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Enhancement of Gulf killifish, Fundulus grandis, fitness and reproduction

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ENHANCEMENT OF GULF KILLIFISH, *FUNDULUS GRANDIS*, FITNESS AND REPRODUCTION

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ABSTRACT

Gulf killifish, *Fundulus grandis*, shows promise for commercial development as a marine baitfish species. Significant markets for *F. grandis* already exist throughout the Gulf Coast, with the vast majority of supply coming from wild-harvests, which can be hampered by seasonal availability and inconsistent fish health and size. The genus *Fundulus* also represents an important group of model vertebrates for biological study. A recent review paper described this genus as the premier teleost model for environmental biology. New information generated as a result of the work contained in this dissertation may be more broadly applicable to sister species of *F. grandis*, enhancing the primary goal of improving reproductive output and fitness in cultured individuals of this species. Results of this dissertation include optimization of culture salinity, female broodfish body size, and dietary lipid composition. Optimal salinity for culture of juvenile *F. grandis* was 12.0‰, with growth incrementally increasing between 0.5, 5.0, 8.0, and 12.0‰. Survival was negatively affected at 0.5‰. Optimal body size for female broodfish was 12-13 g. Minimum size recommended for broodfish was 7 g and per-unit-mass fecundity begins to level off in females greater than 13 g. Fecundity of *F. grandis* was less sensitive to manipulations of dietary lipid content than many other fishes. No difference in fecundity was found among fish fed isonitrogenous diets ranging in lipid content from 4.0 to 13.8%. Excess lipid was mostly stored in the intraperitoneal cavity, rather than being partitioned for reproduction. Dietary lipid composition produced very little effect on overall fecundity in *F. grandis*, despite experimental diets with very different fatty acid (FA) composition. Differences did occur in subsequent larvae at extreme physiological conditions, but these variations were unlikely to have any effect on survival in culture or natural settings. Examinations of FA dynamics across time revealed that *F. grandis* likely utilizes a combination of mobilization from

somatic reserves and *de novo* biosynthesis of long chain polyunsaturated FAs to compensate for dietary FA deficiency. Overall, the characteristic physiological plasticity of *F. grandis* also applied to lipid dynamics.

CHAPTER 1
GENERAL INTRODUCTION

The 38 species that currently constitute the genus *Fundulus* (Eschmeyer, 2013) are distributed throughout North and Central America. Fishes of this genus inhabit diverse ecosystems encompassing extremes of abiotic parameters such as dissolved oxygen, pH, temperature, and salinity (Whitehead, 2010). Close phylogenetic relationships between species occupying very different physicochemical conditions make *Fundulus* an opportune genus in which to study the evolution of physiological plasticity in fishes. Burnett et al. (2007) described the estuarine *Fundulus* species of eastern North America, mummichog *F. heteroclitus* and Gulf killifish *F. grandis* (Actinopterygii, Cyprinodontiformes, *Fundulidae*), as premier field and laboratory models for understanding how teleost fishes interact with their environment on an individual and population-level basis. This dissertation seeks to improve understanding of reproductive and early life stage physiology in *F. grandis*, with the potential to utilize new knowledge to improve standard culture procedures for this species or closely related Fundulidae.

In addition to scientific examination, *F. heteroclitus* and *F. grandis* are economically and ecologically important in their respective native ranges. *F. grandis* is abundant in coastal marshes spanning the northern Gulf of Mexico and around peninsular Florida to the northeastern part of that state (Rozas & LaSalle, 1990; Williams et al., 2008). As a small, generalist-feeding species, *F. grandis* creates an important link in estuarine food webs and has been identified as ecologically significant (Subrahmanyan & Drake, 1975). In northeastern Florida, geographic distributions of *F. grandis* and *F. heteroclitus* overlap (Gonzalez et al., 2009); with the latter extending in range up the east coast of North America to the north shore of the Gulf of St. Lawrence, Canada (Bigelow & Schroeder, 1953). Along the northern Gulf of Mexico coast, *F. grandis* is commonly called cocahoe minnow in Louisiana, bull minnow in Alabama, or mud

minnow in other regions. Wild-harvested individuals are widely marketed as marine baitfish, with seasonal inconsistencies in availability creating uncertain market conditions (Green et al., 2010). A workshop report by Oesterling et al. (2004) identified the *Fundulus* species complex as having the highest potential for successful culture as a marine baitfish species in the southeastern United States. This report renewed interest in aquaculture of *F. grandis*, which dates back more than 35 years (Tatum & Helton, 1977; Trimble et al., 1981; Waas & Strawn, 1982).

Reproductive output has been identified as one of the primary impediments to successful culture of *F. grandis* for the live bait market (Patterson et al., 2013). Fecundity of *F. grandis* on a per-unit body mass basis is six-fold lower than the successfully cultured freshwater baitfish, fathead minnow *Pimephales promelas* (Clemment & Stone, 2004; Kumaran et al., 2007; Green et al., 2010). Work targeted at reducing this reproductive bottleneck could increase the viability of *F. grandis* culture, while also improving the ability of investigators to produce animals for scientific experimentation. Research at Louisiana State University has identified culture techniques to take advantage of unique reproductive strategies in *F. grandis*. For example, *F. grandis* culturists can take advantage of the species capacity to produce embryos that incubate out of water in sufficiently humid environments (Coulon et al., 2012) and can manipulate ambient temperature to manage embryonic development rate during this period (Brown at al., 2011). These techniques offer approaches to managing reproduction in *F. grandis* for synchronous hatching of larger cohorts of larvae. However, previous research has not assessed the potential to influence fecundity or early-life stage development and survival at the level of the individual organism.

Various biotic and abiotic factors have the potential to affect reproduction and early-life stage development in fishes. Parameters investigated by hypothesis-driven, replicated research

studies within this dissertation include salinity, maternal body size, lipid content and fatty acid composition of maternal diets. Previous research with many fishes has revealed the importance of these factors in reproductive physiology and indicated that their optimization in *F. grandis* may prove beneficial for aquaculture producers. For example, in euryhaline teleosts it has been observed that a salinity of 10-12‰ corresponds closely with internal osmolality, and thus serves to reduce energy spent on osmoregulation (Sampaio & Bianchini, 2002; Bernatzeder et al., 2008). Conserved energy near this salinity range can subsequently be redirected to other biological processes (Varsamos et al., 2005). Maternal body size has also been shown to affect reproductive output in fishes. Overall fecundity generally increases as female body mass increases, likely because of an increase in available body cavity area and the bioenergetics of larger fish allocating a higher proportion of available energy for vitellogenesis (Jonsson & Jonsson, 1997). Finally, maternal dietary lipid plays an important role in fitness of larval animals, especially in fishes such as *F. grandis* which hatch with considerable yolk reserves. Lipids can serve as the primary energy reserve for reproduction as well as being physically incorporated into eggs (Kadri et al., 1996; Adams & Huntingford, 1997; Xie et al., 1998). Furthermore, optimized inclusion of lipids in diets fed to aquaculture broodfish has been found to increase fecundity in a number of species (Ling et al., 2006; Grisdale-Helland et al., 2008; Sink & Lochmann, 2008; Bentley et al., 2009). Composition of dietary lipid is another important consideration as fishes have varying abilities to biosynthesize certain classes of fatty acids, and those fatty acids considered essential can vary by species (Tocher, 2003). This dissertation covers all of the above factors in *F. grandis*, in addition to investigating the specifics of fatty acid dynamics during spawning.

When testing potential means of maximizing fecundity in a species, it is critical to consider the reproductive strategy employed, as this trait is highly variable among fishes. A brief review of the reproductive physiology of *F. grandis* will provide insight on how we might expect the parameters listed above to influence fecundity and early-life stage development. The euryhaline *Fundulus* species are thought to be recently derived from marine ancestors, and display a remarkable physiological plasticity (Whitehead, 2010) that extends to reproductive behavior. In addition to air incubation, *F. grandis* is able to reproduce across a broad salinity range, with embryos successfully developing and hatching at salinities from 0 to 80‰ (Perschbacher et al., 1990). Both *F. heteroclitus* (Denoncourt et al., 1978; Samaritan & Schmidt, 1982) and *F. grandis* (Simpson & Gunter, 1956) have been observed to establish reproducing populations in landlocked freshwater environments. Relatively large eggs, small body size, and the single ovary of female *F. grandis* physically constrain the number of embryos which can be produced during a single spawning event. This limitation is overcome by rapid oogenesis (Hsiao & Meyer, 1988) and frequent spawns throughout a protracted spawning season (Green et al., 2010). *F. grandis* is characterized as an income breeding species, with rapid turnover of ingested maternal nutrition for oogenesis, as opposed to capital breeding fishes which may spawn annually, semi-annually, or only once in a lifetime (McBride et al., 2013). Based on these observations, we expect to see successful growth and development across a broad salinity range, an influence of maternal body size on fecundity, and rapid effects of dietary lipid content and fatty acid composition on reproduction and larval fitness.

An extension document published by the LSU Agricultural Center entitled “Cocahoe Minnow Production Manual” is available from the AgCenter’s web site and contains details of culture techniques, supplies, and economic analyses of various production systems (Anderson et

al., 2013). The research chapters in this document examined reproductive and early-life history physiology of *F. grandis* across a number of parameters. Individual experiments were carried out in recirculating aquaculture systems in order to isolate fixed effects. Chapter 2 quantified growth, survival, and condition of juvenile *F. grandis* across a hyposmotic salinity gradient while measuring mRNA transcript abundance for three important transmembrane osmoregulatory proteins in the gill. The impetus for this experiment was to determine how an increased requirement for active osmoregulation affected *F. grandis* while examining the physiological basis for these effects. Chapter 3 described the influence of maternal body size on fecundity and the biology of embryos and newly hatched larvae in *F. grandis*. This experiment generated practical recommendations for broodfish management while illuminating unexpected correlations between maternal body size and embryological development. Chapter 4 identifies reproductive response to lipid nutritional status caused by altering dietary lipid levels available to spawning *F. grandis*. Chapter 5 reports results of the manipulation of dietary fatty acid profiles rather than total content. This examination of the effects of broodfish diets lacking long-chain polyunsaturated fatty acids provided insight on lipid metabolism and physiological mechanisms for managing fatty acid deficiency in actively spawning fish. Finally, Chapter 6 also reports effects of manipulated dietary fatty acid profiles on spawning *F. grandis*, but measured changes in tissues and eggs across time from a common baseline to determine rates of specific fatty acid utilization. This method was able to provide a more complete picture of fatty acid dynamics in spawning *F. grandis*. Chapter 7 contains a summary of the dissertation and its major findings.

CHAPTER 2
EFFECTS OF LOW SALINITY MEDIA ON GROWTH, CONDITION, AND GILL ION TRANSPORTER EXPRESSION IN JUVENILE GULF KILLIFISH, *FUNDULUS GRANDIS*[1]

Introduction

Gulf killifish *Fundulus grandis* are abundant in coastal marshes from northeastern Florida to Veracruz, Mexico (Rozas & LaSalle, 1990; Williams et al., 2008) and constitute an ecologically important portion of the estuarine nekton in these areas (Kneib, 1997). This species is a popular live baitfish along the southern Atlantic and Gulf of Mexico coasts where most marketed fish are taken from wild stocks with seasonally inconsistent availability (Green et al., 2010). Egg production per gram female per day in *F. grandis* is at least 6-fold lower than in fathead minnow *Pimephales promelas*, a successfully cultured freshwater (FW) species accounting for 20% of baitfish sales in the United States (Clemment & Stone, 2004; Kumaran et al., 2007; Green et al., 2010). While fecundity is low, *Fundulus* larvae are highly developed at hatch with fully functional eyes and mouthparts (Armstrong & Child, 1965). Larvae also possess functional gills, which take over the task of osmoregulation from the external epithelia shortly after hatching (Katoh et al., 2000), although the precise ontogeny of ion regulation and oxygen uptake in larval gills remains a subject of discussion (Rombough, 2007). Despite knowledge of the ontogeny of embryonic and larval development, few studies have examined early-life stage growth and survival in *F. grandis*.

F. grandis is able to survive and reproduce across a broad salinity range. Embryos will develop and hatch in salinities ranging from 0-80‰ (Perschbacher et al., 1990) and fish have been collected from waters ranging from 0.05-76‰ (Simpson & Gunter, 1956). Salinity has been shown to influence growth rates across all life history stages in many fish species and

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improved growth at intermediate salinities has often been associated with a lower standard metabolic rate (Bœuf & Payan, 2001). Growth of the euryhaline Southern Brazilian flounder *Paralichthys orbignyanus* was reduced in FW, although survival was unaffected (Sampaio & Bianchini, 2002). Research examining cellular and molecular aspects of osmoregulation and their effects on survival in *F. grandis* is valuable for further development of culture methods in this species. Work with the *Fundulus* genus provided much of the early knowledge base on ion transport in the gills of saltwater fish, with the mummichog *F. heteroclitus* as the primary experimental animal (Wood & Marshall, 1994). *F. heteroclitus* is a closely related congener whose range overlaps *F. grandis* in northeastern Florida (Gonzalez et al., 2009). This species has been the subject of extensive work on osmoregulation (Marshall et al., 2002; Wood & Laurent, 2003; Scott et al., 2004; Scott et al., 2005; Wood & Grosell, 2008; Hyndman & Evans, 2009; Flemmer et al., 2010; Whitehead et al., 2011), while considerably less attention has been devoted to salinity effects on *F. grandis*. Metabolic costs of acclimation to FW in adult *F. grandis* were demonstrated by Kolok and Sharkey (1997) who reported significantly lower critical swimming speeds (U_{crit}; Beamish, 1978) in FW acclimated fish versus fish acclimated to 10‰ brackish-water. In addition to reduced U_{crit}, the authors also observed 40% mortality after a swimming challenge in FW and no mortalities in identical brackish-water trials.

