (±0.44)	0.97	<0.0001
Body area	24°C	1.39 (±0.06)	-1.39 (±0.89)	0.94	<0.0001
	27°C	1.69 (±0.07)	-1.75 (±1.06)	0.94	<0.0001

Table 1.5. Summary of temperature × age interaction statistics (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, *f* = *f* value, *p* = *p* value) for Northern largemouth bass (*Micropterus salmoides salmoides*) morphometric traits obtained from repeated measures mixed-model ANOVAs.

Morphometric trait	**DFN**	**DFD**	***f***	***p***
Total length	10	48.7	7010.17	<0.0001
Body area	10	38.8	410.7	<0.0001
Myotome height	10	48.3	5003.32	<0.0001
Eye diameter	10	43.9	1594.12	<0.0001
Jaw length	10	43.4	485.73	<0.0001
Yolk area	2	12	542.08	<0.0001
Oil droplet area	2	12	2198.86	<0.0001
Condition index	10	60	1479.86	<0.0001

Table 1.6. Summary of temperature effect statistics (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, f = f value) for Northern bass (*Micropterus salmoides salmoides*) morphometric traits (TL = total length, BA = body area, MH = myotome height, ED = eye diameter, JL = jaw length, YA = yolk area, ODA = oil droplet area, CI = condition index) obtained from decomposed ANOVA models run at each fish age.

	Day 2			**Day 4**			**Day 8**			**Day 12**			**Day 20**			**Day 28**		
Trait	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****	**DFN**	**DFD**	***f****
TL	2	12	615.7	2	12	60.9	2	12	863.4	2	12	7958.5	2	12	42850	2	12	39687
BA	2	12	186.2	2	12	28612	2	12	7324.3	2	12	33211	2	12	846.1	2	12	6241.9
MH	2	12	2588.7	2	12	977.5	2	12	1159.6	2	12	12017	2	12	77259	2	12	136696
ED	2	12	1242.5	2	12	1116	2	12	413.5	2	12	8231.9	2	12	16478	2	12	2836.6
JL	2	12	8174.8	2	12	524.2	2	12	2964.3	2	12	2011.3	2	12	521.6	2	12	2681.2
YA	2	12	10199	2	12	5709.2												
ODA	2	12	858.3	2	12	5970.1												
CI	2	12	1572.3	2	12	618	2	12	118.4	2	12	6540.5	2	12	10372	2	12	13119

* $p < 0.001$

Table 1.7. Comparison of relative gene expression (mean ± SEM displayed) between Florida largemouth bass, *Micropterus salmoides floridanus,* and Northern largemouth bass, *M. salmoides salmoides,* at 8 and 28 days post-hatch. Separate t-tests were performed at each temperature to evaluate the effect of subspecies. Beta actin (*actb*) and elongation factor 1α (*ef1*α) served as reference genes and timepoint 0 (initial sample) for Northern largemouth bass was set as the reference group for comparison.

Temperature	**Gene**	**Florida**		**Northern**		***t-statistic***	***p-value***
		Mean	**SEM**	**Mean**	**SEM**		
8 days post-hatch							
21	*gh*	0.674	0.228	1.463	0.611	1.32	0.228
24	*gh*	1.094	0.341	4.186	1.361	2.20	0.085
27	*gh*	1.012	0.315	3.421	0.814	2.76	0.025
21	*igf*	0.648	0.241	9.934	1.194	7.62	0.001
24	*igf*	0.909	0.394	5.562	2.234	2.08	0.106
27	*igf*	0.831	0.165	5.073	1.671	2.53	0.035
21	*hsp70*	1.100	0.089	0.747	0.100	2.64	0.030
24	*hsp70*	1.197	0.118	0.603	0.012	5.03	0.007
27	*hsp70*	0.812	0.081	0.550	0.027	3.05	0.016
28 days post-hatch							
21	*gh*	3.735	2.060	5.285	0.677	0.64	0.541
24	*gh*	4.909	1.353	5.187	1.613	0.13	0.898
27	*gh*	21.920	5.420	6.770	2.290	2.34	0.052
21	*igf*	1.482	0.084	2.140	0.668	0.98	0.382
24	*igf*	1.711	0.281	11.254	5.295	1.80	0.169
27	*igf*	4.289	1.024	19.366	10.731	1.40	0.255
21	*hsp70*	1.075	0.095	0.523	0.059	4.61	0.002

24	*hsp70*	0.721	0.034	0.539	0.024	4.16	0.004
27	*hsp70*	0.725	0.080	0.426	0.049	3.19	0.013

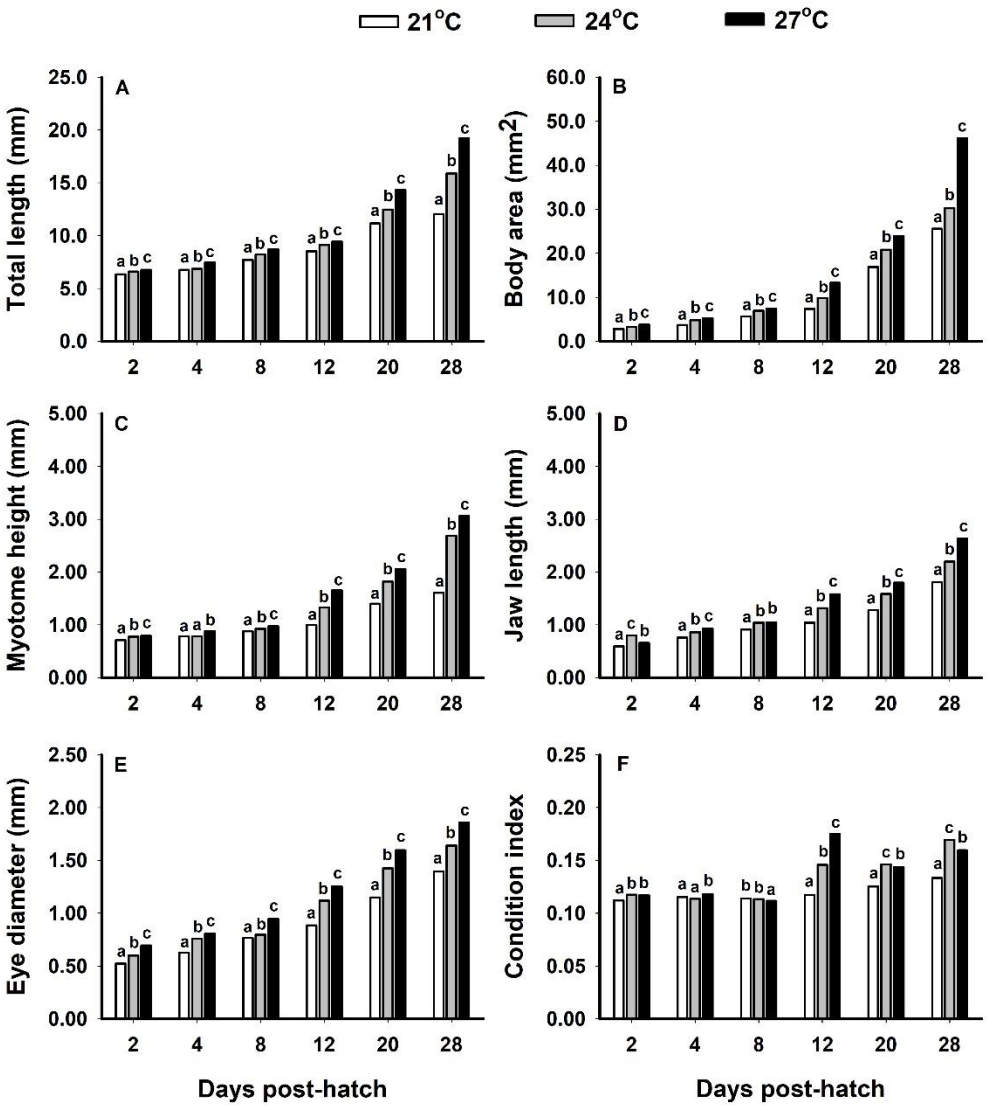
21°C
24°C
27°C
A
Total length (mm)
B
Body area (mm2)
C
Myotome height (mm)
D
Jaw length (mm)
E
Eye diameter (mm)
F
Condition index
Days post-hatch
Days post-hatch

Fig 1.1. Effect of rearing temperature on Florida largemouth bass (*Micropterus salmoides floridanus*) total length (A), body area (B), myotome height (C), jaw length (D), eye diameter (E), and condition index (F). Individual ANOVA models were run at 2, 4, 8, 12, 20, and 28 days post-hatch. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

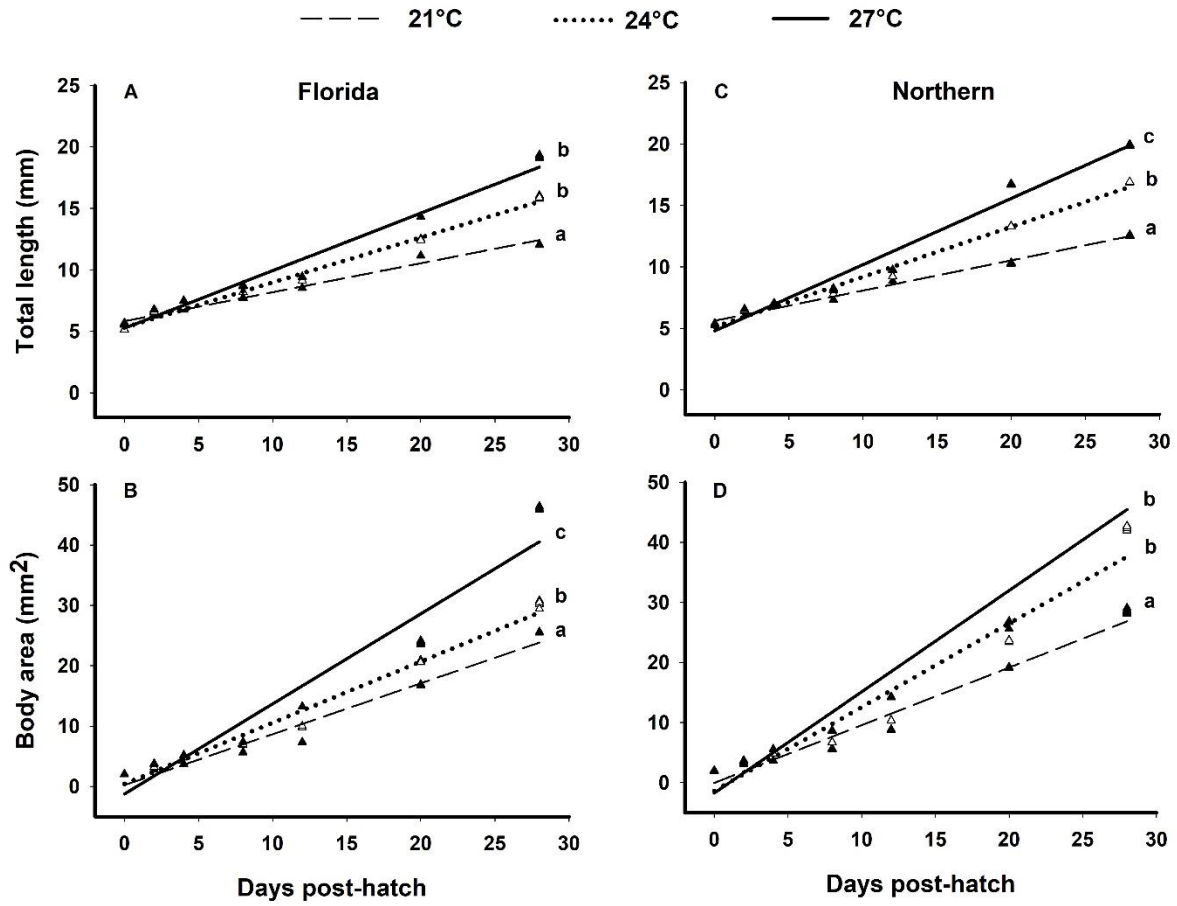

Fig 1.2. Linear regressions were used to model total length (A) and body area (B) for Florida largemouth bass (*Micropterus salmoides floridanus*) and total length (C) and body area (D) for Northern largemouth bass (*M. salmoides salmoides*) at 21, 24, and 27°C. Letters represent significant differences among the slopes across temperatures ($p < 0.05$).

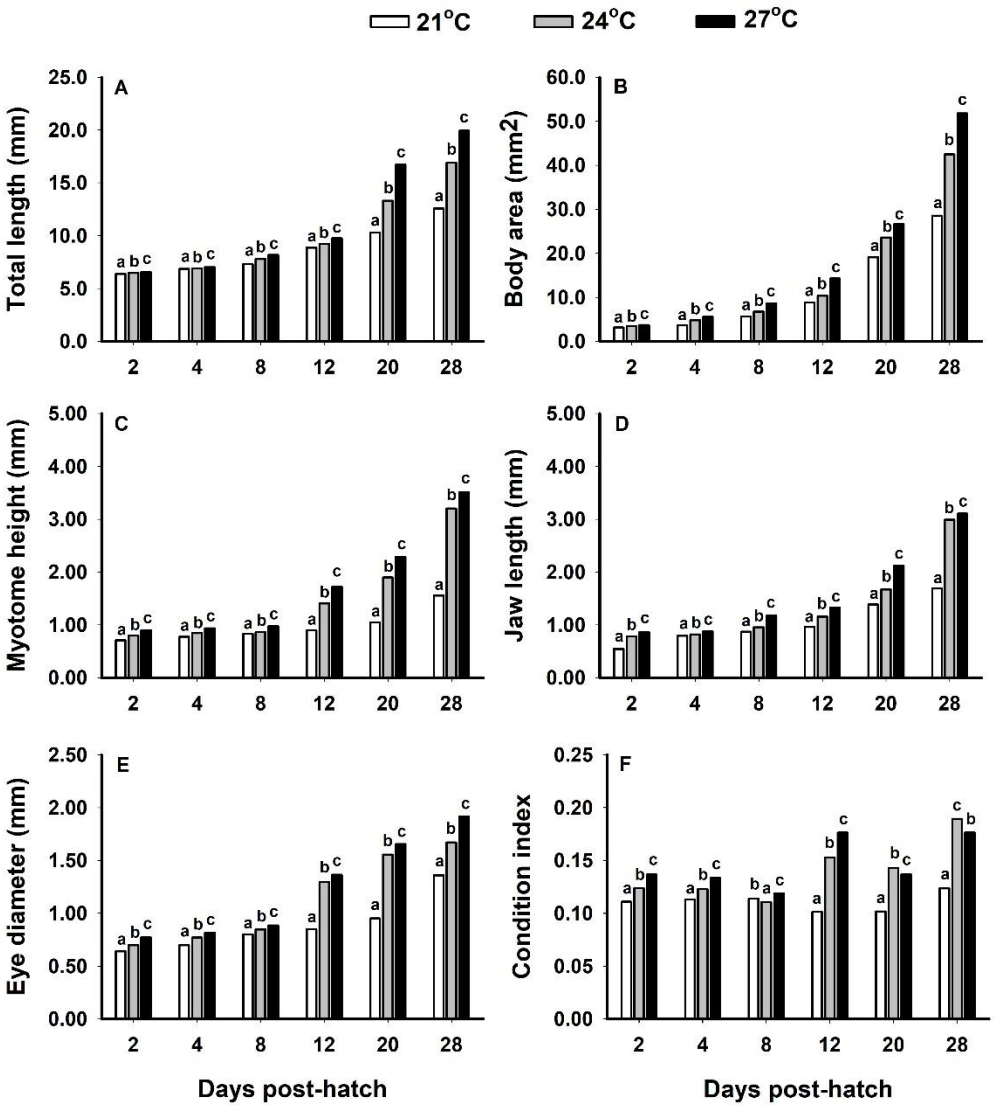
21°C
24°C
27°C
A
Total length (mm)
B
Body area (mm2)
C
Myotome height (mm)
D
Jaw length (mm)
E
Eye diameter (mm)
F
Condition index
Days post-hatch
Days post-hatch

Fig 1.3. Effect of rearing temperature on Northern largemouth bass (*Micropterus salmoides salmoides*) total length (A), body area (B), myotome height (C), jaw length (D), eye diameter (E), and condition index (F). Individual ANOVA models were run at 2, 4, 8, 12, 20, and 28 days post-hatch. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

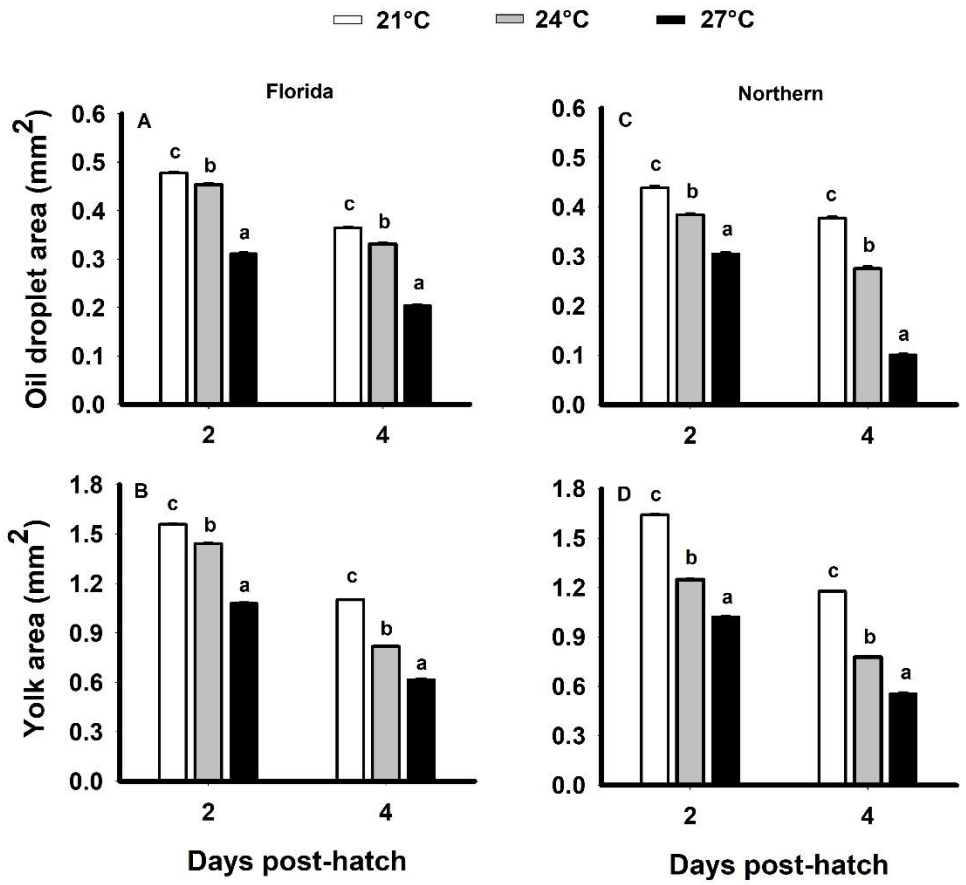

Fig 1.4. Effect of rearing temperature on Florida largemouth bass (*Micropterus salmoides floridanus*) oil droplet area (A), yolk area (B), and Northern largemouth bass (*M. salmoides salmoides*) oil droplet area (C), and yolk area (D). Individual ANOVA models were run at 2 and 4 days post-hatch. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

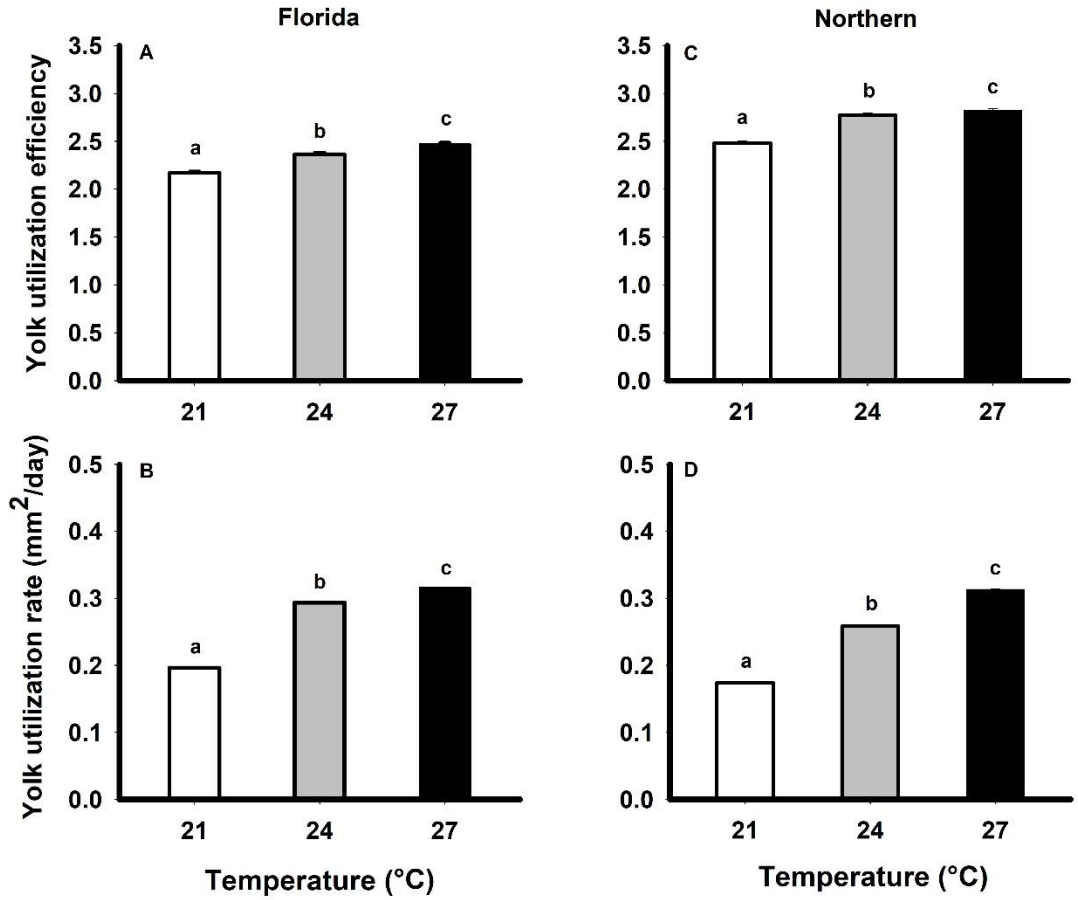

Fig 1.5. Effect of rearing temperature on Florida largemouth bass (*Micropterus salmoides floridanus*) yolk utilization efficiency (A), yolk utilization rate (B), and Northern largemouth bass (*M. salmoides salmoides*) yolk utilization efficiency (C), and yolk utilization

rate (D). Individual ANOVA models were run at 2 and 4 days post-hatch. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