Fish in non-isosmotic media must actively compensate for passive diffusion of salts and water between blood and environment. In teleosts, the salinity range which generally minimizes the osmotic gradient between blood and water is ≈10-12‰ and it has been proposed that energy conserved due to reduced osmoregulatory activity near these salinities is redirected for growth (Varsamos et al., 2005). For example, a series of differential salinity exposures estimated the isosmotic point of *P. orbignyanus* at 10.9‰ (Sampaio & Bianchini, 2002) and the plasma

osmolality of juvenile dusky kob *Argyrosomus japonicus* was consistently around 360 mOsm l^{-1} or 12‰ (Bernatzeder et al., 2008). Once the gills are fully developed, they are the main site of osmotic regulation by excretion of ions in seawater and uptake of ions in FW (Perry, 1997; Evans et al., 2005). Within the gills of young juvenile to adult fish, specialized mitochondria-rich cells (MRC, formerly referred to as chloride cells) perform the ion and water trafficking tasks of osmoregulation. In *F. heteroclitus* embryos, MRC are located predominantly in the yolk-sac membrane, with a gradual migration to the external epithelia beginning several days before hatch and MRC shared between external epithelia, gills, and opercular membrane until ~25 days post hatch at which point the gills and opercular membrane dominate ionoregulation (Katoh et al., 2000). A number of transmembrane proteins expressed in MRC perform various osmoregulatory functions and the direction of water and ion flow are dependent upon their apical or basolateral location in the cell membrane (Marhsall et al., 2002; Hiroi & McCormick, 2007; Bodinier et al., 2009b). Recently described ion transport proteins thought to facilitate absorption of ions in hyposmotic media include ClC-3, a member of the ClC chloride channel family (Tang et al. 2010), the Na^+/H^+ exchangers NHE2 and NHE3 (Ivanis et al., 2008; Yan et al., 2007), Na^+/Cl^- cotransporter (NCC), a cation-chloride cotransporter (Hiroi et al., 2008; Inokuchi., 2009), H^+-ATPase (Lin et al., 2006), and the SLC26 anion exchange family (Bayaa et al., 2009). The precise functions of these newly described ion transport proteins are still open for discussion. Three ion transport proteins found in MRC which cooperate to regulate ionic concentration in teleost blood were chosen for this study: Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Marshall and Singer, 2002; Hiroi & McCormick, 2007). Despite their general classification as secretory proteins involved in hyposmoregulation, NKCC1 and CFTR were

chosen for this experiment in *F. grandis* because of interest in examining their potential transcriptional regulation and because many of the proteins involved in ion absorption in euryhaline fishes have been only recently described and their functions are still a subject of discussion. For example, work on ClC-3, the Cl^- transporter which likely plays an absorptive role in hyposmotic environments (Tang et al. 2010), had not yet been published when the present study was conducted. While NKCC1 and CFTR change distribution within MRC dependent upon osmotic status of the animal (Marshall et al., 2002), NKA remains embedded in the basolateral membrane of MRC where it generates an electrochemical gradient through hydrolysis of ATP. This electrochemical gradient is necessary for the activity of other transmembrane proteins, including NKCC1 and CFTR, whose abundance and location determine the rate and direction of ion transfer (Marshall & Singer, 2002; Bodinier et al. 2009b). Reduced growth rates observed in *P. orbignyanus* from a FW environment may have been partially attributable to the high energetic costs of increased NKA activity (Sampaio & Bianchini, 2002). Reduced NKA activity in isosmotic conditions may provide an energetically favorable environment for other teleost species (Lin et al., 2003; Saoud et al., 2007; Partridge & Lymbery, 2008). Previous work with mRNA expression of these three genes in euryhaline fishes provides some background for the present study. Adults of the *F. grandis* congener *F. heteroclitus* up-regulated NKA mRNA in the gills after transfer from brackish water to FW (Scott et al., 2004; Scott et al., 2005). In the euryhaline European sea-bass, *Dicentrarchus labrax*, CFTR mRNA was down-regulated in the gills after transfer from seawater (SW) to FW (Bodinier et al., 2009b). Similarly, expression of NKCC1 was significantly lower in the gills of FW acclimated *D. labrax* than those acclimated to SW (Lorin-Nebel et al., 2006).

Knowledge of the effects of low salinities on growth of juvenile fish could provide practical information for culture of early-life stage *F. grandis* and gene expression analyses may reveal how the effects of physiological compensation at low salinities manifest at the molecular level. The objective of this study was to evaluate the ability of juvenile *F. grandis* to survive and grow across a hyposmotic salinity gradient and to examine long term expression patterns of genes encoding important ion transporters in the gills.

Methods

Experimental systems and water quality

This study was conducted at the Aquaculture Research Station (ARS; Louisiana State University Agricultural Center, Baton Rouge, LA, USA) in four identical, adjacent recirculation systems maintained at salinities of 0.5, 5.0, 8.0 and 12.0‰ using Crystal Sea Marinemix (Marine Enterprises International Inc., Baltimore, MD, USA) . Replicate tanks were 100-L glass aquaria containing 75 L of recirculating water with aeration provided by a single airstone. Photoperiod was set at 13 h light/11 h dark. Water remained at ambient temperature and was monitored independently in each recirculation system using temperature loggers which recorded temperature every 30 min for the duration of the experiment. Each system was serviced by a bubble washed bead filter and a 25 W ultraviolet sterilizer.

Salinity and dissolved oxygen (DO) were recorded every 48 h. Dechlorinated municipal water was used to compensate for evaporation. Total ammonia nitrogen (TAN) and nitrite nitrogen (nitrite) were measured by the salicylate and diazotization methods, respectively. Titration kits were used to test alkalinity and hardness (reported as $CaCO_3$) of the treatment water. Sodium bicarbonate was added to the systems during the experiment to maintain

alkalinity. An ion meter was used to determine pH. Water quality parameters other than salinity and DO were tested once a week for the duration of the experiment.

Stocking and feeding

Juvenile *F. grandis* were obtained from a population cultured at the ARS and held in a single recirculating tank with aeration and 7.0‰ salinity. Prior to the current study fish were fed a commercial starter diet containing approximately 52% protein and 14% lipid. Groups of juvenile fish were directly transferred from 7.0‰ and grown in triplicate at four different salinities; 0.5, 5.0, 8.0, and 12.0‰. The growth trial lasted 12 weeks with gill samples taken after weeks 1, 3, and 7. We stopped gill sampling for gene expression analysis after week 7 because transcriptional regulation was expected to be complete, with expression levels stable by this point. The growth and body condition trial was extended five weeks past the end of gill sampling to allow a sufficient period for treatment effects to fully manifest. Salinities of 0.5, 5.0, and 8.0‰ were chosen to represent varying degrees of hyposmotic stress. Each of the four systems was randomly stocked in triplicate with fish at a mean mass of 0.50 ± 0.01 g. Seventy-five fish were initially used in each replicate for a density of one fish per L. Initially, fish were fed the commercial starter diet, however necropsy of fish sampled after one week revealed extreme hepatic lipidosis and during weeks 2 through 12 a crumbled commercial diet containing approximately 32% protein and 4% lipid was fed to reduce caloric intake among all treatments. Fish were fed 4% of body mass per day divided into morning and afternoon feedings with the amount fed adjusted following biweekly growth sampling.

Sampling procedures

At stocking, wet mass (nearest 0.0001 g) and total length (TL), or the distance from the tip of the snout to the most distal portion of the caudal fin (nearest mm) were determined for a

random sample of 25 individuals per replicate. After initial stocking the same parameters were measured for a random sample of 20 individuals per replicate every 14 days. Length and mass data were used to calculate growth rates as well as relative condition factor at final sampling. Mean relative condition factor was compared among salinities using the calculations of Le Cren (1951) to quantify animal fitness in terms of body mass per unit length. Because all fish were reared in the same conditions prior to being stocked at various salinities, length and mass data from initial stocking was used to create a log transformed plot with the equation:

$$\log M = \log a + b \log L \quad (1)$$

where M is mass (g), L is TL (cm), b is the slope of the line and log a is its intercept. Subsequently, the values a and n were used as constants in the equation:

$$\widehat{M} = aL^b \quad (2)$$

to calculate the expected mass for an individual based on TL. $\widehat{M}$ was calculated for each fish at final sampling and a relative condition factor (K_n) was determined as a ratio of recorded final mass M to predicted expected mass for each individual using the formula:

$$K_n = \frac{M}{\widehat{M}} \quad (3)$$

Specific growth rate (SGR) was calculated by the formula:

$$SGR = 100(\ln M_2 - \ln M_1)/T \quad (4)$$

where M_1 and M_2 are mean initial and final mass, respectively and T is the number of days in the growth trial.

RNA extraction and cDNA synthesis

Following anesthesia with 60 mg/L MS-222, gills were removed from three fish per replicate after weeks 1, 3, and 7. Gill samples from all replicates of a single salinity were pooled

to provide nine samples for each time and salinity combination. The entire gill lamellae was immediately flash-frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

Five of the nine gill samples from each salinity and date combination were randomly chosen for analysis. Frozen organs were ground to a fine powder using a liquid nitrogen cooled mortar and pestle. Approximately 100 mg of this powdered tissue was immediately transferred into 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), vortexed, and passed 10 times through a 20-1/2 gauge needle to ensure complete homogenization. Total RNA was isolated using the TRIzol reagent according to the manufacturer's instructions with slight modification. To increase RNA purity, the TRIzol treatment was repeated on each sample as described by Galvez et al. (2007). After the second TRIzol isolation, RNA from each sample was dissolved in 50 μl of RNAase-free water with concentration subsequently quantified using a NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to synthesize cDNA from 2 μg of total RNA in a reaction volume of 20 μL. RT-PCR was performed using the Applied Biosystems high capacity cDNA reverse transcription kit with RNAase inhibitor (Life Technologies Inc., Carlsbad, CA, USA).

Quantification of gene expression by real-time PCR

The mRNA encoding genes NKA, NKCC1, CFTR, and elongation factor (EF1α) was quantified in duplicate from gills at each of the four experimental salinities and each of three time periods within salinity. The reference gene EF1α has been frequently used in studies on expression of ion transport proteins in fish (Galvez et al., 2007; Bodinier et al., 2009b; Tipsmark et al., 2011) and was employed here to determine relative changes in target gene mRNA. First strand cDNA was quantified using a NanoDrop 1000 and diluted 20 times in nuclease-free water. Quantitative real-time RT-PCR (qPCR) analyses were performed using an iCycler iQ Real-Time

PCR Detection system and the iQ SYBR Green supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). Primers for EF1α and CFTR were designed using Primer Express software (Applied Biosystems, Calsbad, CA, USA) and were reported in Scott et al. (2004). Primers for NKCC1 and NKA were designed using Primer 3 software v 0.4.0 (National Human Genome Research Institute, USA); NKCC1 primers were reported in Meng (2014) and NKA primers were designed for this study (NKA1F,5'- ACTGCCAAGGCCATTGCTAA-3' and NKA1R,5'-AACGACGCAAGCTTTGGCAT-3'). Standard curves were run for each primer by amplifying serial dilutions of a random mixture of experimental cDNA using thermal cycling protocols described below. Efficiencies for all four primers were between 1.8 and 2.0. Samples contained 4 μL nuclease-free water, [5 μM] forward primer, [5 μM] reverse primer, 10 μL iQ SYBR Green supermix, and 5 μL of 1:20 diluted template cDNA. The cycling conditions were: denaturation and hot-start polymerase activation (95 °C, 4 minutes); 50 cycles (95 °C, 10 s; 60 °C, 30 s); melt curve (95 °C to 65 °C in 0.5 °C increments). Samples not producing expected melt curves were discarded from analysis. Critical threshold (Ct) values were automatically calculated for each sample using algorithms in the qPCR software (Bio-Rad). Ct values were normalized and reaction efficiencies were calculated using the program LinRegPCR data analysis program (version 12.5, download: http://LinRegPCR.HFRC.nl, Ruijter et al., 2009). Efficiencies for all reactions were between 85% and 110% with 100% representing a doubling in PCR product after every cycle. A blank control containing water was included with each reaction to test for environmental contamination. Specific primers for NKA, NKCC1, CFTR, and EF1α (GenBank accession numbers AY057072, AY533706 and AF000271, AY430091, respectively) were developed and tested in a closely related species, *Fundulus heteroclitus*. Ct values for EF1α were minimally variable among different salinity treatments at 21.95 ± 0.32, 21.67 ± 0.20, 21.78

± 0.37, and 22.08 ± 0.42 for salinities of 0.5, 5.0, 8.0, and 12.0‰, respectively. This lack of variation coupled with past utilization as a reference gene in similar applications confirms the functionality of EF1α for this experiment. Relative expression of target genes was calculated for 100 copies of the reference gene using the formula of Rodet et al. (2005):

$$N = 100 \times 2^{(\text{Ct reference gene} - \text{Ct target gene})} \quad (5)$$

Statistical analyses

Data were reported as mean ± standard error of the mean (SEM). Relative condition factor (*Kn*) and growth rates were compared among salinities using one-way analysis of variance (ANOVA) and Ryan-Einot-Gabriel-Welsch (REGWQ) post-hoc tests for pairwise comparisons of treatment groups. Survival across the four salinities from stocking until termination of the experiment was assessed using a Chi-Square analysis with the null hypothesis that survival rates should remain constant across treatments. For gene expression data, normality was tested with the Kolmogorov-Smirnov-test and homoscedasticity was tested with the Fisher-Snedecor *F* test. Then, multiple comparisons among salinity treatments and across time periods at the same salinity were performed using one-way ANOVA and Tukey post-hoc tests for pairwise comparisons of treatment groups. All hypotheses were tested at a significance level of $\alpha=0.05$ and all tests were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA).

Results

Water chemistry

Water chemistry parameters are reported in Table 2.1. Temperature in the four adjacent systems was allowed to fluctuate with ambient lab conditions so inter-system temperatures

Table 2.1. Mean (± SEM) water chemistry parameters throughout a 12-week growth trial on juvenile *Fundulus grandis* reared in recirculating aquaculture systems.

Parameter	Nominal Salinity (‰)			
	0.5	5.0	8.0	12.0
Salinity (‰)	0.54 ± 0.01	5.06 ± 0.02	7.94 ± 0.02	11.99 ± 0.03
DO (ppm)	7.01 ± 0.05	6.93 ± 0.05	6.76 ± 0.06	6.67 ± 0.05
pH	8.24 ± 0.04	8.22 ± 0.05	8.33 ± 0.02	8.12 ± 0.04
Hardness (mg/L)[a]	106.0 ± 3.5	336.6 ± 9.4	863.6 ± 12.4	1047.3 ±16.3
Alkalinity (mg/L)[a]	99.7 ± 2.7	101.2 ± 3.4	99.8 ± 3.2	99.3 ± 2.9
TAN (mg/L)	0.62 ± 0.04	0.64 ± 0.03	0.71 ± 0.04	0.69 ± 0.03
Nitrite (mg/L)	0.14 ± 0.01	0.15 ± 0.02	0.13 ± 0.01	0.16 ± 0.01

[a] Reported as $CaCO_3$

would match their conditions to the greatest extent possible. Among the four systems, mean temperature was 26.80 ± 0.12°C.

Growth, survival and condition

Fish mass of *F. grandis* reared at different salinities is detailed in Figure 2.1. Growth is reported as mean change in g per fish sampled. Mass gain and SGR were significantly lower than those of all other treatment levels in the 0.5‰ salinity while the 12.0‰ treatment had significantly higher values of these parameters than the 5.0‰ treatment (Table 2.2). Survival was lowest in fish reared at 0.5‰, and statistical analysis revealed a significant effect of salinity on survival. Final condition factor (*Kn*) was significantly lower in the 0.5‰ treatment (Table 2.2), indicating that fish reared in this salinity had lower body mass per unit length compared to the experimental population.