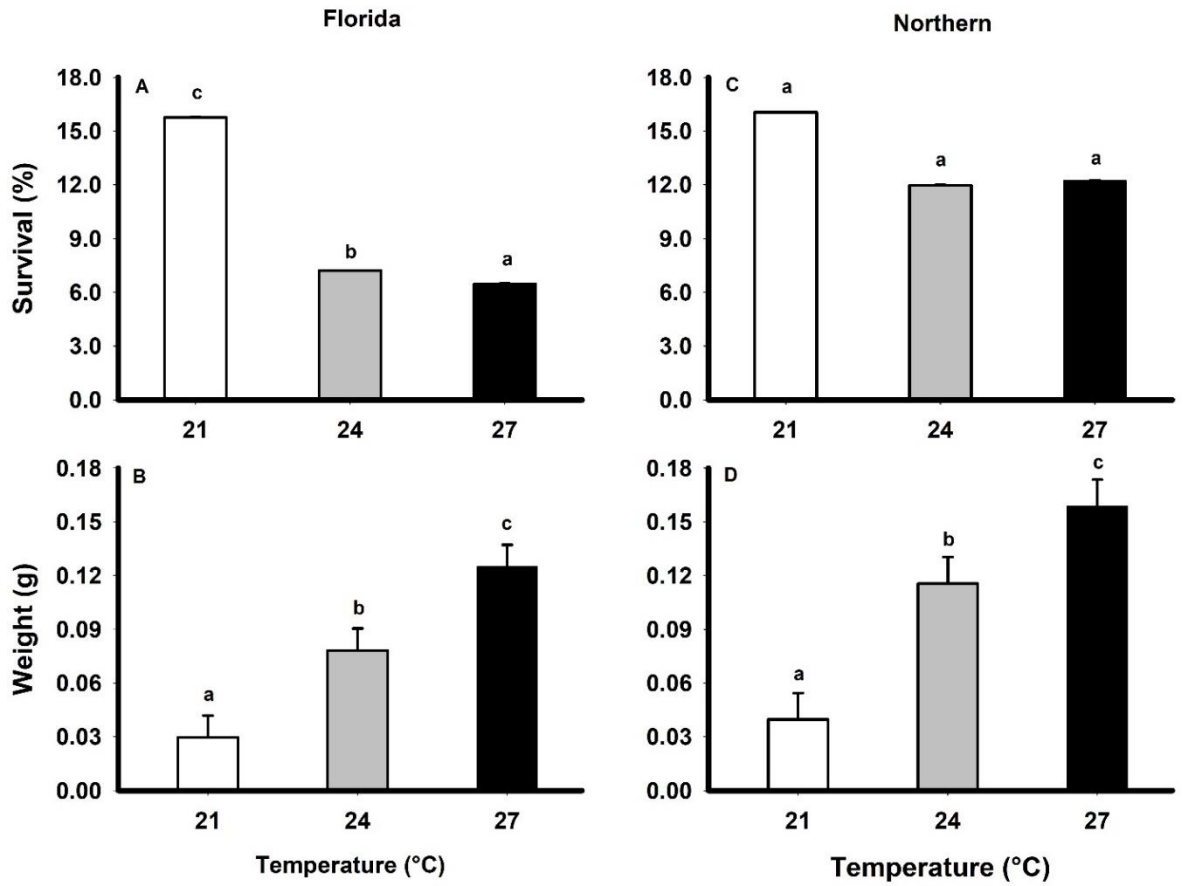

Fig 1.6. Effect of rearing temperature on Florida largemouth bass (*Micropterus salmoides floridanus*) survival (A), individual wet weight (B), and Northern largemouth bass (*M. salmoides salmoides*) survival (C), and individual wet weight (D). These variables were measured at 29 days post-hatch. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

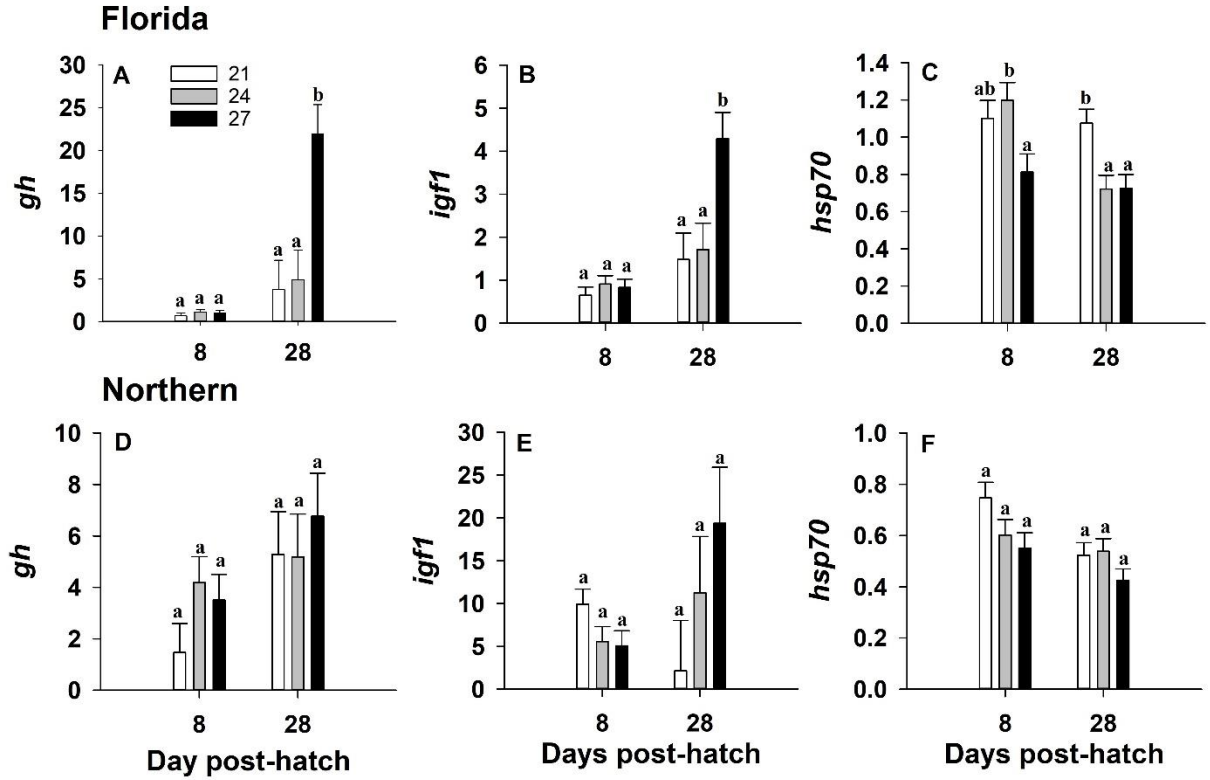

Fig 1.7. Effect of rearing temperature on gene expression (growth hormone (*gh*), insulin-like growth factor 1 (*igf1*), and heat shock protein 70 (*hsp70*)) of Florida largemouth bass (*Micropterus salmoides floridanus;* A-C) and Northern largemouth bass (*M. salmoides*

salmoides; D-F) at 8 and 28 days post-hatch. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003). Beta actin (*actb*) and elongation factor 1α (*ef1*α) were used as reference genes and all data was normalized to initial (day 0) values for the respective fish species.

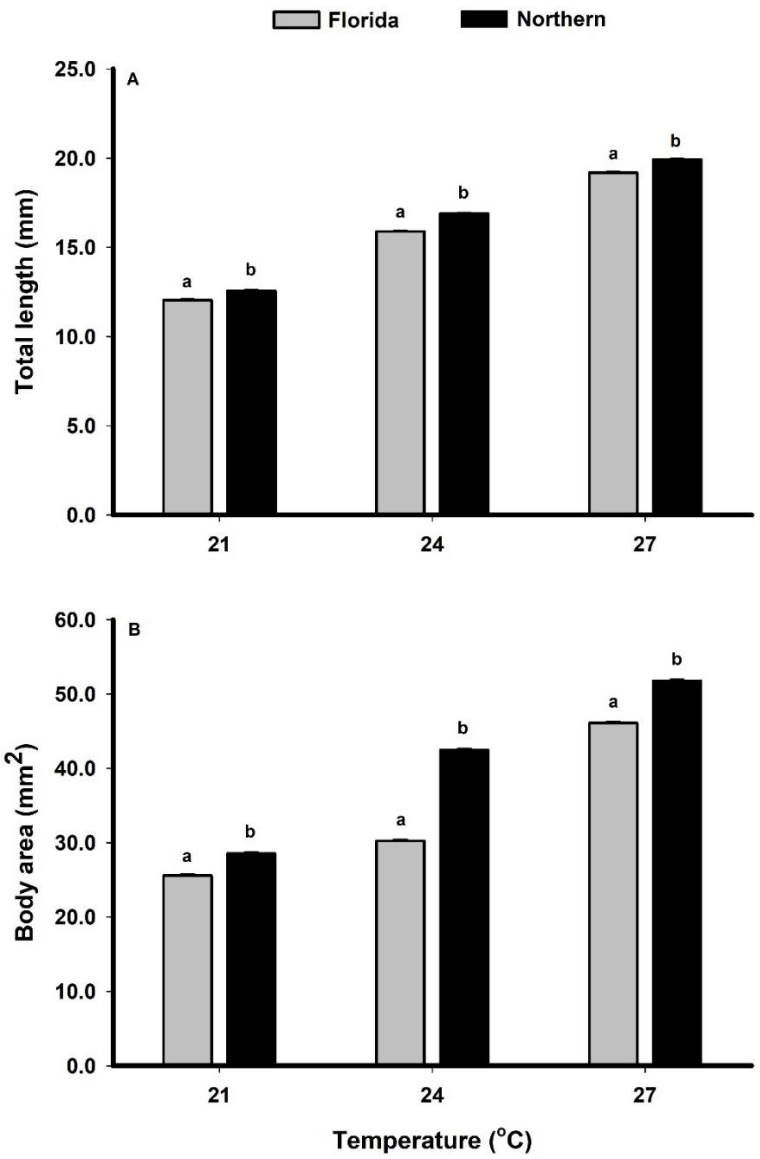
Florida
Northern
A
Total length (mm)
25.0
20.0
15.0
10.0
5.0
0.0
a
b
a
b
a
b
21
24
27
B
Body area (mm2)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
a
b
a
b
a
b
21
24
27
Temperature (°C)

Fig 1.8. Effect of subspecies (Florida largemouth bass, *Micropterus salmoides floridanus* vs. Northern largemouth bass, *M. salmoides salmoides*) on total length (A) and body area (B) at 29 days post-hatch. Two-factor ANOVA models were run and interactions between subspecies and temperature were detected for both traits. Thus, separate t-tests were performed at each temperature to evaluate the effect of subspecies. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent standard error.