Relative expression of NKA, NKCC1, and CFTR

Fish reared at a salinity of 0.5‰ had significantly higher NKA mRNA quantity at week 1. After week 1 there were no significant differences in relative expression of NKA mRNA between salinities. From week 1 to week 3, fish from both the 0.5 and 12.0‰ treatments showed

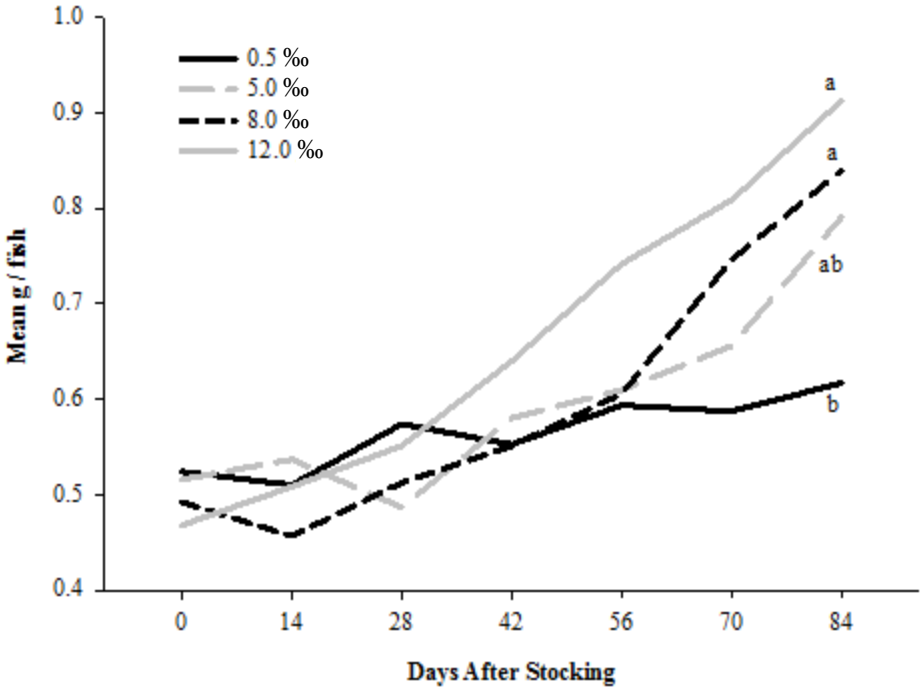

Figure 2.1. Fish mass of juvenile *Fundulus grandis* reared in 0.5, 5.0, 8.0, and 12.0‰ for 84 days. Letters denote statistical significance at final sampling (REGWQ; $p<0.05$).

Table 2.2. Growth, survival, and condition data for juvenile *Fundulus grandis* reared in 0.5, 5.0, 8.0, and 12.0‰ for 84 days. Letters denote statistical significance across parameters (REGWQ; $p < 0.05$).

Parameter	Nominal Salinity (‰)			
	0.5	5.0	8.0	12.0
Initial mass (g)	0.53 ± 0.02^{a}	0.51 ± 0.02^{a}	0.49 ± 0.02^{a}	0.46 ± 0.02^{a}
Final mass (g)	0.62 ± 0.03^{b}	0.79 ± 0.05^{ab}	0.84 ± 0.05^{a}	0.91 ± 0.07^{a}
Mass gain (g)	0.09 ± 0.03^{c}	0.28 ± 0.03^{b}	$0.35 + 0.04^{ab}$	0.45 ± 0.03^{a}
SGR[#] (%/day)	0.18 ± 0.06^{c}	0.51 ± 0.04^{b}	0.63 ± 0.07^{ab}	0.81 ± 0.02^{a}
Survival (%)	59.3	86.5	96.3	89.7
Initial *Kn*[†]	1.053^{a}	1.010^{a}	0.999^{a}	0.989^{a}
Final *Kn*[†]	0.976^{b}	1.091^{a}	1.096^{a}	1.104^{a}

[#]Specific growth rate
[†]Relative condition factor (LeCren 1951)

a statistically significant decrease of NKA mRNA of ≈68% while fish from the intermediate salinities did not show a significant change (Figure 2.2). NKCC1 mRNA quantity was not

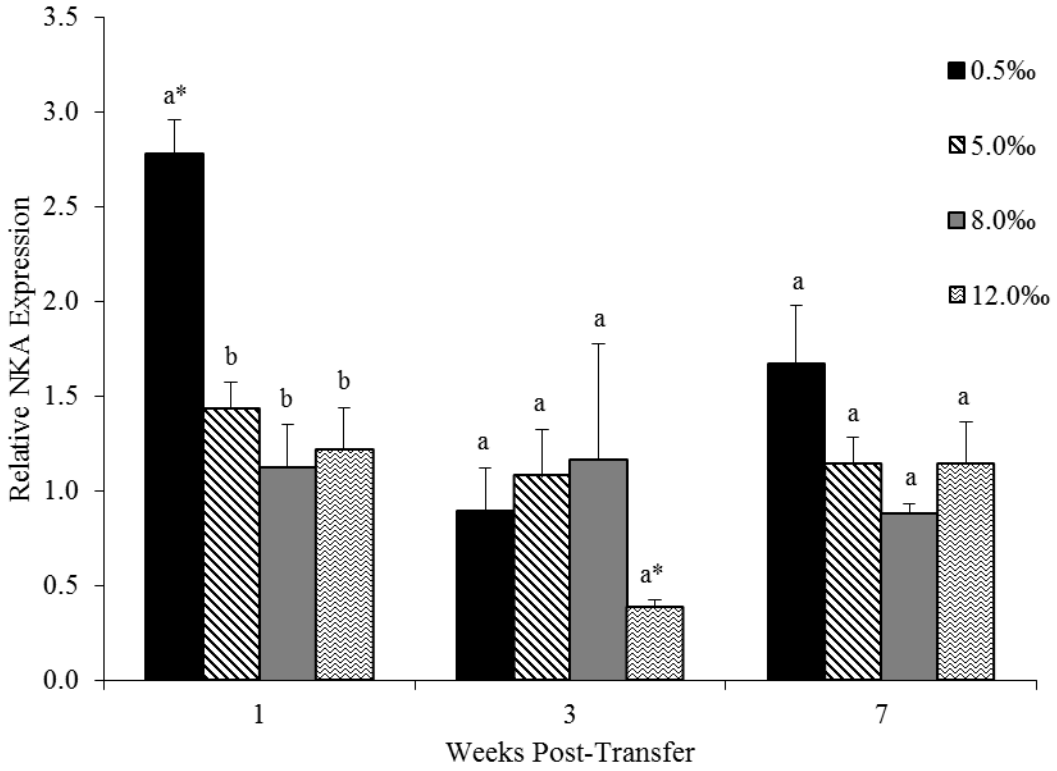

Figure 2.2. Relative expression of NKA in gills after direct transfer from 7.0‰ (n=5 for each point). The reference gene is EF1α. Letters denote statistical significance across salinities at the same time point while an asterisk (*) denotes significance within salinity compared across time points (Tukey HSD; $p<0.05$).

significantly different between salinities at week 1. In week 3 NKCC1 relative expression values were lower in fish reared at 0.5 and 12.0‰ compared with fish from the 5.0 and 8.0‰ treatments, however only the difference at 5.0‰ was statistically significant. Significant NKCC1 decrease of ≈45% from week 1 to week 3 was observed in fish reared at 0.5‰. Over the same time period, non-significant decreases were observed in the higher salinities (Figure 2.3). No significant differences in relative expression of CFTR mRNA were observed between salinities or over time (Figure 2.4).

Discussion

The growth, survival, and body condition data in this study demonstrate the indirect metabolic expense to *F. grandis* juveniles maintaining ion balance in a hyposmotic environment

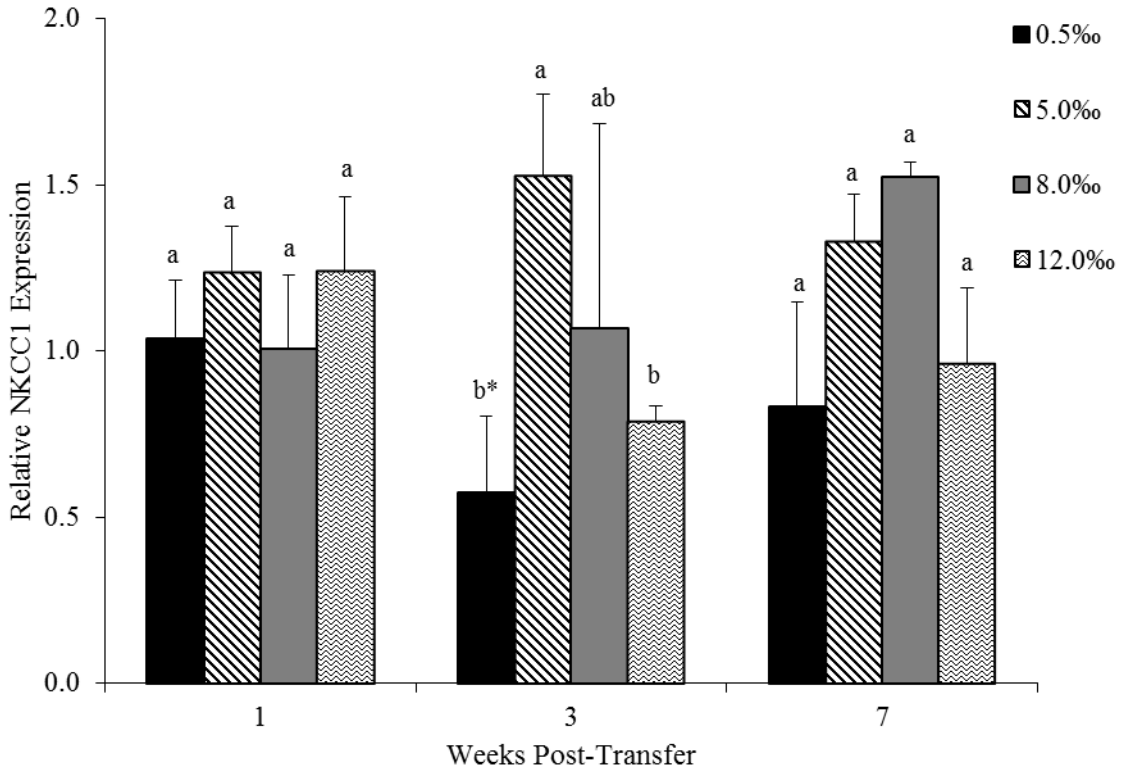

Figure 2.3. Relative expression of NKCC1 in gills after direct transfer from 7.0‰ (n=5 for each point). The reference gene is EF1α. Letters denote statistical significance across salinities at the same time point while an asterisk (*) denotes significance within salinity compared across time points (Tukey HSD; $p<0.05$).

over extended time periods. We observed significantly reduced body condition factor in a recirculating culture setting at low salinity and this effect appears to persist for some marsh dwelling species in the wild. Sailfin molly *Poecilia latipinna* and western mosquitofish *Gambusia affinis* share habitat with *F. grandis*, and individuals of these two species collected from coastal Louisiana marshes demonstrated significantly reduced body condition factors at FW (0.53 ± 0.09‰) compared with brackish (9.32 ± 0.35‰) sites (Martin et al., 2009). Osmoregulatory ability in *F. grandis* may vary by life-stage, as other work has shown significant effects of ontogeny on salinity tolerance in euryhaline teleost species (Watanabe et al., 1985; Varsamos et al., 2005; Bodinier et al., 2009a; Bodinier et al., 2010). Perschbacher et al. (1990) and Kolok and Sharkey (1997) have published experiments that elucidate the adverse

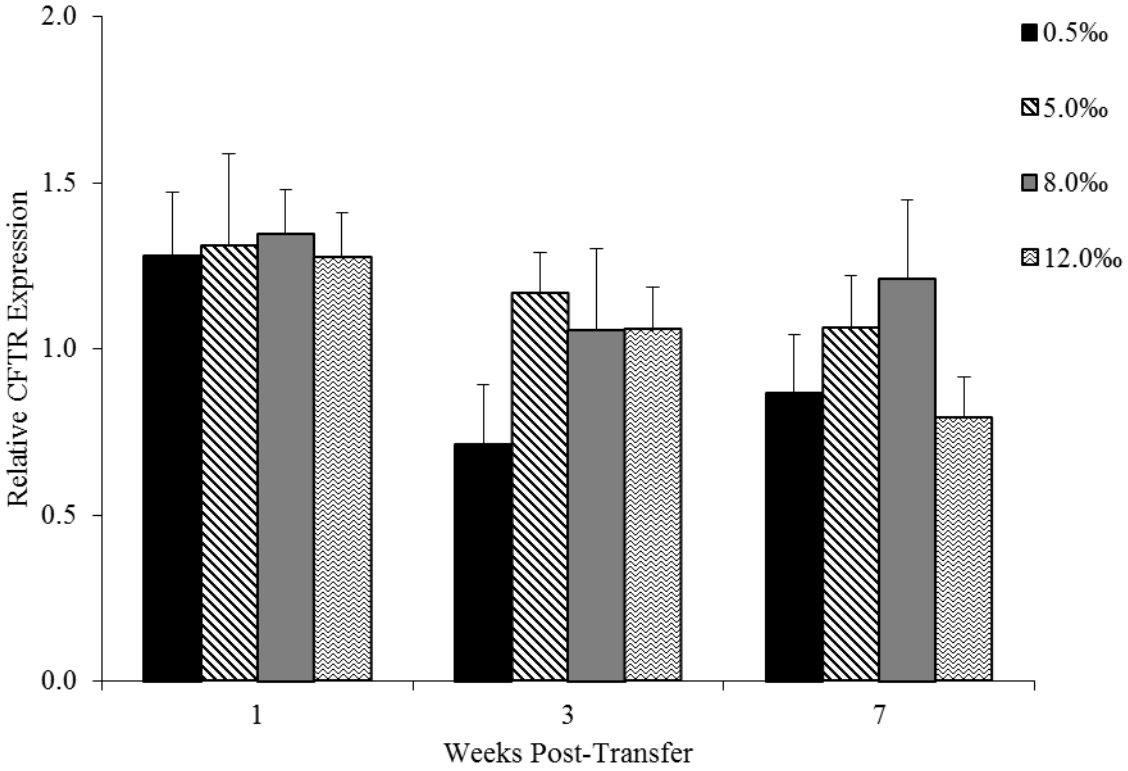

Figure 2.4. Relative expression of CFTR in gills after direct transfer from 7.0‰ (n=5 for each point). The reference gene is EF1α. There was no statistically significant difference across salinities at a time point or within salinities across time points.

physiological effects of hyposmoregulation in *F. grandis* observed for the present study. Perschbacher et al. (1990) found no significant differences in hatching percentage of *F. grandis* eggs across a salinity range from 0 to 35‰ with the exception of a 10‰ trial where fungus associated with that salinity group may have been the cause of low hatch rates. In the same study, larvae in the 0‰ salinity group experienced 40% mortality from hatching until 2.5 weeks post hatch. This level of mortality was significantly greater than larvae reared at 5.0‰, the next incremental salinity level. Kolok and Sharkey (1997) observed 40% mortality in FW acclimated *F. grandis* challenged in a critical swimming speed experiment while fish acclimated and exercised in brackish water (10‰) experienced no mortalities. In the current study, mean mortality across the three 0.5‰ replicates was 40.7% over a 12-week period.

Phenotypic and physiological differences may exist among a subset of individuals which hinder their ability to successfully maintain ion balance in FW (Whitehead et al., 2011). Previous work has identified differential intraspecific adaptations to FW acclimation in juvenile *D. labrax* resulting in mortality for that proportion of individuals unable to properly coordinate ion conservation (Nebel et al., 2005). In the kidneys of non-acclimating *D. labrax*, NKA abundance and activity was reduced, and fish were unable to produce hypotonic urine. *Dicentrarchus labrax* attempted to compensate for this inability to conserve ions at the kidney by increasing active absorption of ions at the gills. In this scenario, NKA abundance and activity increased in the gills in those individuals unable to regulate at the kidney, and an excessive proliferation of branchial MRC was observed, possibly to the point of interfering with gas exchange (Nebel et al., 2005). The individual level differences in osmoregulatory ability observed in *D. labrax* may exist in *F. grandis* but have yet to be identified.

In *F. grandis* larvae and juveniles, a critical salinity where osmoregulation creates difficulty maintaining homeostasis appears to exist somewhere below 5.0‰. Whitehead et al. (2011) reported that *F. heteroclitus* experienced an energetically taxing transition in salinities at or below 1‰ which caused wild populations to adapt and reside in osmotic niches on either side of a ≈0.5‰ salinity barrier. Parent fish of the cultured *F. grandis* population in this experiment were taken from coastal Louisiana brackish water marshes, and thus faced no selective pressure towards FW adaptation. Inland FW populations of *F. grandis* have been documented (Campbell et al., 1980), and fish from these populations may have an improved ability to maintain internal ion concentrations in a hyposmotic environment. The present study produced satisfactory growth and body condition in juvenile *F. grandis* at salinities of 5.0‰ or greater but a significant reduction in survival, SGR, and body condition occurred at 0.5‰. Further work with *F. grandis*

in the 0.5 to 5.0‰ salinity range could identify an inflection point where modification of gill morphology and energy used on osmoregulation reduces the ability to effectively culture this species.

Much of the energy used in osmoregulation is devoted to synthesis of ion trafficking proteins and active maintenance of an electrochemical gradient by NKA (Hwang & Lee, 2007). While cells of the kidney, intestines, and opercular epithelia are known to function in ion transfer between animal and environment (Nebel et al., 2005; Scott et al., 2005; Scott et al., 2006) gene expression was examined in the gills because this organ has been identified as the primary site of osmoregulation in juvenile and adult fishes (Katoh et al., 2000). Due to its necessity in maintenance of an intercellular electrochemical gradient in non-isosmotic condition, NKA can be used as an index or marker of overall ionoregulation in the gills (Evans et al., 2005; Bodinier et al., 2010; Ostrowski et al., 2011). In the present study relative NKA expression was significantly higher at 0.5‰ relative to the higher salinities at 7 days after direct transfer from 7.0‰. Scott et al. (2005) reported a statistically significant two-fold increase in activity of NKA in the gills of *F. heteroclitus* at 12 h, 3 d, and 7 d after transfer from 10% SW to FW. In a separate study on *F. heteroclitus*, NKA was significantly upregulated by two-fold at 4 d post transfer from brackish water to FW and expression levels had dropped by 30 d post transfer (Scott et al., 2004), a pattern consistent with observations of *F. grandis* NKA expression in the present study. NKCC1 is generally thought to be the secretory isoform of the protein while NKCC2 or other forms may be active in Cl^- uptake in some fishes (Hwang & Lee, 2007; Hiroi et al., 2008). At one week, relative expression of NKCC1 in *F. grandis* was not elevated or depressed at any salinity. Marshall et al. (2002) suggested that modifications of NKCC in *F. heteroclitus* are relatively slow following a salinity transfer. Statistically significant down-regulation of the gene

encoding NKCC1 was observed at week 3 for the 0.5‰ salinity as could be expected for an ion secretory protein in the gills of a FW acclimating fish. Scott et al. (2004) observed downregulation of NKCC1 after FW transfer in *F. heteroclitus*, although the change in expression level occurred quickly and was more transient than observed in the present study. CFTR has been immunolocalized to the basolateral membrane of MRC in freshwater killifish suggesting a potential mechanism of Cl^- exit into the blood (Marshall et al., 2002). However, CFTR is associated with hyposmoregulation in most euryhaline and marine species and down-regulation of the gene or decreases in protein abundance upon FW transfer have been observed in *D. labrax* and Hawaiian goby, *Stenogobius hawaiiensis* (McCormick et al., 2003; Lorin-Nebel et al., 2006; Bodinier et al., 2009a;). In *Fundulus*, the method of Cl^- regulation may be different. Scott et al. (2004) observed relocation of CFTR proteins within MRC after transfer from brackish water (10‰) to FW and SW. It is also possible that the absence of variation in expression of CFTR mRNA is due to the existence of other chloride channels such as ClC-3 or the SLC26 family (Bayaa et al., 2009; Tang et al., 2010). More recent evidence suggests that *F. grandis* may not actively uptake Cl^- from surrounding media in hyposmotic conditions. Rather, the animal may obtain this ion from intracellular cytoplasm in the short term and diet in the long term, coupled with a tightening of leaky para-cellular junctions to reduce loss to the environment (Meng, 2014). For both CFTR and NKCC1, a reduced interval until first sampling may have produced observable gene regulation results. It is possible that sampling fish 24 or 72 hours after transfer would have shown regulation in these genes, and that this regulation is complete at 7 d. Future work could utilize more frequent sampling early in the exposure period to examine possible expression changes for CFTR and NKCC1 in *F. grandis*. Across all three genes, the intermediate salinities of 5.0 and 8.0‰ did not produce a statistically significant difference in

expression from each other or over time. Transfer from 7.0‰ to either of these intermediate salinities may not represent a biologically significant change in osmolarity strong enough to produce observable variations in transcriptional regulation of the ion trafficking proteins examined in the current study.

When analyzing relative expression of genes from fish in the 0.5‰ salinity treatment it is necessary to consider the 40.7% mortality observed at that salinity. Although mortalities were not individually recorded, they were observed throughout the duration of the experiment. If differential intraspecific acclimation exists in *F. grandis* at near-FW conditions as discussed in section 4.2 for juvenile *D. labrax* (Nebel et al., 2005), sampling which occurred at later dates would have had an increased likelihood of choosing an individual with good ability to ionoregulate in FW as those with poor ability were less likely to survive. Relative expression of NKA at 0.5‰ strongly downregulated from week 1 to week 3 and stabilized at week 7. Statistically significant downregulation was also observed in NKCC1 at 0.5‰ from week 1 to week 3 with stabilization at week 7. The general trend of stabilization in gene expression over time observed at 0.5‰ salinity may be partially attributable to mortality of over expressing individuals attempting to compensate for an inability to maintain osmotic homeostasis for other reasons, such as renal ion excretion. In addition, more persistent modifications in protein abundance as an end result of transient increases in mRNA transcription may have already occurred by week 3. The present study examined mRNA transcription in *F. grandis* gills over a longer time frame than that utilized by many past experiments. Most researchers have tested short-term effects of salinity acclimation on osmoregulatory gene expression because after 7 to14 days mRNA transcriptional activity is assumed to stabilize.

It is clear that *F. grandis* has the ability to hyperosmoregulate, but the metabolic challenges of doing so produce adverse effects on growth. Elevated expression of NKA at 0.5‰ salinity shows how this metabolic demand can manifest on a cellular and molecular level. In Louisiana, where saltwater intrusions are becoming more frequent and persistent (Shaffer et al., 2009), *F. grandis* populations which reside on the FW side of a ≈0.5‰ salinity barrier could be replaced by less FW tolerant conspecifics. In a culture situation without ready access to saline water a balance must be reached between the expenses of maintaining ideal water ion concentrations and reduced growth observed as salinity decreases. This study casts doubt on the ability to successfully grow *F. grandis* at salinities of 0.5‰ or lower, although more FW tolerant populations may exist or may be possible to produce through selective breeding. Further work should be able to determine a critical salinity below 5.0‰ where culture is no longer feasible.

CHAPTER 3
INTRASPECIFIC VARIATION IN REPRODUCTIVE POTENTIAL WITH MATERNAL BODY SIZE IN GULF KILLIFISH, *FUNDULUS GRANDIS* [2]

Introduction

A matrix of regulatory, economic and biological factors were used to identify Gulf killifish *Fundulus grandis* as a member of the marine baitfish group with the greatest potential for commercial culture and live-marketing to anglers in the southeast U.S. (Oesterling et al., 2004). Reproductive limitations due to low fecundity are the primary impediment to development of successful commercial culture of Gulf killifish. On a per unit body mass basis, fecundity of cultured *F. grandis* is less than one-sixth of the fathead minnow *Pimephales promelas*, a successfully cultured freshwater species which accounts for 20% of baitfish sales in the U.S. (Clemment & Stone, 2004; Kumaran et al., 2007; Green et al., 2010). Culture methods directed at optimizing reproduction in *F. grandis* and congeners such as Seminole killifish *F. seminolis* and mummichog *F. heteroclitus* would facilitate increased production of this species group.

In a given fish species, overall fecundity generally increases with increasing female mass due to an increase in body cavity area available for oocyte formation and a greater amount of energy available for egg production (Jonsson & Jonsson, 1997). Volume or mass of individual eggs has also been shown to increase with increasing maternal size in many fishes including brown trout *Salmo trutta* (Jonsson & Jonsson, 1999), Atlantic cod *Gadus morhua* (Kraus et al., 2000), mottled sculpin *Cottus bairdi* (Grossman et al., 2002), and walleye *Sander vitreus* (Johnston & Leggett, 2002). More recently a study of bullhead *Cottus gobio* found that intraspecific variation in egg size was linked to maternal age whereby, older, larger females produced eggs of reduced size relative to younger, smaller fish (Abdoli et al., 2005). These

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results involving effects of female age on egg size may have broader implications for other species. The correlation between female body size and egg size is still a subject of discussion and some species exhibit no such association (Braga-Goncalves et al., 2011). For species which have a positive correlation of body mass or age with egg size, larger eggs require a longer developmental period before hatch (Pauly & Pullin, 1988; Gillooly et al., 2002; Kolm & Ahnesjö, 2005).

Intraspecific variations in embryo characteristics and fecundity associated with female body size have important implications for individual phenotypes in subsequent life history stages and broader scale species population dynamics (Heming & Buddington, 1988; Gisbert et al., 2000; Einum & Fleming, 2002). Long standing theory states that for eggs which incubate in water, size is limited by the ability to obtain oxygen through diffusion because oxygen requirements are determined by egg volume while capacity to acquire oxygen is determined by chorion surface area, which increases proportionally less than volume (Krogh, 1959). However, research has found that embryos derived from larger eggs are not necessarily at a competitive disadvantage during aquatic incubation (Einum et al., 2002). Post-hatch, larvae from larger eggs likely have a competitive advantage over their smaller counterparts. Larvae emerging from larger eggs are expected to be bigger or possess more endogenous nutritional resources, leading to higher survival due to increased swimming performance and resistance to starvation (Bagenal, 1969; Einum & Fleming, 1999; Heath et al., 1999; Kolm & Ahnesjö, 2005).

In addition to interest in production of *F. grandis* and other Fundulidae for use as live bait (DiMaggio et al., 2009), fishes of the genus *Fundulus* have been increasingly cultured for use as model organisms in physiology and environmental biology research (Burnett et al., 2007). The natural habitat, physiology and genetics of these two species are highly similar and

experimental results may be at least partially transferrable between species. Most reproductive studies of *F. grandis* have focused on fractional semi-lunar spawning periodicity or environmental impacts on reproductive potential (Hsiao & Meier, 1989; Nordlie, 2000; Landry et al., 2007; Takemura et al., 2010; Brown et al., 2011; Coulon et al., 2012; Gothreaux & Green, 2012; Tiersch & Yang, 2012). As previously introduced, maternal effects on reproductive potential in fishes are of critical importance and have received extensive study in many species, however, maternal effects have not been thoroughly examined in *Fundulus*.

The objective of the present study was to elucidate the effects of maternal body size on reproductive investment in a captive population of *F. grandis* and examine how those variations may affect embryo incubation and the morphometrics of newly hatched larvae. The results of this study are expected to improve ability to predict fecundity using body size in *F. grandis*. Positive relationships of body size to total fecundity, gonadosomatic index (GSI), individual egg size, and duration of incubation period were expected. Larvae hatched from larger eggs were expected to possess traits which would position them for higher survival rates in either captive culture or natural settings.

Methods

Experimental animals and system

All animals were handled in accordance with European Union directive 2010/63/EU. Adult female *F. grandis* were graded into three size classes based on body mass. Mean body mass for each size group was 18.0 ± 0.3, 12.9 ± 0.3, and 6.9 ± 0.3 g for nominal large, medium, and small classes, respectively. Mass of the small size class was chosen based upon necropsies in our laboratory which suggested this was the lowest mass at which female *F. grandis* reliably reached sexual maturity. Female size classes were randomly assigned in quadruplicate to

adjacent glass aquaria in a recirculation system. Individual aquaria were wrapped in black polyethylene plastic sheeting to prevent inter-replicate interactions among experimental animals. A 2:1 female to male sex ratio was achieved in each replicate by stocking six females with three males, which were randomly selected from a group of males with mean body mass of 14.1 ± 0.2 g. Replicate tanks were 100-L glass aquaria each containing a single airstone and 75-L of water at a salinity of 12‰, which was maintained using Crystal Sea Marinemix (Marine Enterprises International Inc., Baltimore, MD, USA) and dechlorinated water to compensate for evaporation. The experimental system was serviced by a bubble washed bead filter and 50-W ultraviolet sterilizer. Dissolved oxygen (DO) was monitored every 48 h with a YSI 85 meter (YSI Inc., Yellow Springs, OH, USA). Total ammonia nitrogen and nitrite nitrogen were measured by the salicylate and diazotization methods, respectively while alkalinity (as $CaCO_3$) was measured by titration kit (Hach Co., Loveland, CO, USA) and pH was measured with an Oakton Ion 700 meter (Oakton Instruments, Vernon Hills, IL, USA). Water chemistry parameters other than DO were measured weekly. All water chemistry parameters remained within acceptable limits for *F. grandis* (Tatum et al., 1982). Fish were fed to satiation twice daily with a "Tilapia Grower" diet containing 34% protein and 6% lipid (Burris Aquaculture Cargill Animal Nutrition, Franklinton, LA, USA). Satiation was defined as lack of further diet consumption after three minutes with uneaten diet present in the tank.