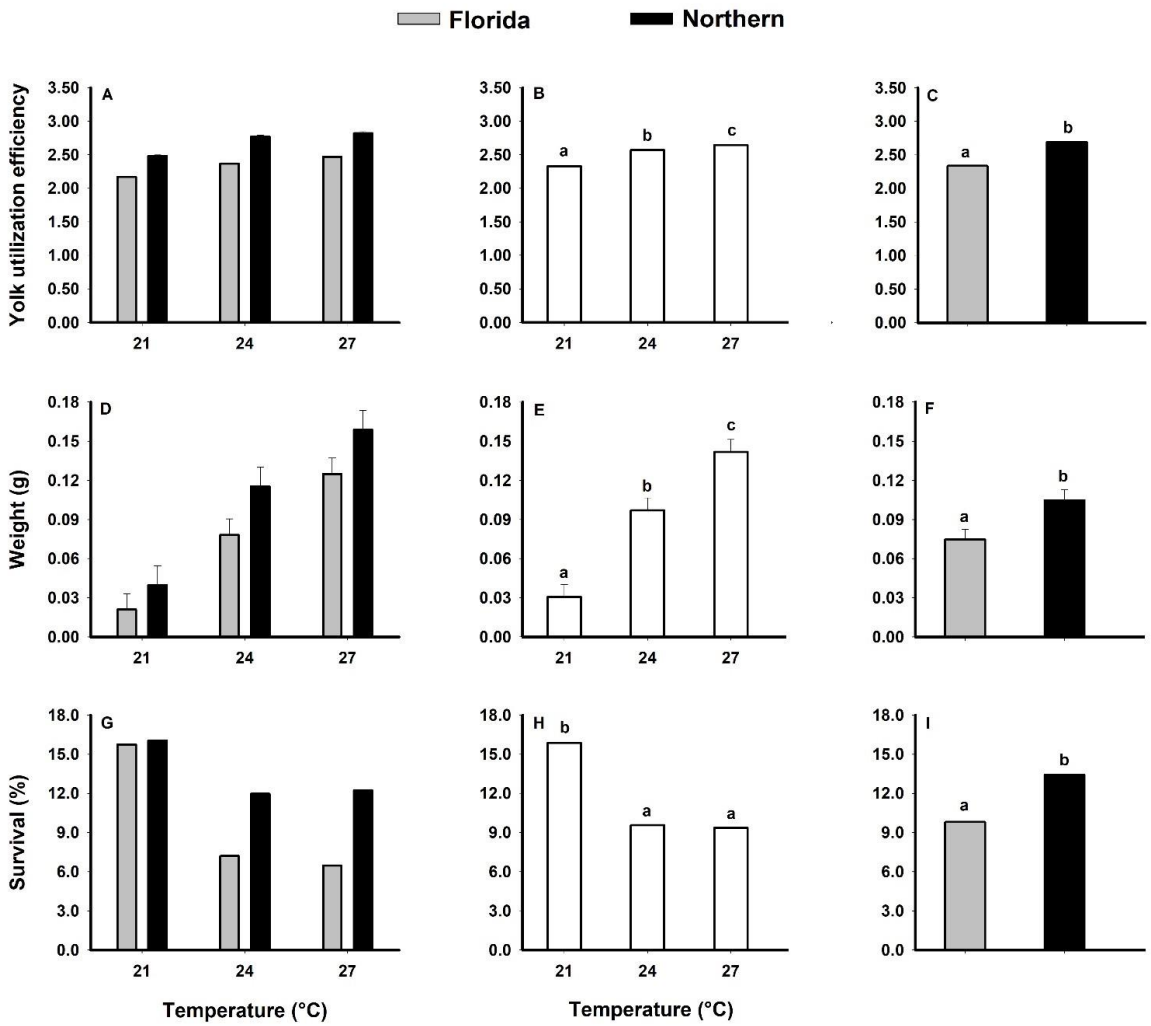

Fig 1.9. Effect of subspecies (Florida largemouth bass, *Micropterus salmoides floridanus* vs. Northern largemouth bass, *M. salmoides salmoides*) on yolk utilization efficiency (A-C), survival (D-F), and wet weight (G-I). A series of two-factor ANOVA models were

used to compare progeny from Florida and Northern largemouth bass at each rearing temperature. Letters represent significant differences among temperatures and subspecies ($p < 0.05$). Error bars represent standard error.

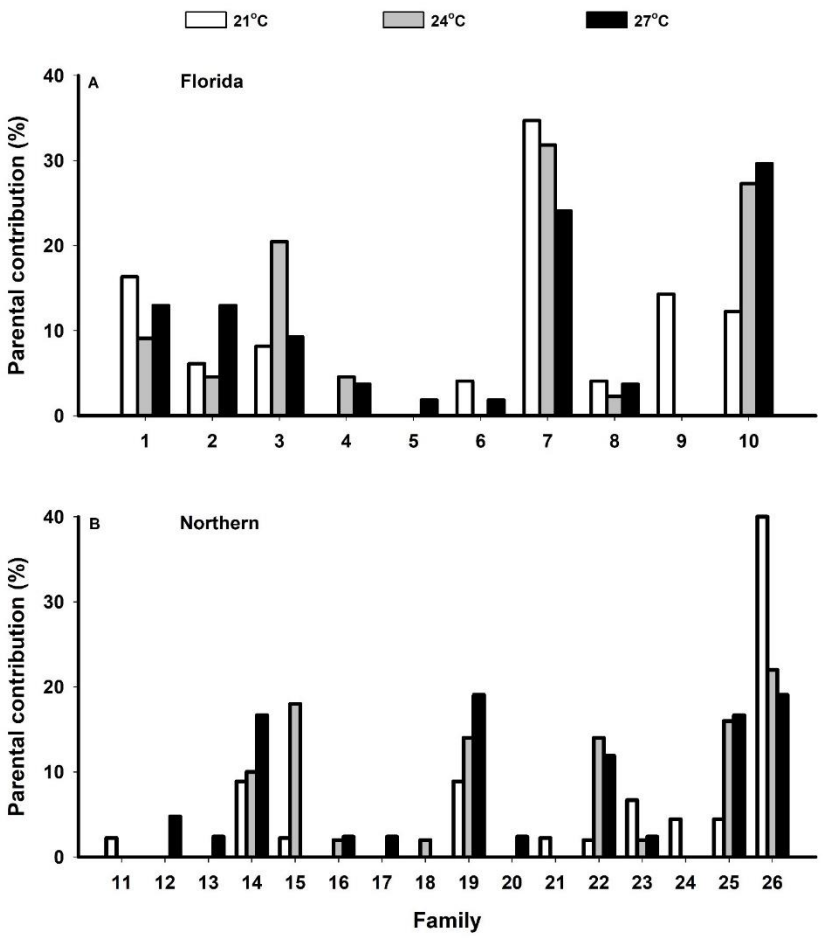

Fig 1.10. Parent pair assignment for Florida largemouth bass (*Micropterus salmoides floridanus*) (A) vs. Northern largemouth bass (*M. salmoides salmoides*) (B) at 21, 24, and 27°C. A 192 SNP panel designed for largemouth bass (LMB) was utilized, along with previous broodstock genotype data (n = 151 Northern LMB, n = 108 Florida LMB). Quality of paternal and maternal contributions to

progeny assignment followed a confidence of >95%, with values below this threshold being removed from analysis. A unique ID was assigned to progeny samples allowing for parent pair contribution to be quantified for both subspecies at the temperature regimes. Parent contribution values represent a parent pair contribution (%) to all progeny studied under the corresponding subspecies and temperature regime.

Chapter 2

Development of first-feeding protocols for largemouth bass (*Micropterus salmoides*)

Abstract

Largemouth bass (LMB), *Micropterus salmoides* is an economically important sport fish species with great potential for indoor recirculation aquaculture systems (RAS) in the United States. Unfortunately, knowledge about optimal dietary requirements during the early life history stages (i.e. egg to first feeding) in LMB remains unresolved. Thus, the objectives of this study were to determine the necessity of rotifers and *Artemia* enrichment for indoor RAS culture in LMB. In total, four dietary treatments were tested: treatment 1 received rotifers with enriched *Artemia*, treatment 2 received rotifers with non-enriched *Artemia*, treatment 3 received only enriched *Artemia*, while treatment 4 received only non-enriched *Artemia*. Starting at 5 DHP, all treatments received a micro-diet. LMB were then randomly sampled at 2 to 24 days post-hatch (dph) for total length (TL), body area (BA), myotome height (MH), jaw length (JL), eye diameter (ED), oil droplet area (ODA), and yolk area (YA). Condition index, yolk utilization efficiency (YUE), and yolk utilization rate (YUR) were calculated, and wet weight and survival were recorded at 25 dph. The current study showed that larvae fed rotifers exhibited a significant increase in morphometric development (TL, ED, MH, JL, BA, and CI). In addition, larvae that were provided rotifers were more efficient and faster at converting yolk reserves to body size. This illustrates that the rotifer diet allowed LMB larvae to transition to exogenous energy sources faster and increase development. However, adding rotifers did not significantly impact the weight or survival of LMB. *Artemia* enrichment also did not significantly increase any of the LMB performance traits. Together, these data improves understandings of LMB dietary requirements

during critical early life history stages to minimize losses and increase hatchery production efficiency.

2.0. Introduction

Largemouth bass (LMB), *Micropterus salmoides*, is arguably the most sought-after recreational fish species in the United States (USFWS, 2006). Production of LMB in North America began around the 1970's, with the goal of supplementing natural populations (Wallus and Simon, 2008; Cooke and Philipp, 2009). This goal remains, however, LMB has recently been a focus species for aquaculture as a food fish (Wang et al., 2019). LMB possesses a high tolerance to stress, fast growth rate, and excellent flesh quality (Park et al., 2015; Park et al., 2017; Wang et al., 2020). In 2017, global LMB production was 415,490 metric tons, with China contributing roughly 99% to this production (Hussein et al., 2020). In comparison, the United States produced 3900 metric tons of LMB in 2018 (USDA, 2018). LMB is among the top five cultured fish species in the United States, behind catfish (channel catfish, *Ictalurus punctatus* and the *I. punctatus* × *I. furcatus* hybrid), trout (*Oncorhynchus sp.*), tilapia (*Oreochromis sp.*), and hybrid striped bass (*Morone chrysops* × *M. saxatilis*). Among these species, LMB ranks highest in the market price for food-size at $5.79 per pound, with catfish, trout, tilapia, and hybrid striped bass averaging $0.97-3.78 per pound (USDA, 2019).

Traditional LMB production follows procedures dating back to the 1930's and utilizes earthen ponds (Snow, 1968), with a multi-phase approach. Broodstock are first brought from outdoor earthen ponds to an indoor facility, where they spawn on spawning-mats (Tidwell et al., 2019). Following fertilization, spawning-mats are brought to nursery ponds, where fry will utilize pond productivity (i.e., zooplankton) until they reach a size (38 – 51 mm) suitable for feed training.

Fingerlings are then transferred to an indoor facility where they undergo feed training on commercial diets. Finally, feed-trained LMB are stocked in grow-out ponds until reaching market size. The entire process can take up to two years, which is one of the reasons LMB command such a high market price. The success of this procedure is highly variable due to fluctuating environmental factors, exposure to predation, and unpredictability of nursery pond productivity (Skudlarek et al., 2013). Traditional LMB production has used nursey ponds to supplement live feed requirements (Tidwell et al., 2000), however, transitioning LMB production to intensive indoor RAS could eliminate the uncertainty of pond productivity and allow more control of water parameters.

Arguably one of the most critical periods during early life history is the transition from endogenous to exogenous feeding. Yolk reserves provide nutrients necessary for organ and tissue development (Kovác, 2002), formation of the digestive system (Kamler, 2002), and anatomical features aiding in prey capture (Yúfera and Darias, 2007). Once endogenous resources have been depleted, timing of exogenous feeding is crucial. Poor transition to exogenous resources has been linked with slower growth rates, nutritional deficiencies, deformities, and lower survival (Blaxter and Ehrlich, 1974; Gisbert et al., 2002). While some species, (i.e., rainbow trout, channel catfish), can successfully be raised on artificial diets from first feeding (Lovell, 1989), LMB require a co-feeding period of both live prey and an artificial diet. Rotifers, *Brachionus spp*, and *Artemia* are the two most common live feeds utilized in larviculture (Csargo et al., 2013). Unfortunately, the live feed is labor intensive, has high production costs, vulnerable to culture collapse, and can be a vector for disease (Walford et al., 1991). Additionally, their application remains challenging due to variable quality in size, hatching rates of cysts, and fluctuations in price (Lavens and Sorgeloos, 2000). Many studies have focused on early diet regimes of other warmwater species; however,

there is limited information on the capabilities of LMB to ingest and assimilate artificial diets (Skudlarek et al., 2013; Coyle and Matthews, 2019). Thus, optimizing an early weaning protocol that reduces live feed duration and speeds the transition to artificial diets would allow for better control of nutrient quality and feed delivery (Fuller, 2020).