Spawning and reproductive assessment

Photoperiod and water temperature manipulation were used to induce spawning during a two week period. Water was warmed at a rate of 0.5° C per day from an initial temperature of 19°C to a spawning temperature of 26° C. Simultaneously, day length was increased by 15 minutes per day using laboratory light timers, from an initial photoperiod of 11.5 h light:12.5 h

dark to a spawning photoperiod of 13 h light:11 h dark. Spawning temperature and day length were chosen to approximate environmental conditions experienced during peak spawning periods for captive populations held outdoors (Green et al., 2010). After initial manipulations, water temperature and day length remained constant throughout the experimental spawning period. Spawning substrates were made by inserting 7x15 cm rectangles of Spawntex (Blocksom and Co., Michigan City, Indiana) into 15 cm lengths of 9 cm diameter polyvinyl chloride tubes. Each replicate received one substrate horizontally oriented on the bottom of the tank immediately following photoperiod and temperature manipulation. Embryos were collected from substrates every 72 h for six weeks beginning 10 January 2011.

The number of embryos from each replicate was recorded at each collection date and embryos were photographed under a dissecting microscope using MetaVue 6.1 image processing software (Molecular Devices, Sunnyvale, CA, USA). A micrometer was photographed with each set of embryos at an identical magnification for image analysis. All eggs from each replicate at each collection were incubated in separate plastic weigh boats containing ~25 mL of recirculation system water held at room temperature (~22.5° C). Incubation water was changed at 48-h intervals to reduce the accumulation of metabolites. Days-to-hatch for each embryo were determined by calculating the number of days between collection and hatch. Newly hatched larvae were immediately bottled in 10% neutral buffered formalin and refrigerated for future photographic processing with methods identical to those used for embryos. After the six week spawning period three randomly selected females from each replicate were euthanized with an overdose of tricaine methanesulphonate. Whole body wet mass and gonad mass were determined for calculation of GSI (I_G) using the formula

$$I_G = \frac{W_g}{W} \quad (6)$$

where W is total body mass inclusive of gonads (g) and W_g is gonad mass (g).

Image analyses

Morphometric analysis of embryonic and larval images was performed using Image Tool 2.0 software (The University of Texas Health Science Center, San Antonio, TX, USA). For embryonic analysis, software was calibrated using the micrometer and instructed to detect the length of the longest (polar) axis and shortest (equatorial) diameter for each embryo. Chorion volume was subsequently calculated using the formula for the volume of a prolate spheroid

$$V = \left(\frac{\pi}{6}\right) a^2 b \quad (7)$$

where V is the volume in units cubed, a is the equatorial diameter, and b is the length of the polar axis. Larval images were measured using identical software and calibration methods. Figure 3.1 illustrates orientation of larvae and location of morphometric measurements. Standard length (SL), or length from the most distal point on the head to the tip of the caudal peduncle, and the two dimensional area of the body cavity (BCA) containing the yolk-sac and internal organs were measured. BCA was calculated as the area inside the traced outline represented in Figure 3.1. BCA is used rather than yolk volume because it is not possible to discern between the yolk and internal organs, but serves as a useful proxy for endogenous nutritional resources. In order to best elucidate differences between size class treatments and avoid variation associated with length of incubation period, a modal hatch period was utilized whereby the first third and last third of larvae to hatch from each replicate on a given collection date were not included in image analysis.

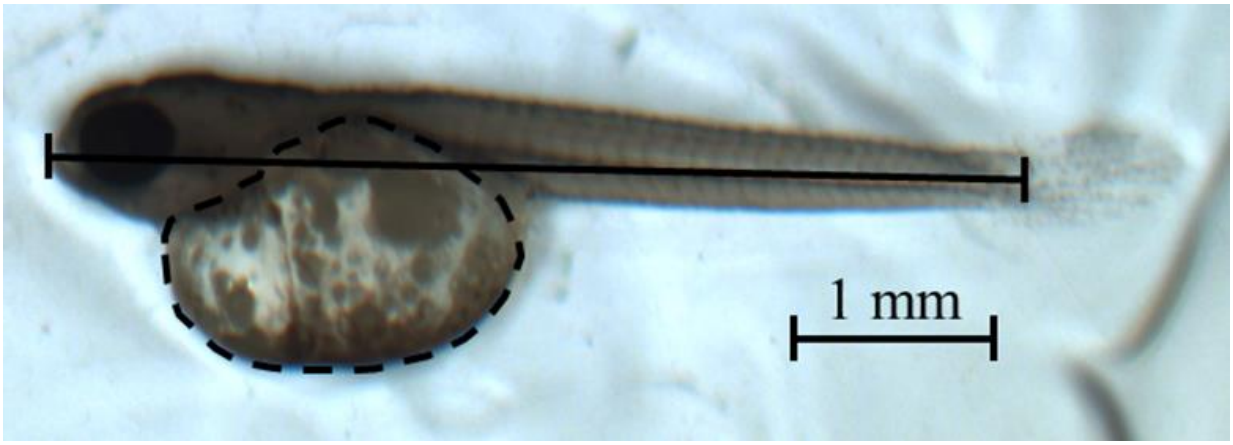

Figure 3.1. Example determination of newly hatched larval morphometrics. Solid line represents standard length and two-dimensional area inside dashed line represents body cavity area.

Statistical analyses

Statistical tests were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA). Means are presented as mean ± SEM. Normality of GSI data was assessed with the Shapiro-Wilk test. GSI data were transformed as arcsin(square root *y*) and periodic fecundity count data were transformed as square root(*y*+1). Correlation of GSI to female body mass was tested with simple linear regression. Differences in periodic fecundity of body size groups were analyzed by mixed model repeated measures analysis of variance (ANOVA) with the Tukey HSD *post-hoc* test. Survival to hatch of embryos across treatment levels was analyzed by logistic regression. Mean values of time to hatch, chorion volume, larval SL, and larval body cavity area were compared across treatment levels using a generalized linear mixed model ANOVA (proc GLIMMIX) with the Ryan-Einot-Gabriel-Welch (REGWQ) *post-hoc* test. All statistical tests were performed at a significance level of $\alpha = 0.05$.

Results

Fecundity and GSI

Periodic fecundity was significantly affected by female broodfish body size (repeated measures ANOVA, $F_{\text{body size}} = 32.42$, $p < 0.001$; $F_{\text{body size x collection date}} = 1.48$, $p = 0.06$). Large females had significantly higher fecundity than medium and small females while fecundity in the medium size class was significantly greater than the small size class (Tukey HSD *post-hoc*, $p \leq 0.010$ for all pairwise comparisons). Figure 3.2 displays these periodic fecundity data and illustrates the characteristic cyclic nature of fractional spawning in *F. grandis*. Mean GSI values for the female broodfish size classes were 4.66 ± 0.43, 4.54 ± 0.60, and 2.13 ± 0.39 for large, medium, and small females, respectively. GSI data were not normally distributed (Shapiro-Wilk, p=0.048), therefore transformed GSI values were plotted against female body mass (Figure 3.3). Correlation of GSI to female body mass was weak but statistically significant (simple linear regression, $R^2 = 0.405$, $t = 4.74$, $p < 0.001$).

Eggs and newly hatched larvae

Table 3.1 contains data describing mean egg volume, time to hatch, and percent hatch by maternal size class. Egg volume was significantly influenced by female body mass (proc GLIMMIX ANOVA, $F = 19.33$, $p < 0.001$). Time to hatch for those eggs surviving to hatch also had a significant positive relationship with female body mass (proc GLIMMIX ANOVA, $F = 30.49$, $p < 0.001$). No significant differences existed in survival to hatch for embryos from different maternal body mass treatments (logistic regression, Wald $X^2 = 0.48$, $p = 0.788$). SL and BCA of newly hatched larvae were significantly different among maternal body size treatment levels (proc GLIMMIX ANOVA, $F = 12.22$, $p < 0.001$ and $F = 8.43$, $p < 0.001$, respectively) (Figure 3.4).

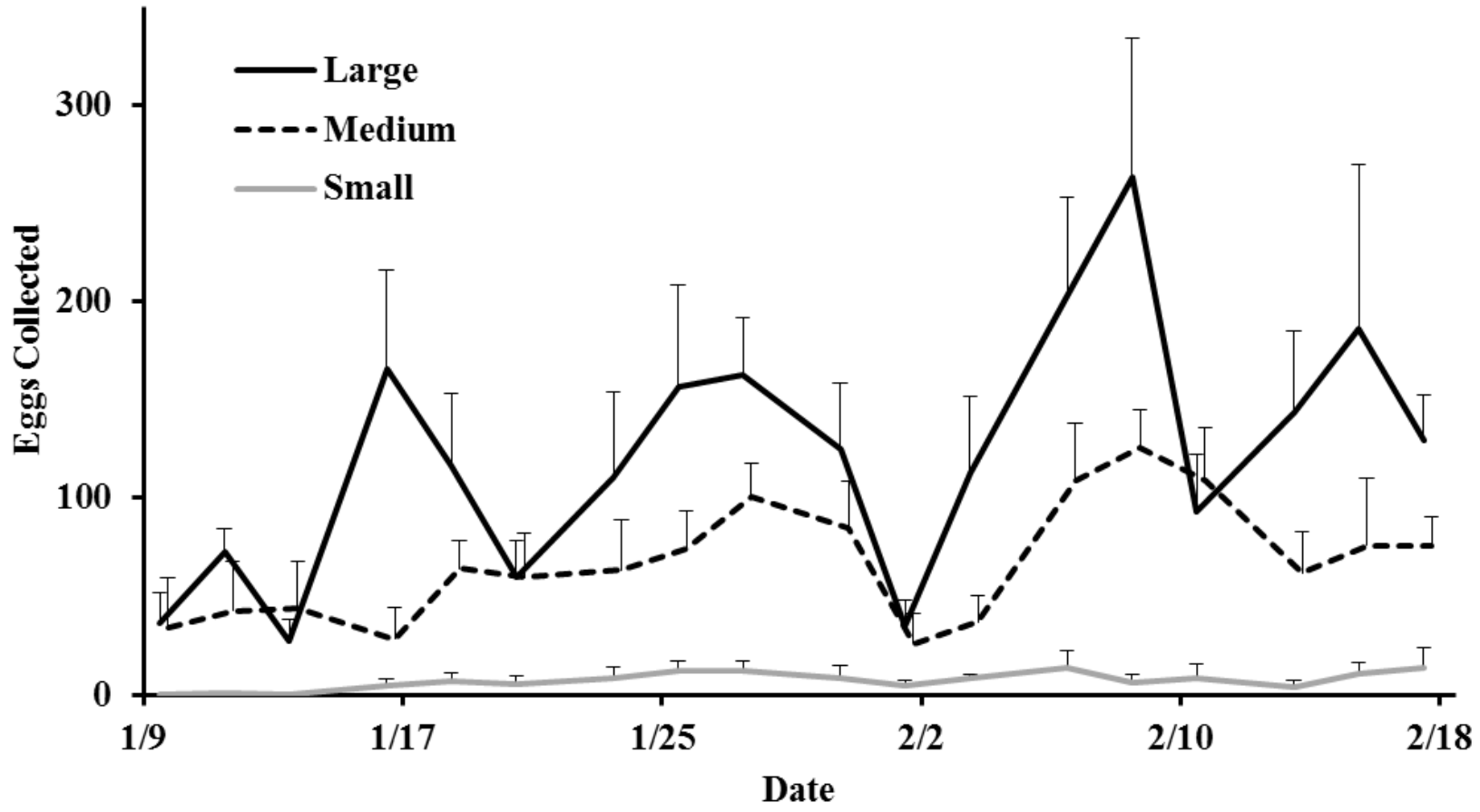

Figure 3.2. Mean (±SEM) eggs collected from the four replicate tanks in small, medium, and large size classes at each sampling point. Statistically significant differences in fecundity existed among all three size classes (Tukey HSD; $p<0.05$).

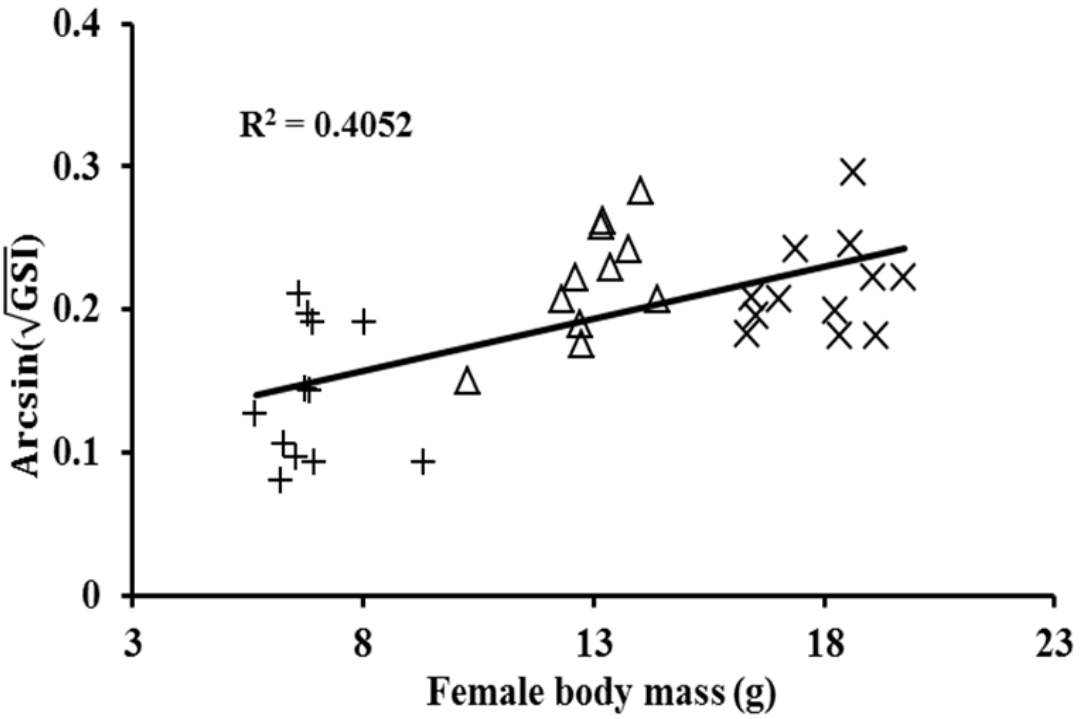

Figure 3.3. Linear regression of female broodfish body mass and arcsin(square root GSI) for a subsample of fish from each replicate tank at the conclusion of a six-week spawning period. Symbols denote individuals belonging to small (+), medium (Δ), and large (×) size classes. The equation of the line is y = 0.0073x + 0.0986.

Discussion

Results from the present study were largely consistent with previous findings regarding maternal body size effects on reproduction (Einum & Fleming, 2002; Johnston & Legget, 2002; Koç et al., 2007). As expected, total fecundity and egg volume significantly increased with increasing female body size. In other fishes, a decrease in egg size and lack of significant increase in fecundity have been shown to occur among older females (Abdoli et al., 2005). If such a relationship exists in *F. grandis*, the maternal age at which it occurs was not tested in this experiment. Fish in this study were not aged as the focus was on maternal body size rather than age. With a mean SL of 114.8 ± 0.7 mm the large size class from this study was ≈30% smaller

Table 3.1. Mean ± SEM chorion volume (mm^3), time to hatch (days), and percent survival to hatch for embryos produced by *Fundulus grandis* during a six-week spawning period. Letters denote statistical significance among experimental female size classes (REGWQ; $p<0.05$). No significant difference was detected in probability of survival to hatch among female size classes (Wald $X^2=0.48$; $p=0.788$).