Given the present information on LMB, the objectives of this study were to determine the necessity of rotifers and *Artemia* enrichment for indoor RAS culture of LMB. Furthermore, these approaches will advance LMB dietary conditions to improve hatchery production efficiency and genetic enhancement programs for food production.

2.1. Materials and Methods

2.1.1 Animal care

Protocols for fish experimentation were reviewed and approved by the Auburn University Animal Care and Use Program (IACUC# 2020-3772).

2.1.2. Broodstock facility

Florida LMB were raised in a concrete raceway (27 × 3 × 1 m) at Red Hills Fishery in Boston, Georgia, USA (30.8478ºN, -83.7606ºW), where they were fed live goldfish to satiation. Spawning was induced by following a 10 h photoperiod for four weeks, followed by an 8 h photoperiod for three to four weeks, a 10 h photoperiod for two weeks, and finally a 14 h photoperiod for two weeks. Spawning mats (Spawntex, Pentair Aquatic Eco-Systems, Apopka, FL, USA) were utilized and evenly distributed along the raceway during the spawning event. Florida LMB broodstock were represented by 42 males and 51 females, with length and weight

ranging from 324 to 470 mm and 0.49 to 1.87 kg, respectively. The spawning season took place from (15 October 2021 to 31 November 2021), where water temperature and dissolved oxygen (DO) ranged from 19.4 to 23.1°C. Mean nitrite was 0.019 mg/L, mean nitrate was 0.8 mg/L, and mean total ammonia nitrogen (TAN) was 0.07 mg/L at embryo collection.

2.1.3. Embryo collection and rearing

Embryos were transported from Red Hills Fishery to Auburn University E.W. Shell Fisheries Center (32.6526ºN, -85.4859ºW) on 15 November 2021 in 114 L coolers (Coleman, Chicago, IL, USA) containing 40 L of raceway water. In total, Florida LMB embryos were represented by 11 spawning mats.

Aquaria water temperature was set to 26.0ºC ± 0.5ºC upon embryo arrival, reflecting the water temperature of the coolers and slowly decreased (over ~4 h) to 21ºC ± 0.5ºC. Spawning mats were suspended 15 cm below the water surface in 75 L aquaria. At peak hatch, the water flow rate was adjusted to slowly raise water temperature to the desired study temperature of 27.0ºC ± 0.5ºC (see Chapter 1). Two RAS were utilized, with each system containing eight aquaria. Each RAS contained a UV filter (Emperor Smart DC2305, Pentair Aquatic Eco-Systems, Apopka, FL, USA), bead filtration system (Bubble Bead Filter XS10000**,** Aquaculture Systems Technology, Baton Rouge, LA, USA), bag filter (Pall x-100, Pall Corporation, Port Washington, New York, USA), 0.5 hp pump (PerformancePro Cascade, Cascade Pump Company, Santa Fe Springs, CA, USA), 17 × 75 L aquaria, three 795 L circular blue tanks, two 190 L sump tanks, heat-pump (AquaLogic Delta Star DSHP-9, Aqua Logic Inc, San Diego, CA, USA), or in-line heater (AquaLogic Titanium Evo Z31E, Aqua Logic Inc, San Diego, CA, USA). In addition, systems were equipped with diffused air, a water flow rate of ~7 L/min, and maintained at 24.0 ± 0.5ºC.

Temperature and DO were checked twice daily (08:00 and 16:00; YSI model 58 with 550A probe; YSI, Yellow Springs, OH, USA). In addition, other water quality parameters were tested twice weekly using a spectrophotometer (D/R 2000 Direct Reading, Hach, Colorado, USA) and pH meter (pH30 meter, Oakton Instruments, Vernon Hills, IL, USA). Nitrite and nitrate levels were kept between 0 to 0.02 mg/L, ammonia 0 to 0.05 mg/L, pH 7.2 to 7.7, alkalinity 95 to 125 mg/L $CaCO_3$, and hardness 80 to 90 mg/L $CaCO_3$. The ambient temperature in the facility was kept at 21^{o}C ± 0.5^{o}C and rearing of offspring took place under a 12 h light/12 h dark photoperiod at ~250 lux.

2.1.4. Larval and juvenile rearing

Embryos were monitored every five hours, starting at 52 h before hatch. Once >50% hatch was obtained, larvae were evenly distributed to study aquaria and water level was reduced to 17 L (~88 larvae/L). Each RAS had eight experimental aquaria with an initial stocking density of 1,500 larvae proportionally represented by each spawning mat. In total, six spawning mats were utilized, with 250 larvae stocked from each spawning mat.

2.1.5. Experimental design and dietary regimen

LMB were fed six times per day (0600, 1000, 1200, 1600, 2000, 0000) with rotifers (*B. plicatilis*, Reed Mariculture, Campbell, CA, USA), Premium Grade A *Artemia* nauplii (Brine Shrimp Direct, Ogden, UT, USA), Otohime micro-diet (Marubeni Nisshin Feed, Tokyo, Japan), Skretting starter feed (Skretting, Tooele, UT, USA), or combinations of these diets (Fig. 1). In total, there were four dietary treatments with four replicate aquaria per treatment. Treatment 1 LMB received rotifers with enriched *Artemia,* Treatment 2 received rotifers with non-enriched

Artemia, Treatment 3 received only enriched *Artemia*, while Treatment 4 received only non-enriched *Artemia*. *Artemia* were enriched with ORI-N3 at 0.35 g/L (50% protein, 17% lipids, 68% moisture, 90 mg/g n-3 HUFA, and >35 DHA/EPA, Skretting, Tooele, UT, USA) at peak hatch and every 12 h. Starting at 0 dph, larvae were fed 2 *Artemia*/mL and 5 rotifers/mL. At 5 DHP all treatments received Otohime A_1 micro-diet at 0.01g/L per feeding. The rotifer feedings were reduced by 1/mL each day to slowly wean LMB off rotifers, starting at 8 DPH. By 12 DPH, the fish were weaned off rotifers. To supplement the reduction in rotifers, *Artemia* was increased to 5/mL at 8 dph. From 9 to 12 DPH, LMB were slowly transitioned onto Otohime B_1 and Otohime B_2 diets, where they were fed only Otohime B_2 by 12 DPH. Otohime B_2 was maintained until 20 DPH, where LMB reached an adequate size to the begin Skretting starter feed. This final feeding regime was maintained until 24 DPH, when the experiment was terminated.

Rotifers were cultured in a 190 L conical tank at 23°C, a salinity of 17 ppt, and fed a diet of ORI-ONE (Skretting, Tooele, UT, USA) and Nanno 3600 (Reed Mariculture, Campbell, CA, USA) at 0.4 g/million rotifer and 1.3 mL/million rotifer, respectively. *Artemia* were cultured in 12 L hatching cones at 26°C and a salinity of 35 ppt. To ensure proper prey densities, 1 mL samples were taken daily from the rotifer and *Artemia* cultures and counted in duplicate. Rotifer culture followed the batch method, where ~170 L of culture were harvested every five days. *Artemia* were cultured for 24 h, collected using a mesh screen, and rinsed with distilled water before feeding.

2.1.6. Data collection

Morphology

Fish were sampled at 2, 4, 8, 12, 20, and 24 DPH. For each sampling day, 20 random individuals (ten for morphology and ten for molecular analyses) were randomly selected from each replicate and temperature. For this study, 0 DPH is defined as when >50% of eggs have hatched. Fish were euthanized with 200 ppm MS-222 (tricaine methanesulphonate; Argent Laboratories Inc., Redmond, WA, USA) and digital images were obtained using a Zeiss stereomicroscope (sterREO Discovery V12) equipped with 0.5 to 1.0 × objectives and ZEN 2.5 imaging software (blue edition). Measurements were extracted using ImageJ (Version 1.46r) software. Total length (TL, distance from tip of snout to tip of tail), body area (BA, body area excluding fin-fold area and yolk sac), myotome height (MH, body height measured posterior to anus), jaw length (JL), eye diameter (ED), oil droplet area (ODA), and yolk area (YA) and were obtained for each individual. The condition index was calculated by dividing MH by TL (Koslow et al., 1985). Yolk utilization efficiency (YUE) was calculated by dividing the increase in BA from 2 to 4 DPH by the corresponding decrease in YA. In comparison, the yolk utilization rate (YUR) was calculated by the reduction of YA from 4 to 2 DPH divided by the corresponding time interval (Hardy and Litvak, 2004; Politis et al., 2017). At 25 DPH, wet weights (± 0.001 g) were determined for 50 fish per aquaria and tank survival was determined.

Statistical analysis

Data were analyzed using SAS software (v.9.1; SAS Institute Inc., Cary, NC, USA). Residuals were evaluated for normality using Shapiro–Wilk tests and homoscedasticity using plots of residuals vs. predicted values to ensure they met model assumptions. Data were transformed ($\log_{10}$ or arcsine square root), when necessary. To examine the impact of rotifers and *Artemia* on morphometric traits (TL, ED, MH, JL, BA, CI, ODA, and YA), we analyzed data using a series of

repeated measures ANOVAs. If a significant higher-order interaction was detected, separate t-tests were performed at each age to evaluate the effect of rotifers or *Artemia*. The effect of rotifers and *Artemia* on YUE, YUR, survival, and wet weight were determined using a series of two-way ANOVA models. Alpha was set at 0.05. Tukey's post-hoc analyses were used to compare least-squares means between treatments.

2.2. Results

Repeated measures factorial ANOVA models indicated significant Age × Rotifer interactions for TL, EY, MH, JL, and BA (Table 2.1.). Therefore, the saturated ANOVA models were broken down into a series of t-tests to look at the effect of rotifer treatment at each sampling day for these morphometric traits (Fig. 2.2.). There was a significant impact of rotifers for most morphological traits at each dph (all $p \leq 0.0497$), where fish increased in size when offered the rotifer diet. However, no higher-order interactions were significant for CI ($p > 0.097$; Table 2.1., Fig. 2.2.F); the dietary regimen and age main effects were interpreted. Here, LMB were in better condition when fed the rotifer diet. Overall, *Artemia* enrichment did not significantly increase any of the morphometric traits (Table 2.1.).

The Age × Rotifer interaction was significant for both YA ($p < 0.0001$) and ODA ($p < 0.0001$) (Table 2.1.). Therefore, these two saturated models were decomposed into separate t-tests at 2 and 4 dph, where both yolk traits decreased when larvae were offered rotifers. Interactions were not significant for YUE ($p = 0.872$) or YUR ($p = 0.453$), as such main effects were interpreted (Table 2.3.). Here, larvae fed rotifers were most efficient at converting their yolk reserves to body

size and utilized their yolk at the fastest rate, compared to those not fed rotifers (Fig 2.4.). *Artemia* enrichment did not significantly impact any of the yolk-related traits (Table 2.3.).

Rotifers or *Artemia* enrichment did not significantly impact LMB's weight ($p = 0.142$) or survival ($p = 0.275$) up to 25 dph.