Female Size Class	Embryo Parameter		
	Chorion Volume (mm^3)	Time to Hatch (days)	Percent Survival to Hatch
Small 6.9 ± 0.3 g	4.50 ± 0.06^a	14.1 ± 0.2^x	40.2 ± 3.2
n	68	129	79
Medium 12.9 ± 0.3 g	4.77 ± 0.04^b	17.1 ± 0.6^y	40.5 ± 7.0
n	221	1138	705
Large 18.0 ± 0.3 g	4.95 ± 0.03^c	19.8 ± 0.4^z	39.0 ± 1.8
n	268	2048	1290

than the maximum size of 150 mm SL reported in wild *F. grandis* by Subrahmanyam and Drake (1975). Although Lipcius and Subrahmanyam (1986) found no *F. grandis* specimens greater than two years old in north Florida marshes and suggested that few individuals spawn for more than one season in the wild, we have observed annual spawning by captive populations lasting 4+ years (Green, unpublished data). It is possible that the significant increases in egg volume and fecundity observed in *F. grandis* across all size classes in this study would not exist for older and larger individuals, as has been demonstrated in other fishes (Abdoli et al., 2005). In the present study, mean GSI of the large size class of females was not

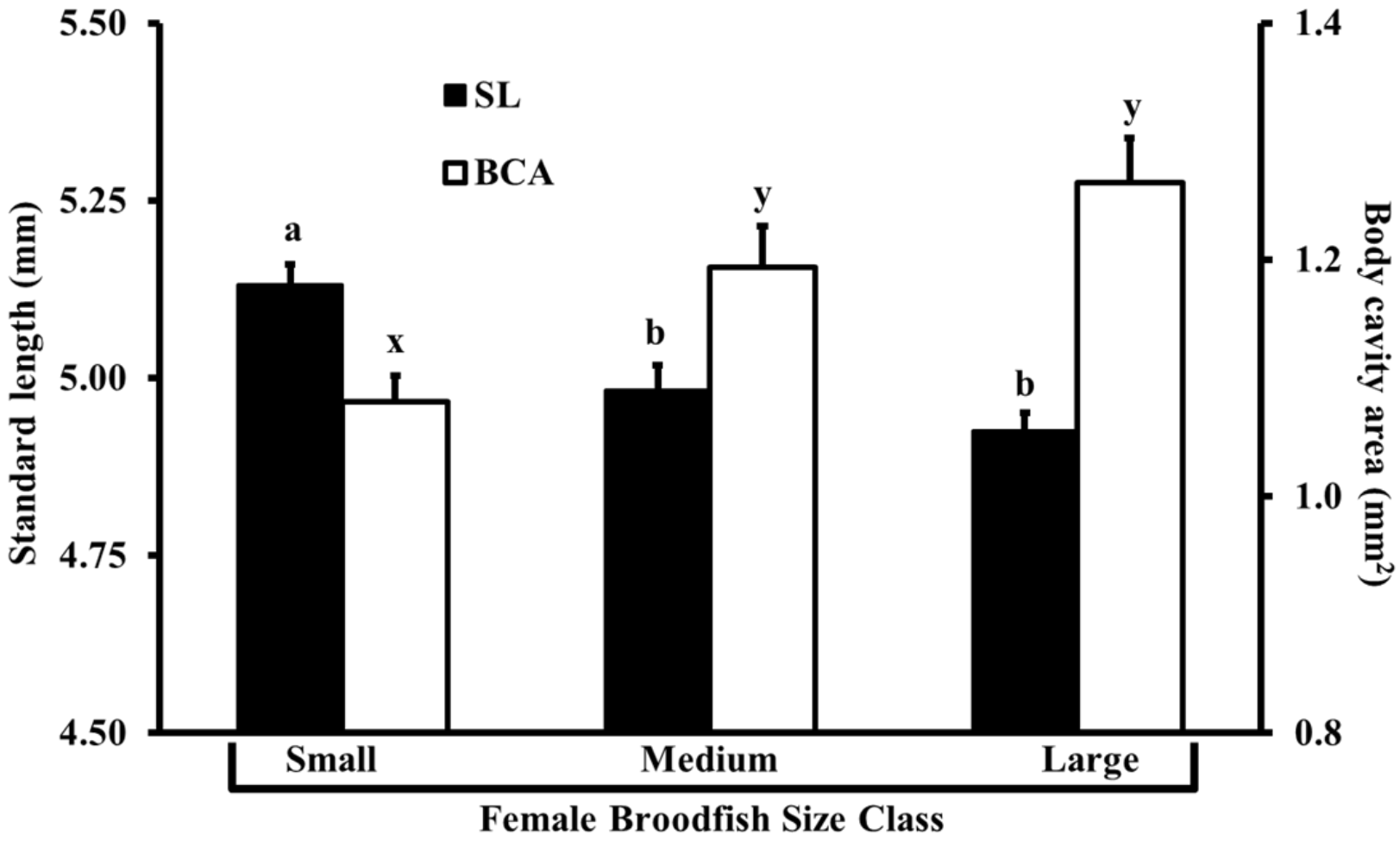

Figure 3.4. Mean (± SEM) standard length (SL; black bars) and body cavity area (BCA; white bars) of newly hatched larvae across female broodfish size classes. Larvae included in analysis were those hatching in the middle third of larvae from a given collection date in terms of incubation duration. *A priori* comparisons were applied separately to each morphometric and letters denote statistical significance (REGWQ; $p<0.05$)

significantly higher than that of the medium size class, while this morphometric increased by over 100% in medium females relative to their small counterparts.

The potential plateau of GSI in females between medium and large size classes accounts for the low R^2 value and weak regression correlation (Figure 3.3). Lack of increase in GSI between medium and large size classes did not prevent large females from producing significantly more eggs than medium females (Figure 3.2). Although the amount of body cavity volume devoted to reproduction was proportionally similar for medium and large fish, females with a larger body size had a greater volume in which to produce eggs, rendering the expected increase in overall fecundity (Jonsson & Jonsson, 1997). Females in the small size class devoted proportionally less of their body cavity area to reproduction than larger females. Similar patterns have been observed in Arctic charr *Salvelinus alpinus* (Adams & Huntingford, 1997), Atlantic salmon *Salmo salar* (Jonsson & Jonsson, 2003), bullhead *Cottus gobio* (Abdoli et al., 2005), and chub *Leuciscus cephalus* (Koç et al., 2007), among other species. In addition to individual species-specific observations, a period following the onset of sexual maturity when reproductive effort increases until the animal reaches a point of size-specific reproductive optimization, observed here as a plateau in GSI, has been modeled theoretically and described empirically using meta-analysis of many species (Beverton, 1992; Charnov, 2008). Results of the present study indicate that in a captive population of *F. grandis* the minimum body mass necessary for size-specific reproductive optimization occurs between ~ 7 and 13 g.

When making interspecific comparisons of maternal effects on reproduction, it is important to bear in mind the larger context of variability in reproductive strategies of fishes (Murua et al., 2003). *F. grandis* is a fractional spawning species with a highly protracted spawning season. In captive *F. grandis* held outdoors, mean egg volume has been shown to

decrease significantly as the ~8 month spawning season progresses (Green et al., 2010). Total fecundity also varies throughout the spawning season and is significantly correlated with water temperature (Gothreaux & Green, 2012). Thus, reproductive analyses of fractional spawning species are confounded with factors whose influences reduced or eliminated in species whose reproduction involves a single annual spawning event. In the present study, spawning occurred in January and February and was induced by photoperiod and temperature manipulation. These months are not part of the natural spawning period for *F. grandis*, and the maternal effects observed here potentially vary from those which would be observed in natural settings because of environmental factors or the artificially induced timing of reproduction.

While environmental effects on reproduction were not the focal point of this study, it should be noted that indoor laboratory spawning conditions appeared to have a negative impact on the fecundity of experimental animals. Groups of *F. grandis* held in outdoor pool mesocosms at an identical 2:1 female to male sex ratio, also spawning on horizontally oriented Spawntex substrate, produced a range of 0.72 – 0.90 eggs g female^{-1} day $^{-1}$ when mean female mass was ~11 g (Green et al., 2010). In the present study, broodfish with mean mass 12.9 ± 0.3 g produced 0.40 ± 0.09 eggs g female^{-1} day^{1}. Although *F. grandis* in the present experiment continued to exhibit semilunar spawning periodicity as expected from past work (Hsiao and Meier, 1989), a number of factors may contribute to the reduction in fecundity observed when laboratory held fish are compared to captive groups spawning outdoors. In addition to the lack of live prey items and total reliance on a prepared diet in the laboratory, there are environmental differences between the two culture conditions which likely alter behavior and nutritional status and thus fecundity. Also, captive populations of fishes can experience rapid evolutionary shifts in egg size and fecundity whereby natural selection favoring large eggs is replaced by fecundity

selection driving a rapid shift towards small eggs (Heath et al., 2003). However, with limited data available from wild populations of *F. grandis* or its congeners, a comparative examination is beyond the scope of this work. The present study was expected to improve ability to predict fecundity in laboratory spawning populations of *F. grandis* using body mass. While Figure 3.5 is not useful for statistical comparison, the equation for the power function which fits the plot of female mass against eggs produced per day could provide a viable tool for predicting fecundity in laboratory spawning *F. grandis*. An unrelated replicated experiment performed in a different location, using a recirculation system with many dissimilar features, including smaller, circular, plastic tanks and spawning substrate elevated on open platforms. Females in the unrelated study had a mean body mass of 8.16 ± 0.14 g and over the course of a six week spawning period these females produced 0.85 eggs $fish^{-1}$ day^{-1} (Patterson and Green, 2014). The equation in Figure 3.5 predicts a fecundity of 0.81 eggs $fish^{-1}$ day^{-1} for females of this mass, a discrepancy of less than 5% from observed empirical data. The predictive ability of the power function in Figure 3.5 requires further validation but has potential value for *Fundulus* cultured in laboratory recirculating systems as a model species in a variety of fields (Burnett et al., 2007).

As discussed previously, volume of individual *F. grandis* eggs exhibited an expected relationship to maternal size. However, eggs from even the smallest group of females, which had a mean diameter of 2.11 ± 0.01 mm, were larger than the eggs of ~75% of fishes included in a review of 384 marine and freshwater teleost species by Kamler (2005). Making this relatively large egg size in *F. grandis* more atypical is its occurrence in a warm-water teleost which is native exclusively to lower latitudes (~31°N to ~21°N; Williams et al., 2008), as egg diameter in fishes generally increases towards the poles, reaching a mean of 2.8 mm in Antarctic species (Knox, 1994). The occurrence of large eggs in *F. grandis* may be at least partially explained by

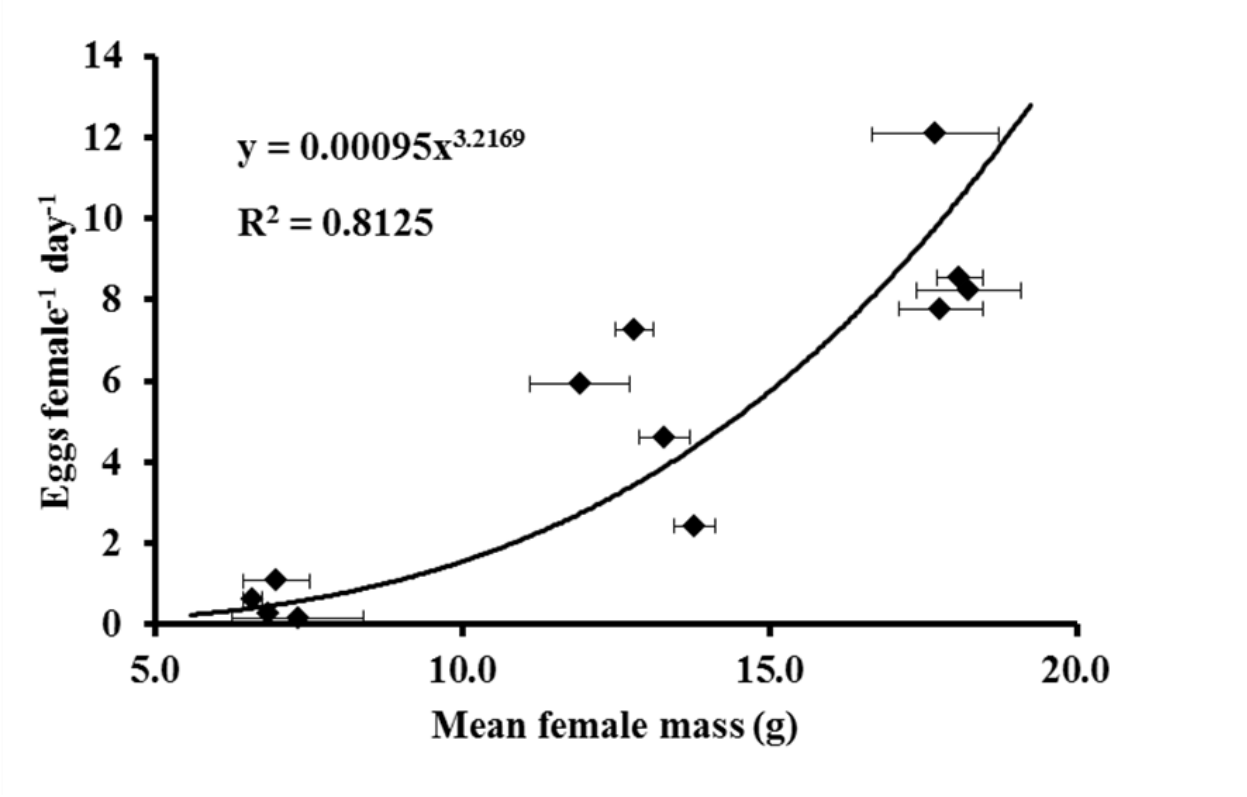

Figure 3.5. Power function describing the relationship between female body mass and fecundity. Egg production was normalized as eggs female^{-1} day^{-1} from a six-week spawning period. Data points denote replicate tanks and error bars represent SEM of female body mass.

a physiological characteristic uncommon among fishes; the ability of embryos to develop out of water in a sufficiently humid environment. Eggs of *F. grandis* are likely naturally exposed to air for extended periods during tidal fluctuations, and in culture settings have been shown to undergo complete development during periods of up to 19 days out of water (Coulon et al., 2012). Exposure of the chorion to relatively oxygen rich atmospheric air may eliminate the egg volume to surface-area ratio problem which is thought to limit the size of eggs which develop in water (Krogh, 1959). In addition to increasing oxygen availability for development, air incubation of relatively large eggs, in an intertidal area, by a low fecundity species, is a strategy which allows synchronous hatching by larger cohorts of highly precocial larvae possessing

functional eyes, mouthparts, and substantial residual yolk. In this situation, maternal effects on embryos appear as differences in body size and endogenous nutritional resources of larvae.