2.3. Discussion

The current study showed that LMB larvae fed rotifers exhibited a significant increase in morphometric development and yolk characteristics. More specifically, larvae that were provided rotifers were more efficient at converting their yolk reserves to body size and did so faster. This illustrates that the rotifer diet allowed LMB larvae to transition to exogenous energy sources and increase development quickly. During early life development, one of the most critical stages is the transition from endogenous to exogenous feeding, a key driver to proper behavioral, morphological, and physiological development (Yúfera and Darias, 2007). Proper development is aided by exogenous lipids, proteins, carbohydrates, vitamins, and amino acids crucial for metabolic energy (Divya et al., 2011; Schrama et al., 2018). Failure to provide these essential nutrients during transition often leads to swimming impairments, morphological deformities, and mortality (Gwak and Tanaka, 2001; Dou et al., 2002). Few fin fish larvae accept an artificial diet at first feeding, with catfish and salmonids being the exception; thus, live feed is necessary for LMB (Lovell, 1998). In a similar species, the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), larvae offered a diet regimen of rotifers during first-feeding exhibited an increase in early development and acceptance of formulated dry diet (Ludwig, 2003). Similarly, earlier studies found LMB larvae that were offered various live diets (i.e., rotifers, cladocerans, copepods, and diptera larvae) selected more rotifers from 1 to 4 DPH (Wickstrom and Applegate, 1989).

Consequently, *Artemia* may be too large for LMB larvae to ingest during the early exogenous transition. Thus, rotifers provide the proper live feed size and nutritional requirements. *Artemia* is a preferred live feed for other larval fish species; however, one must consider live prey size and stage of larval development when optimizing larval fish diets(Bengston et al., 1991). *Artemia* nauplii are typically between 400 to 500 μm, where S-type rotifers are 100 to 120 μm and L-type rotifers are 130 to 340 μm (Conceição et al., 2010; Hagiwara et al., 2014; Le et al., 2018). Largemouth bass gape size at 0 to 4 DPH is between 500 to 1,000 μm. However, to prevent damage to the esophagus, larvae tend to select prey 25 to 50% of their gape (Timmerman et al., 2000; Yúfera and Darias, 2007). Thus, offering rotifers as a first-feed followed by *Artemia* or formulated diet may provide better prey receptivity and nutrient boost to improve the performance of LMB during early life stages.

Moreover, our study found LMB that were reared on just an *Artemia* diet at first-feeding resulted in lower morphometric performance, final weights, survival, efficiency at utilizing yolk reserves, and utilization rate. A series of experiments have previously been conducted on first-feeding protocols for LMB, where six candidate diets were evaluated during the exogenous transition (Skudlarek et al., 2013). Results suggested an *Artemia* diet was advantageous and produced higher survival and final weights than larvae that received no *Artemia*. Notably, the current study is among very few studies that have compared rotifers and *Artemia* as a first-feed, with limited information available on larval LMB nutritional requirements (Tidwell et al., 2002). *Artemia* has been extensively researched as the first-feed for many marine and freshwater species; however, the paucity of nutrients (Chakraborty, 2007) requires more research into a suitable first-feed for LMB. As such, the findings of this study illustrate the important role rotifers could serve as a first-feed for LMB and could significantly benefit early life performance.

In conclusion, the introduction of rotifers during the exogenous transition followed by a co-feeding protocol significantly improved intensive larviculture of LMB. Information gained from this study provides insights to improve efficiency and success of LMB larviculure utilizing RAS technology.

References

Bengtson, D.A., Leger, P., Sorgeloos, P., 1991. Use of *Artemia* as a food source for aquaculture. In: Artemia biology, Browne, R.A., Sorgeloos P., Trotman C.N.A, (Eds.) CRC Press, Boca Raton, Florida, pp. 255–285.

Blaxter, J.H.S., Ehrlich, K.F., 1974. Changes in behaviour during starvation of herring and plaice larvae. The Early Life History of Fish. Springer-Verlag Berlin, 575-588.

Chakraborty, R., 2007. Variation in fatty acid composition of Artemia salina Nauplii enriched with microalgae and Backer's Yeast for use in larviculture. Journal of Agriculture and Food Chemistry 55, 4043-4051.

Csargo, I.J., Brown, M.L., Ward, M.J., 2013. Evaluation of four commercial grower diets for production of largemouth bass. Journal of Applied Aquaculture 25, 35-49.

Conceição, L.E.C., Yúfera, M., Makridis, P., Morais, S., Dinis, M.T., 2010. Live feeds for early stages of fish rearing. Aquaculture Research 41, 613–640.

Cooke, S., Philipp, D.P., 2009. Centrarchid Fishes. Diversity, Biology and Conservation. Wiley-Blackwell

Coyle, S.D., Matthews, M.D., 2019. Production of feed trained largemouth bass fingerlings: nursery phase through feed training. In: Tidwell, J., Coyle, S., Bright, L.A (eds.). Largemouth bass aquaculture. 5M Publishing Ltd. Sheffield, UK., 91 -129.

Divya, S.P., Kumar, T.T.A., Rajasekaran, R., Balasubramanian, T., 2011. Larval rearing of clownfish using *Brachionus plicatilis* rotifer as starter food. Science Asia 37, 179-185.

Dou, S., Masuda, R., Tanaka, M., Tsukamoto, K., 2002. Feeding resumption, morphological changes and mortality during starvation in Japanese flounder larvae. Journal of Fish Biology, 60, 1363–1380.

Fuller, A.S., 2020. Minimizing the time fed rotifers maximizes hybrid striped bass larval growth in recirculating aquaculture systems. North American Journal of Aquaculture 82, 208-214.

Gisbert, E., Merino, G., Muguet, B., Bush, D., Piedrahita, R.H., Conklin, D.E., 2002. Morphological development and allometric growth patterns in hatchery-reared California halibut larvae. Journal of Fish Biology 61, 1217-1229.

Gwak, W.S., Tanaka, M., 2001. Developmental change in RNA: DNA ratios of fed and starved laboratory-reared Japanese flounder larvae and juveniles, and its application to assessment of nutritional condition for wild fish. Journal of Fish Biology, 59, 902–915.

Hagiwara, A., Wullur, S., Marcial, H.S., Narisato, H., Sakakura, Y., 2014. Euryhaline rotifer *Proales similis* as initial live food for rearing fish with small mouth. Aquaculture 432, 470–474.

Hardy, R.S., Litvak, M.K., 2004. Effects of temperature on the early development, growth, and survival of Shortnose Sturgeon, *Acipenser brevirostrum*, and Atlantic Sturgeon, *Acipenser oxyrhynchus*, yolk-sac larvae. Environmental Biology of Fishes 70, 145–154.

Hussein, G.H.G., Chen, M., Qi, P.P., Cui, Q.K., Yu, Y., Hu, W.H., Tian, Y., Fan, Q.X., Gao, Z.X., Feng, M.W., Shen, Z.G., 2020. Aquaculture industry development, annual price analysis and out-of-season spawning in largemouth bass *Micropterus salmoides*. Aquaculture 519, 734901

Kamler, E., 2002. Ontogeny of yolk-feeding fish: an ecological perspective. Reviews in Fish Biology and Fisheries 12, 79-103.

Koslow, J.A., Brault, S., Dugas, J., Fournier, R.O., Hughes, P., 1985. Condition of larval cod (*Gadus morhua*) off southwest Nova Scotia in 1983 in relation to plankton abundance and temperature. Marine Biology 86, 113–121.

Kovác, V., 2002. Synchrony and herochrony in ontogeny (of fish). Journal of Theoretical Biology 217, 499-507.

Lavens, P., Sorgeloos, P., 2000. The history, present status and prospects of the availability of Artemia cysts for aquaculture. Aquaculture 181, 397-403.

Lee, I.S., Ohs, C.L., Broach, J.S., DiMaggio, M.A., Watson, C.A., 2018. Determining live prey preferences of larval ornamental marine fish utilizing fluorescent microspheres. Aquaculture 490, 125–135.

Ljubobratovic, U., Kosanovic, D., Demény, F.Z., Krajcsovics, A., Vukotic, G., Stanisavljevic, N., Golic, N., Jeney, G., Lukic, J., 2020. The effect of live and inert feed treatment with lactobacilli on weaning success in intensively reared pike-perch larvae. Aquaculture 516.

Lovell, T., 1998. Nutrition and feeding of fish. New York: Van Nostrand Reinhold.

Ludwig, G.M., 2003. Tank culture of larval sunshine bass, *Morone chrysops* (Rafinesque) × *M. saxatilis* (Walbaum), at three feeding levels. Aquaculture Research 34, 1277-1285.

Olivotto, I., Buttino, I., Borroni, M., Piccinetti, C.C., Malzone, M.G., Carnevali, O., 2008. The use of the Mediterranean calanoid copepod *Centropages typicus* in Yellowtail clownfish (*Amphiprion clarkii*) larviculture. Aquaculture 284, 211–216.

Park, J., Renukdas, N., Luna, T., Roy, L.A., 2015. The effects of biomass density on size variability and growth performance of juvenile largemouth bass, *Micropterus salmoides*, in a semi-closed recirculating system. Journal of World Aquaculture 46, 283-291.

Park, J., Renukdas, N., Luna, T., Roy, L.A., 2017. Maximum yield approximation and size distribution patters of stocker size largemouth bass, *Micropterus salmoides* reared in a semi-closed indoor system. Aquaculture Research 48, 780-791.

Politis, S.N., Mazurais, D., Servili, A., Zambonino-Infante, J.L., Miest, J.J., Sørensen, S.R., Tomkiewicz, J., Butts, I.A.E., 2017. Temperature impacts gene expression and morphological development of European eel, *Anguilla anguilla* larvae. PLos One 12(8).

Roo, J., Hernández-Cruz, C.M., Mesa-Rodriguez, A., Fernández-Palacios, H., Izquierdo, M.S., 2019. Effect of increasing n-3 HUFA content in enriched Artemia on growth, survival and skeleton anomalies occurrence of greater amberjack *Seriola dumerili* larvae. Aquaculture 500, 651–659.

Schrama, J.W., Haidar, M.N., Geurden, I., Heinsbroek, L.T.N., Kaushik, S.J., 2018. Energy efficiency of digestible protein, fat and carbohydrate utilization for growth in rainbow trout and Nile tilapia. British Journal of Nutrition 119, 782-791.

Skudlarek, N., Coyle, S.D., Tidwell, J.H., 2013. Development of first-feeding protocols for indoor larviculture of largemouth bass (*Micropterus salmoides*). Journal of Applied Aquaculture 25, 9-23.

Snow, J.R., 1968. Some progress in the controlled culture of the Largemouth Bass, *Micropterus salmoides*, (Lac.). Southern Division of the American Fisheries Society Annual Meeting.

Tidwell, J.H., Coyle, S. D., Webster, C.D., 2002. Centrarchids: Largemouth bass, Micropterus salmoides. Edited by: C. D. Webster, C. Lim. In *Nutrient requirements and feeding of finfish for aquaculture*, CABI Publishing, Wallingford, UK, 374-380.

Tidwell, J., Coyle, S., Bright, L.A., 2007. Effects of different types of dietary lipids on growth and fatty acid composition of largemouth bass. North American Journal of Aquaculture 69, 257-264.

Tidwell, J.H., Coyle, S.D., Bright, L.A., 2019. Largemouth bass aquaculture. 5M Publishing, United Kingdom.

Tidwell, J.H., Coyle, S.D., Woods, T.A., 2000. Species profile-largemouth bass. SRAC Publication No. 732. Stoneville, MS: Southern Regional Aquaculture Center.

Timmerman, C.M., Annett, C.A., Bailey, C.F., 2000. Determination of factors limiting prey size swallowed by larval and small juvenile largemouth bass. Transactions of the American Fisheries Society 129, 618-622.

United States Department of Agriculture, 2018. 2017 Census of Aquaculture.

United States Department of Agriculture, 2019. 2018 Census of Aquaculture.

United States Fish and Wildlife Service, 2006. National Survey of Fishing, Hunting, and Wildlife-Associated Recreation. US Department of the Interior, US Department of Commerce, US Census Bureau.

Walford, J., Lim, T.M., Lam, T.J., 1991. Replacing live feed with microencapsulated diets in the rearing of seabass (*Lates calcarifer*) larvae: do the larvae ingest and digest protein-membrane microcapsules? Aquaculture 92, 225-235.

Wallus, R., Simon, T.P., 2008. Reproductive biology and early life history of fishes in the ohio river drainage: Elassomatidae and Centrarchidae 6. CRC Press, Boca Raton, FL.

Wang, D., Yao, H., Li, Y.H., Xu, Y.J., Ma, X.F., Wang, H.P., 2019. Global diversity and genetic landscape of natural populations and hatchery stocks of largemouth bass *Micropterus salmoides* across American and Asian regions. Scientific Reports 9.

Wang, Y. Ni, J., Nie, Z., Gao, J., Sun, Y., Shao, N., Xu, G., 2020. Effects of stocking density on growth, serum parameters, antioxidants status, liver and intestine histology and gene expression of largemouth bass (*Micropterus salmoides*) farmed in the in-pond raceway system. Aquaculture Research, 1-13.

Wickstrom, G.A., Applegate, R.L., 1989. Growth and food selection of intensively cultured largemouth bass fry. The Progressive Fish-Culturist 51, 79-82.

Yúfera, M., Darias, M.J., 2007. The onset of exogenous feeding in marine fish larvae. Aquaculture 268, 53-63.

Tables

Table 2.1. Summary of diet treatment effect (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, *f* = *f* value, TL = total length, ED = eye diameter, MH = myotome height, JL = jaw length, BA = body area, YA = yolk area, ODA = oil droplet area, CI = condition index, *p* = *p* value) for Florida largemouth bass (*Micropterus salmoides floridanus*) morphometric traits obtained from a repeated measures factorial ANOVA.

			TL	**ED**	**MH**	**JL**	**BA**	**YA**	**ODA**	**CI**
Treatment	**DFN**	**DFD**	***f***	***f***	***f***	***f***	***f***	***f***	***f***	***f***
Artemia	1	72	0.78	0.08	0.44	0.23	1.23	0.08	1.34	0.08
Rotifers	1	72	270.97**	123.84**	130.27**	139.05**	233.12**	29.16**	326.71**	28.58**
Artemia×Rotifers	1	72	0.19	0.12	0.08	0	0.15	0.08	1.32	0.84
Age	5	72	2768.54**	500.78**	590.33**	788.83**	3757.78**	236.4**	430.78**	65.63**
Age×Rotifers	5	72	18.97**	6.43**	3.21*	7.89**	17.13**	22.14**	31.55**	1.95
Age×*Artemia*	5	72	0.31	0.31	0.22	0.68	0.42	0.01	1.36	0.25
Age×*Artemia*×Rotifers	5	72	0.69	0.18	0.44	0.95	0.46	0.03	0.8	0.34

* $p < 0.001$

** $p < 0.0001$

Table 2.2. Summary of diet treatment effect (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, *f* = *f* value, *p* = *p* value) for Florida largemouth bass (*Micropterus salmoides floridanus*) final weight and survival obtained from a two factor ANOVA.

			Weight		**Survival**	
Treatment	**DFN**	**DFD**	***f***	***p***	***f***	***p***
Artemia	1	11	0.5	0.493	0.2	0.6597
Rotifers	1	11	1.32	0.2745	0.58	0.4634
Artemia×Rotifers	1	11	0.16	0.6973	0.64	0.4393

Table 2.3. Summary of diet treatment effect (DFN = numerator degrees of freedom, DFD = denominator degrees of freedom, *f* = *f* value, YUE = yolk utilization efficiency, YUR = yolk utilization rate, *p* = *p* value) for Florida largemouth bass (*Micropterus salmoides floridanus*) yolk utilization efficiency and yolk utilization rate obtained from a two factor ANOVA.

			YUE		**YUR**	
Treatment	**DFN**	**DFD**	***f***	***p***	***f***	***p***
Artemia	1	11	1.83	0.201	0.04	0.8403
Rotifers	1	11	5.54	0.0365	29.61	0.0001
Artemia*Rotifers	1	11	0.06	0.8047	0.66	0.4318

Figures

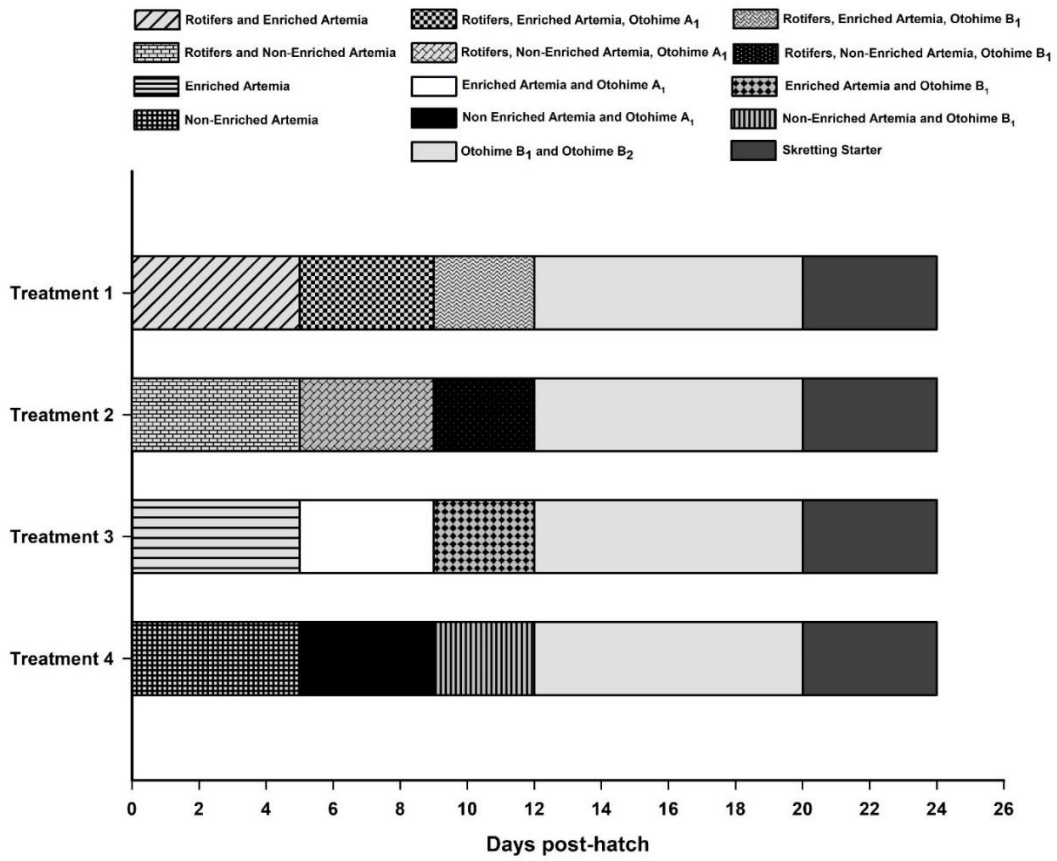
Rotifers and Enriched Artemia
Rotifers, Enriched Artemia, Otohime A1
Rotifers, Enriched Artemia, Otohime B1
Rotifers and Non-Enriched Artemia
Rotifers, Non-Enriched Artemia, Otohime A1
Rotifers, Non-Enriched Artemia, Otohime B1
Enriched Artemia
Enriched Artemia and Otohime A1
Enriched Artemia and Otohime B1
Non-Enriched Artemia
Non Enriched Artemia and Otohime A1
Non-Enriched Artemia and Otohime B1
Otohime B1 and Otohime B2
Skretting Starter
Treatment 1
Treatment 2
Treatment 3
Treatment 4
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Days post-hatch

Fig 2.1. Various diet regimens for larval Florida largemouth bass (*Micropterus salmoides floridanus*). Treatment 1 largemouth bass received rotifers with enriched *Artemia,* Treatment 2 received rotifers with non-enriched *Artemia*, Treatment 3 received only enriched *Artemia,* while Treatment 4 received only non-enriched *Artemia*. All treatments received formulated dry diets.

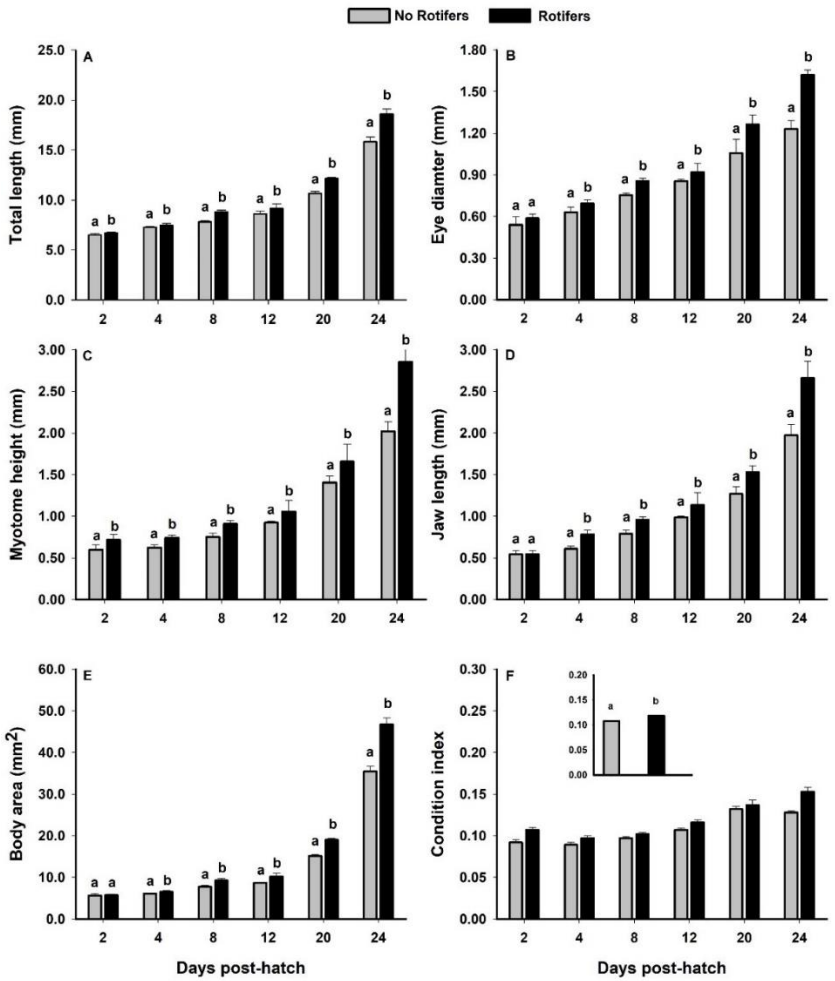

Fig 2.2. Effect of diet regimen on Florida largemouth bass (*Micropterus salmoides floridanus*) total length (A), eye diameter (B), myotome height (C), jaw length (D), body index (E), and condition index (F). Individual ANOVA models were run at 2, 4, 8, 12, 20,

and 24 days post-hatch. Letters represent significant differences among diet treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