Eggs in the present study were incubated in water, and eggs from different female size classes had an expected significant positive relationship between incubation duration and egg size (Table 3.1). For eggs developing out of water, this discrepancy in oxygen availability may be eliminated by the ability of fully developed embryos to delay hatching until immersion (Perschbacher et al., 1995). However, differences in the morphometrics of newly hatched larvae in the present study (Figure 3.4) raised unexpected questions about the development of embryos within different sized eggs. In previous studies on aquatic incubation of *F. grandis* eggs, environmental variables such as lower temperature or higher salinity resulted in slower development and increased time to hatch. In these experiments, longer aquatic incubation resulted in larvae with decreased body cavity area at hatch and a longer body length (Brown et al., 2011; Brown et al., 2012). In the present experiment, environmental variables were controlled to elucidate maternal effects only, and larger eggs with extended incubation times produced larvae with significantly more body cavity area, but a significantly shorter body length at hatch. This unexpected relationship may be explained by multiple factors. First, larger eggs would be expected to contain more yolk from the beginning of development (Kamler, 2005), and because no differences in environmental variables were present to affect the rate of yolk utilization across egg sizes, this difference remains perceptible in newly hatched larvae. Second, the decrease in body length of newly hatched larvae from larger eggs may be explained by the oxygen availability problem previously discussed for aquatically incubated eggs. Although environmental oxygen availability should not have changed across treatments, egg size variations may have reduced the amount of oxygen available to developing embryos in larger eggs. Lower

oxygen availability may have slowed the rate of development, leading to shorter body length at hatch and an increase in incubation time necessary to reach a suitable stage for hatching. Einum et al. (2002) found that in Atlantic salmon, oxygen consumption increased relatively slowly with increasing egg size and theorized that this was due to a lower demand for oxygen in larger embryos relative to what was predicted based on embryo size. It is also possible that this lack of increase in respiration in proportion to embryo size resulted from the inability of the larger embryo to acquire enough oxygen across the chorion, leading the embryo to oxyconform and decrease metabolic rate.

Impacts of maternal variation of reproductive potential in Gulf killifish were largely similar to those observed in other fishes. However, maternal effects on newly hatched larvae varied from expected results. All of this information has important implications in both the culture and ecology of the species. Practical culture recommendations for *F. grandis* were established. Maximum egg production is attained with broodfish females of a body size $\geq$ 13 g, and Figure 3.5 may prove useful as a predictor of egg production by female body mass in recirculating systems. Further work should examine the maternal-larval relationship for air incubated eggs, as air and aquatic incubation appear to have very different sets of developmental considerations. Biochemical mechanisms leading to shorter larvae with more endogenous nutritional resources hatching from larger eggs were only discussed theoretically and could be examined experimentally.

CHAPTER 4
PHYSIOLOGICAL AND REPRODUCTIVE RESPONSE TO VARYING QUANTITATIVE LIPID INCLUSION IN DIETS FOR GULF KILLIFISH *FUNDULUS GRANDIS*[3]

Introduction

Investigations of Gulf killifish *Fundulus grandis* culture to supply marine live-bait markets date back at least 35 years (Tatum & Helton, 1977; Trimble et al., 1981; Waas & Strawn, 1982). Following a report by Oesterling et al. (2004) listing the *Fundulus* species group as having the greatest potential for successful development as a cultured live bait in the southeastern U.S., interest in Gulf killifish was renewed (Green at el., 2010; Phelps et al., 2010; Brown et al., 2011; Gothreaux & Green, 2012; Patterson et al., 2012). Additionally, Gulf killifish have been increasingly cultured in laboratory systems for use as a model species (Burnett et al., 2007). This species has many desirable characteristics for aquaculture including tolerance to a wide range of environmental salinity and oxygen levels, and capacity to spawn in captivity without artificial hormonal stimulation (Griffith, 1974; Perschbacher et al., 1990; Martínez et al., 2006; Green et al., 2010). Gulf killifish embryos also have the ability to complete development out of water in a humid environment with hatch delayed until immersion in water, a characteristic which allows controlled hatching of large cohorts of larvae (Coulon et al., 2012).

A primary impediment for successful commercial development of Gulf killifish culture has been low fecundity. Egg production in Gulf killifish was approximately one-sixth that of the established commercial baitfish, fathead minnow *Pimephales promelas* (Clemment & Stone, 2004; Green et al., 2010). Low fecundity increases the difficulty of obtaining sufficient larvae to support a commercial scale operation and requires high resource allocation for maintenance of

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large numbers of broodfish. Dietary components have a substantial impact on fecundity and gonad development, especially in fractional or continuous spawning species with brief vitellogenic periods (Izquierdo et al., 2001; Coldebella et al., 2011). Gulf killifish utilize a fractional spawning strategy and vitellogenesis of a single oocyte can occur in as little as two days (Hsiao & Meier, 1988), but no published information currently exists on broodfish nutrition in this species.

Lipids play many vital roles in reproduction and early development of fishes. Egg yolk lipids can be sourced from the diet, recently mobilized stores in the body, or *de novo* synthesis (Quintero et al., 2011). Teleost vitellogenins, which form the bulk of fully-grown oocytes, only contain about 20% lipid (Yaron & Sivan, 2006). However, lipids play a disproportionately important role in reproduction due to both their high energy density and necessity in critical cellular functions. Lipid served as the primary energy reserve for reproduction in Arctic charr *Salvelinus alpinus* (Adams & Huntingford, 1997), Atlantic salmon *Salmo salar* (Kadri et al., 1996), and southern catfish *Silurus meridionalis* (Xie et al., 1998). In early development, embryos and yolk-sac larvae of warm-water marine fishes have been shown to use lipid as the predominant energy source while selectively retaining certain long-chain polyunsaturated fatty acids which are essential as membrane components or in neural and visual development (Vetter et al., 1983; Ostrowski & Divakaran, 1991; Vázquez et al., 1994).

Dietary lipid requirements of broodfish and effects of dietary protein-to-lipid ratios have not been established in Gulf killifish and previous information on nutrition in Gulf killifish culture is focused on grow-out phases of production (Waas & Strawn, 1982; Perschbacher & Strawn, 1991). Therefore, the present study was designed to examine the physiological and reproductive effects of dietary lipid content for captive-spawning Gulf killifish and their larvae.

Methods

Experimental Diets

Five practical diets were formulated and analyzed for proximate composition (Table 4.1).

Table 4.1. Formulation and analyzed proximate composition of experimental diets fed to *Fundulus grandis* in the lipid content experiment.

Ingredient (g kg^{-1})	Diet				
	1	2	3	4	5
Soybean Meal	448.6	456.8	465.0	473.3	481.6
Corn Grain	200.0	200.0	200.0	200.0	200.0
Wheat Flour	192.2	158.8	124.8	90.8	56.9
Fishmeal	100.0	100.0	100.0	100.0	100.0
Dicalcium Phosphate	30.0	30.0	30.0	30.0	30.0
Menhaden Fish Oil	---	25.3	51.0	76.6	102.3
Carboxymethylcellulose	20.0	20.0	20.0	20.0	20.0
Vitamin Pre-Mix	5.0	5.0	5.0	5.0	5.0
Mineral Pre-Mix	2.5	2.5	2.5	2.5	2.5
Choline Chloride	0.9	0.9	0.9	0.9	0.9
Vitamin C (Stay-C)	0.6	0.6	0.6	0.6	0.6
Ethoxyquin	0.2	0.2	0.2	0.2	0.2
Proximate Analysis					
Protein (% dry matter)	32.93	32.56	32.47	32.60	33.55
Lipid (% dry matter)	4.01	6.29	8.81	11.21	13.76
NFE (% dry matter)	41.82	40.04	38.08	36.21	32.94
Fiber (% dry matter)	1.70	1.60	1.60	1.55	1.85
Ash (% dry matter)	8.89	8.89	8.64	8.52	8.50
Moisture (%)	10.91	10.82	10.41	9.92	9.40
Energy (kJ g^{-1})	16.61	17.11	17.90	18.20	19.00
P:E ratio[a]	19.83	19.03	18.14	17.91	17.66

[a]Protein:Energy ratio

Diets (1-5) were designed to be isonitrogenous with increasing levels of lipid supplementation as fish oil (FO) in amounts ranging from 0% to ~10% dry mass in increments of ~2.5%. Fatty acid composition of menhaden FO used in this study is contained in Table 4.2. During diet

Table 4.2. Composition of selected fatty acids from menhaden fish oil utilized in formulation of experimental diets fed to *Fundulus grandis* in the lipid content experiment.

Fatty Acid	% Mass (g 100 g fatty acids^{-1})
14:0 (Myristic)	7.33
15:0 (Pentadecanoic)	0.62
15:1 (Pentadecenoic)	0.03
16:0 (Palmitic)	14.52
16:1n-7 (Palmitoleic)	10.77
18:0 (Stearic)	2.66
18:1n-9 (Oleic)	8.34
18:2n-6 (Linoleic)	1.72
18:3n-3 (α-Linolenic)	1.21
18:3n-6 (Linolenic)	0.37
20:1n-9 (Gadoleic)	1.25
20:4n-3 (Eicosatetraenoic)	1.34
20:4n-6 (Arachidonic)	2.35
20:5n-3 (EPA)	12.47
22:5n-3 (Docosapentaenoic)	2.42
22:6n-3 (DHA)	10.73
∑ n-3	31.37
∑ n-6	3.76

preparation, dry ingredients were homogenized mechanically for 1 h before being placed in a mixer for addition of menhaden FO blended with ethoxyquin, an antioxidant. The diet without

supplemental oil had ethoxyquin added directly to the dry ingredients. Subsequently, choline chloride was dissolved in water and 25% dry ingredient mass of water was added during continuous mixing. The diets were pressed through a 3mm dye at room temperature and strands were broken into approximately 3mm x 6mm pellets and dried. This process created a non-extruded, sinking pellet of suitable size for consumption by adult Gulf killifish. Proximate analyses were conducted in duplicate. Experimental diets were analyzed to determine percent moisture (Association of Official Analytical Chemists [AOAC], 2005, procedure 930.15). Gross energy was quantified using a bomb calorimeter (IKA-Werke Model C5000, Staufen, Germany). Composition on a percent dry matter basis was also determined for protein (AOAC, 2005, procedure 990.03), crude lipid (AOAC, 2005, procedure 954.02), fiber (AOAC, 2005, procedure 962.09), and ash (AOAC, 2005, procedure 942.05). Nitrogen-free extract (NFE), predominantly soluble carbohydrate, was calculated as NFE = 100- [%protein + %crude lipid + %fiber + %ash].

Recirculation system and maintenance

Two recirculation systems designed to accommodate replicated experiments were joined in order to provide a sufficient number of spawning units. Each cylindrical spawning unit held a water volume of 30 L with continuous flow-through water circulation and aeration provided by a submerged airstone. The system was serviced by a sumps containing substrate for nitrifying bacteria as well as a bead filters and UV sterilizers. Culture water was maintained at a salinity of 8.0 g L^{-1} using Crystal Sea Marinemix (Marine Enterprises International Inc., Baltimore, MD, USA) and evaporative loss was replaced with dechlorinated municipal water. Salinity, dissolved oxygen, total ammonia nitrogen, nitrite nitrogen, pH, and alkalinity (as $CaCO_3$) were monitored weekly and remained within acceptable values for Gulf killifish (Tatum et al., 1982).

Stocking, feeding, and spawning

Animal handling procedures were conducted using methods approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee under protocol #09-103. Experimental diets were tested in quadruplicate by randomly assigning each of the five diets to four replicated spawning units. Six females from a population of individual body mass 7.81 ± 0.13 g (mean ± SEM) were stocked at random into each of the replicated units. Three males from a population of individual body mass 6.73 ± 0.24 g (mean ± SEM) were stocked per replicate to create a 2:1 female to male sex ratio, an appropriate sex ratio for reproduction as indicated by previous research in our laboratory.

Because all fish received a common diet before being placed in replicate tanks, replicates were fed their assigned experimental diets twice daily to satiation for 16 weeks prior to the start of the experiment. Satiation was defined as lack of further diet consumption after three minutes with uneaten diet present in the tank. This protracted incorporation phase ensured that nutritional status of all animals at the initiation of spawning was fully attributable to experimental diets. Spawning was initiated by gradually increasing day length and water temperature using laboratory light timers and submersible heaters throughout the final six weeks of the incorporation phase. Light cycles were 11 h light : 13 h dark initially and increased to 14 h light : 10 h dark before spawning substrate was added. Culture water was gradually warmed from 15 °C and maintained at 27 °C during spawning. Spawning substrates were 10 x 15 cm strips of Spawntex (Blocksom and Co., Michigan City, IN, USA) attached to wire frames which provided a stable base while elevating the spawning substrate to 8 cm below the water surface. A six-week spawning period was initiated by placing a single substrate in each culture unit. Eggs were collected twice weekly. Duration of the spawning period was chosen to allow three

semilunar cycles, based on an observed reproductive cycle of 13.6 days in Gulf killifish under controlled laboratory conditions (Hsiao & Meier, 1988). Animals were fed to satiation twice daily with per-replicate consumption monitored by recording the difference between diet mass at the start and end of each week.

Reproductive assessment

Eggs were counted to determine periodic fecundity, here used to describe the total number of eggs deposited on a spawning substrate at each collection normalized for female broodfish body mass. Embryos were subsequently incubated in culture-system water (8.0 g L^{-1}) at room temperature for three days, at which point viability rate was determined using a dissecting microscope to observe neurulation. Eggs were photographed through the dissecting microscope using MetaVue 6.1 image processing software (Molecular Devices, Sunnyvale, CA, USA). For calibration, a standard micrometer was photographed with each set of eggs. As embryo development continued, eggs were monitored daily for hatching with newly hatched larvae preserved in 10% neutral-buffered formalin and refrigerated for future photographic analysis of quantitative morphometrics. At the conclusion of the spawning period all surviving females were euthanized with an overdose of tricaine methane sulphonate buffered to a pH of 7 with sodium bicarbonate. Whole body wet mass and tissue masses were quantified for calculation of body composition indices (I_T) using the formula;

$$I_T = (\frac{W_t}{W}) \times 100 \quad (8)$$

where W is total body mass (g) and W_t is tissue mass (g). This method was used to calculate gonadosomatic index (GSI), hepatosomatic index (HSI), and intraperitoneal (IP) fat ratio (IFR) for individual females. Livers were wrapped in aluminum foil and sealed under ultra-pure nitrogen in amber bottles before storage at -80 °C. Liver total lipid (TL) content was quantified

using a Folch extraction (Folch et al., 1957) and three randomly selected livers per treatment level were histologically sectioned and stained with hematoxylin and eosin at the LSU School of Veterinary Medicine (Baton Rouge, LA, USA) to visualize patterns of lipid deposition. Males were euthanized by the same procedure as females and testes were extracted, weighed and crushed in 1:20 w/v Hanks' balanced salt solution (HBSS) at 500 mOsmol/kg osmolality. Dilution with HBSS yielded sperm concentrations of approximately 5-7 $\times$ 10^8 cells ml^{-1} of crushed testes solution as calculated using measured absorbance at 400 nm on a NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and application of a standard curve developed for this species (DaCosta & Yang, personal communication). Progressive sperm motility, which includes only those cells actively moving forward, was determined by computer-assisted sperm analysis (CASA) (Hamilton Thorne Inc., Beverly, MA, USA) following the procedure and settings of Tiersch and Yang (2012) for Gulf killifish collected from 10‰ salinity.