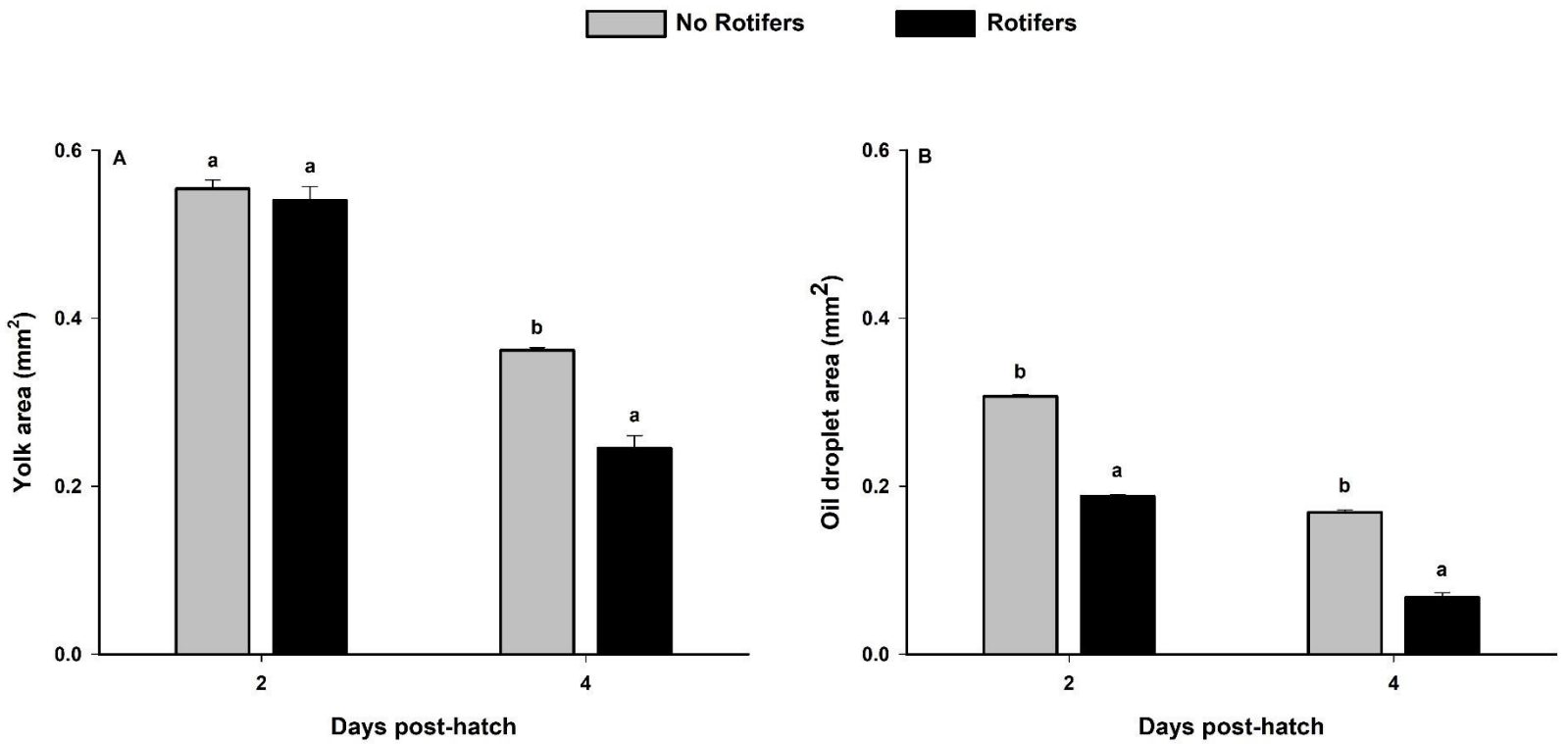

Fig 2.3. Effect of diet regime on Florida largemouth bass (*Micropterus salmoides floridanus*) yolk area (A) and oil droplet area (B). Individual ANOVA models were run at 2 and 4 days post-hatch. Letters represent significant differences among diet treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

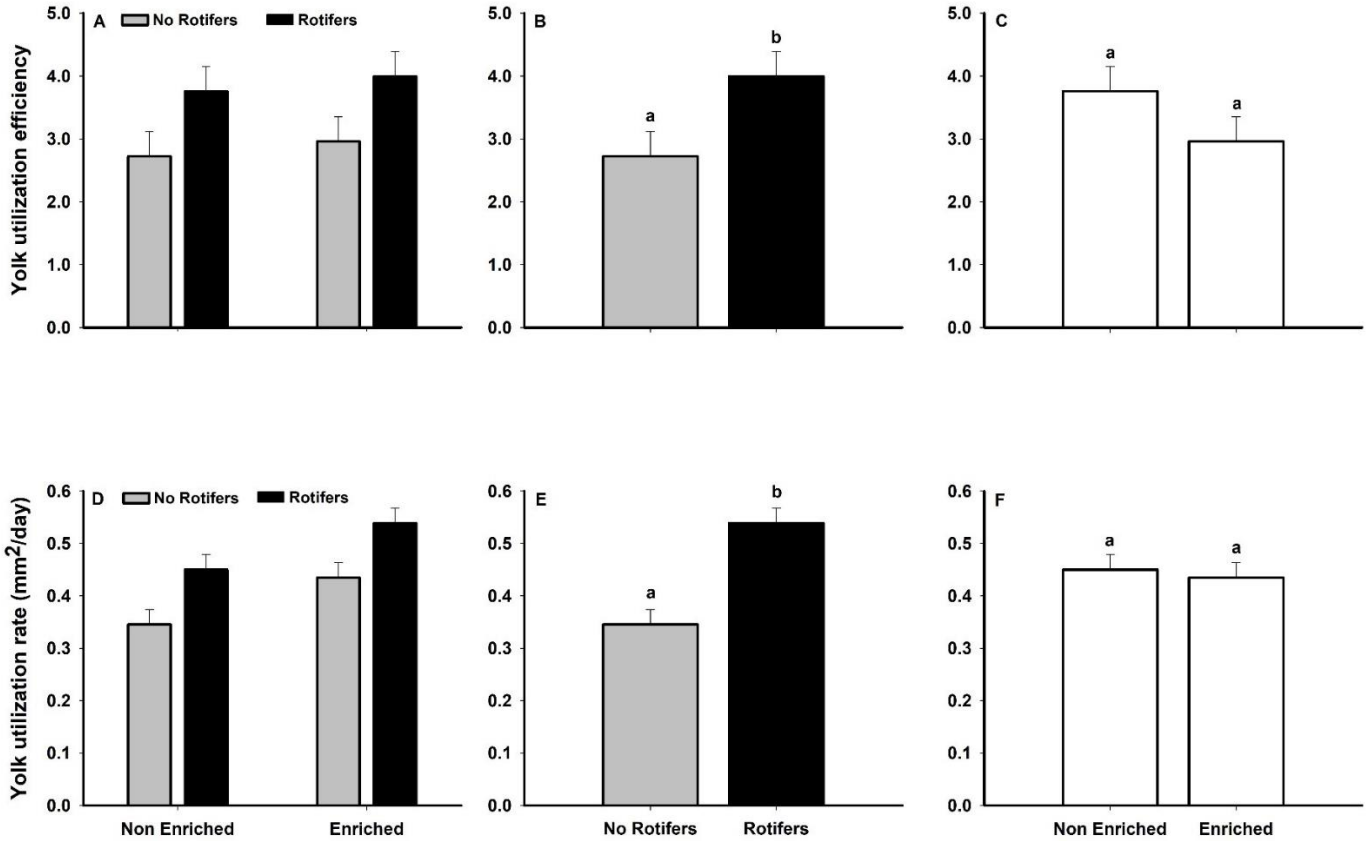

Fig 2.4. Effect of diet regimens on yolk utilization efficiency (A-C) and yolk utilization rate (D-F) of Florida largemouth bass (*Micropterus salmoides floridanus*). A series of three-factor ANOVA models were used to compare yolk metrics for various diet treatments. Letters represent significant differences among temperature treatments ($p < 0.05$). Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).

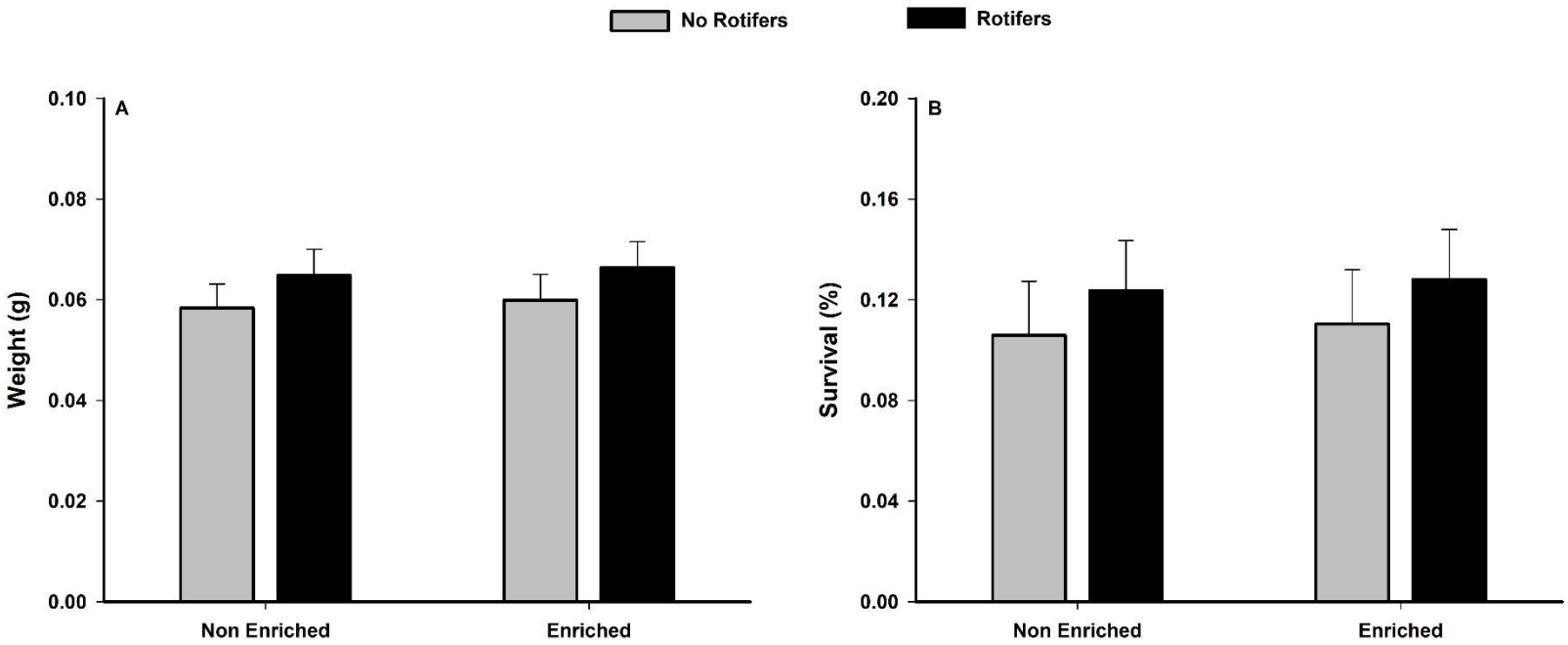

Fig 2.5. Effect of diet regimens on Florida largemouth bass (*Micropterus salmoides floridanus*) individual wet weight (A) and survival (B). These variables were measured at 25 days post-hatch. Error bars represent least square means standard error (Proc Mixed; SAS Institute, 2003).