Image analysis

Egg and larval morphometrics were quantified using Image Tool 2.0 software (The University of Texas Health Science Center, San Antonio, TX, USA). Polar axis and equatorial diameter were measured, and egg volume was calculated using the formula for the volume of a prolate spheroid;

$$V = \left(\frac{\pi}{6}\right) a^2 b \qquad (9)$$

where V is the volume, a is the equatorial diameter, and b is the length of the polar axis. Larval images were measured using methods identical to those used for eggs. Standard length (SL), or length from the most distal point on the head to the tip of the caudal peduncle, and the two dimensional area of the body cavity (BCA) containing the yolk-sac and internal organs were measured.

Statistical analyses

Statistical tests were performed using Statistical Analysis Software Version 9.1 (SAS Institute Inc., Cary, NC, USA). Means are presented as mean ± SEM. Per replicate female body mass at stocking was tested across treatment groups by general linear model analysis of variance (ANOVA). Prior to ANOVA, the assumption of normality in GSI, HSI, and IFR data were assessed with the Shapiro-Wilk test. Percentage data (GSI, HSI, liver TL, IFR, and sperm motility) were transformed as arcsin(square root *y*) and periodic fecundity count data were transformed as square root(*y*+1) for analysis. Differences in periodic fecundity were analyzed by mixed model repeated measures ANOVA with the Tukey HSD *post-hoc* test. Fertilization rates and progressive sperm motility percentage were analyzed as binomial response variables by logistic regression using proc LOGISTIC. Mean values of consumption, egg volume, larval SL, and larval BCA were compared across treatment levels using general linear model ANOVA with the Ryan-Einot-Gabriel-Welch *post-hoc* test. All statistical tests were performed at a significance level of $\alpha = 0.05$.

Results

Consumption and reproductive performance

Consumption and reproductive performance data are contained in Table 4.3. Although fish fed diet 5 consumed less than other experimental groups, there was no statistically significant difference in mean g diet consumed kg fish^{-1} day^{-1} or mean kJ consumed kg fish^{-1} day^{-1} due to high within treatment variation ($F_{4,15} = 2.25$, $p = 0.1127$) and ($F_{4,15} = 1.52$, $p = 0.2474$), respectively. Total female body mass per replicate was not significantly different across treatment groups at stocking ($F_{4,15} = 1.07$, $p = 0.4043$). Per unit-mass fecundity was not significantly affected by dietary lipid content ($F_{4,15} = 0.45$, $p = 0.7682$) while embryo viability

Table 4.3. Mean diet consumption and reproductive performance parameters (± SEM) of Gulf killifish fed varying dietary lipid levels as fish oil (see Table 4.1). No significant differences in parameter values were observed across treatment levels.

Parameter	Diet				
	1	2	3	4	5
g consumed kg fish^{-1} day^{-1}	43.8 ± 2.6	44.2 ± 1.1	44.9 ± 1.6	44.5 ± 1.5	38.4 ± 2.0
kJ consumed kg fish^{-1} day^{-1}	727 ± 43	756 ± 18	803 ± 27	810 ± 28	729 ± 38
Eggs g female^{-1}	4.6 ± 1.0	4.3 ± 0.6	4.0 ± 0.7	4.7 ± 0.4	3.6 ± 0.8
Egg volume (mm^3)	4.71 ± 0.04	4.86 ± 0.23	4.57 ± 0.10	4.54 ± 0.05	4.73 ± 0.06
Sperm motility (%)	44.3 ± 1.3	45.7 ± 2.2	49.7 ± 4.9	42.7 ± 3.7	44.3 ± 2.3
Embryo viability (%)	24.6 ± 2.6	24.6 ± 3.4	21.0 ± 6.0	22.7 ± 4.3	29.4 ± 5.3

rates were low and not significantly different across all treatments (Wald Chi-Square = 3.6648, p = 0.0556). Dietary lipid content also had no significant effect on mean egg volume ($F_{4,15}$ = 0.86, p = 0.5118). Progressive sperm motility of males was within the expected and acceptable range and highly similar across all lipid treatment levels (Wald Chi-Square = 0.3886, p = 0.5330).

Larval Morphometrics

Larval morphometrics of newly-hatched fish derived from adults fed different lipid levels are shown in Figure 4.1. SL of newly hatched larvae was significantly different across dietary lipid treatment levels ($F_{4,295}$ = 3.77, p = 0.0052) with larvae resulting from broodfish fed diet 2 having greater SL than other treatments. Larval BCA showed a significant treatment effect ($F_{4,295}$ = 8.12, p < 0.0001). Diets 4 and 5 produced larvae with numerically increased BCA, although diets 2 and 4 were not statistically different. SL of newly hatched larvae was

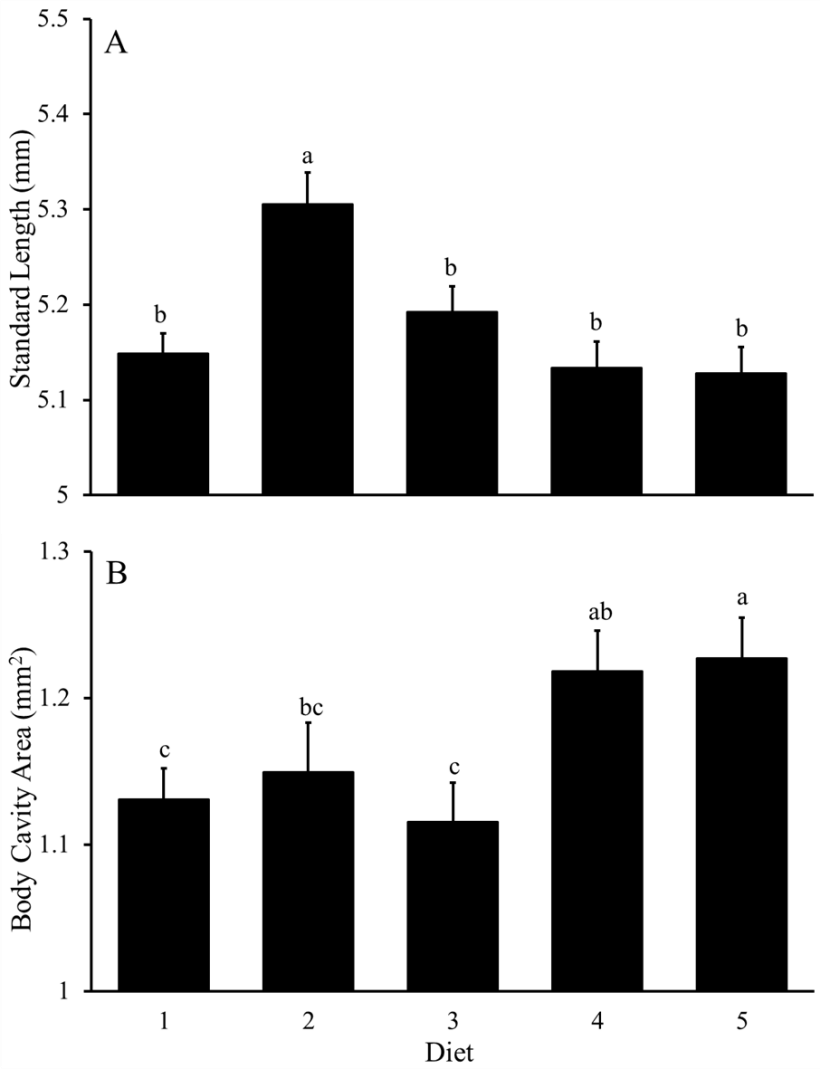

Figure 4.1. Mean morphometrics ± SEM standard length (A) and body cavity area (B) of newly hatched larvae produced by *Fundulus grandis* broodfish fed varying dietary lipid levels as fish oil (see Table 4.1). Letters denote significances across treatment levels within each morphometric variable (REGWQ; $p<0.05$).

significantly different across dietary lipid treatment levels ($F_{4,295} = 3.77$, $p = 0.0052$) with larvae resulting from broodfish fed diet 2 having greater SL than other treatments.

Broodfish Body Composition Indices

During the feeding and reproductive phase of the experiment some individuals exhibited dominance in feeding and spawning behavior. Dominance behaviors lead to some animals

receiving inadequate nutrition. During necropsies, it was determined that IP fat and gonads were potentially severely reduced in these individuals although all fish were included in analyses. Data for body composition indices are contained in Table 4.4. Female broodfish had significantly

Table 4.4. Mean body composition indices (± SEM) of female Gulf killifish broodfish fed varying dietary lipid levels as fish oil. Data were collected at the conclusion of a 16-week feeding and 6-week spawning period during which broodfish consumed diets 1-5 (see Table 4.1). Superscript letters denote significant difference in parameters across treatment levels.

Parameter	Diet				
	1	2	3	4	5
Body mass (g)	8.4 ± 0.4	8.4 ± 0.4	7.8 ± 0.2	8.6 ± 0.3	7.9 ± 0.2
Gonadosomatic Index	4.0 ± 0.7^{a}	2.7 ± 0.5^{b}	1.8 ± 0.3^{b}	2.7 ± 0.2^{b}	2.6 ± 0.3^{b}
Hepatosomatic Index	2.4 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.7 ± 0.1	2.3 ± 0.1
IP Fat Ratio	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	1.3 ± 0.2^{a}	1.1 ±0.2^{ab}
Liver TL (% wet mass)	61.1 ± 3.6	62.1 ± 2.4	63.1 ± 3.3	67.7 ± 2.2	66.6 ± 2.1

different concentrations of IP fat across dietary lipid treatment levels ($F_{4,98} = 6.66$, $p < 0.0001$), with diet 4 producing individuals with an increased proportion of IP fat compared to those fed diets 1 through 3. Gonadosomatic index varied significantly across treatment levels ($F_{4,87} = 3.65$, $p = 0.0085$), with only fish fed diet 1 having significantly increased mean GSI values relative to all of the groups fed higher lipid content diets. Neither HSI nor liver TL content varied significantly across treatment levels ($F_{4,101} = 1.40$, $p = 0.2401$) and ($F_{4,88} = 0.91$, $p = 0.4629$), respectively. Histological preparations of hepatic tissue were produced for a limited number of individuals from all dietary lipid content groups as a means to visualize variations in lipid

deposition. Figure 4.2 contains representative histological liver sections showing varying degrees of lipid deposition. Panels A, B, and C of Figure 4.2 were sections from individuals

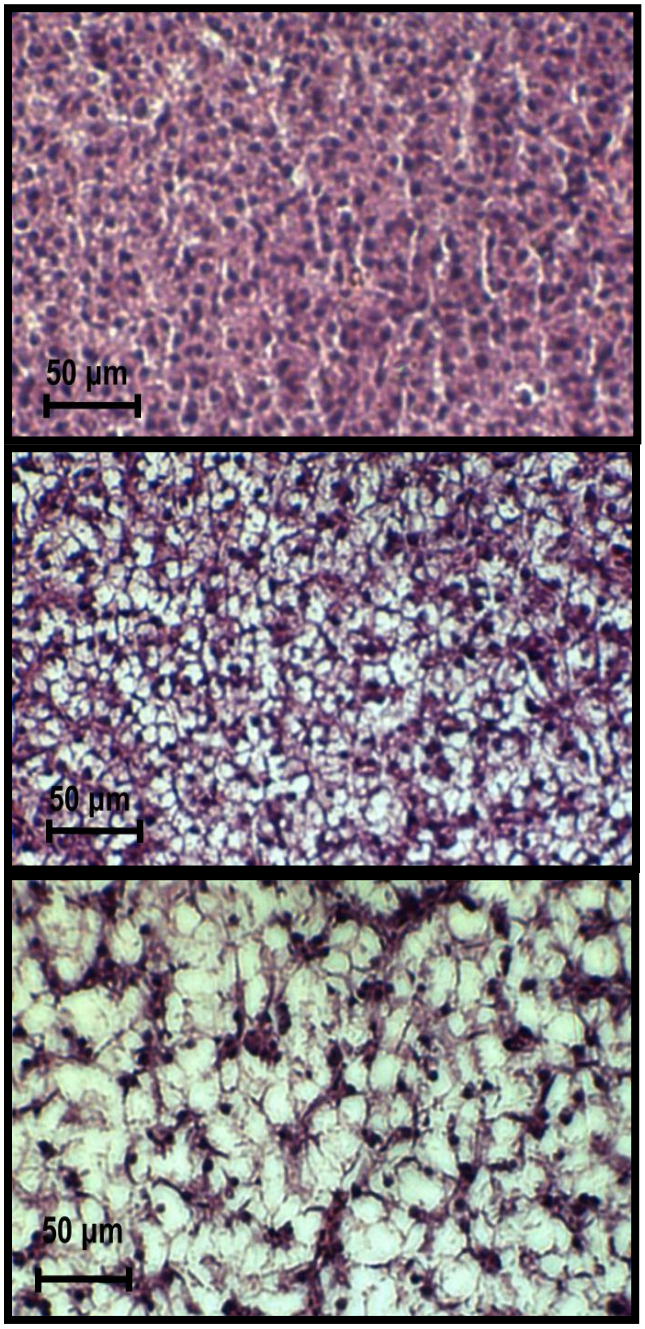

Figure 4.2. 40x magnification. Histological sections of livers from female *Fundulus grandis* obtained in the quantitative lipid inclusion experiment. Sections represent (A) normal liver, (B) moderate lipid accumulation, and (C) severe steatosis with all sections coming from fish fed diet 3. Staining was done using hematoxylin and eosin with nuclei appearing dark purple, liver tissue stained pink, with white where lipid vacuoles existed before dissolution by histological fixation.

taken from the same dietary treatment, and represent the high level of intra-treatment variability. Visual inspection of these sections supplemented quantitative TL analyses with qualitative confirmation that a higher degree of variation existed within treatment levels or replicates than across dietary lipid levels.

Discussion

Lipids in growth and reproduction of fishes

The effects of lipid content in aquaculture diets on aquatic animal growth performance are well studied (Lee et al., 2000; Grisdale-Helland et al., 2008; Hansen et al., 2008; Schulz et al., 2008; Kjær et al., 2009; Green et al., 2011), and the beneficial protein-sparing effect of optimized dietary lipid concentration has been long established and well documented (Bromley, 1980; Beamish & Medland, 1986; Vergara et al., 1999; Lee & Kim, 2001). Optimization of dietary lipid levels has been found to improve fecundity in cultured fishes belonging to diverse taxa such as green swordtail *Xiphophorus helleri* (Ling et al., 2006), black sea bass *Centropristis striata* (Bentley et al., 2009), Atlantic cod *Gadus morhua* (Grisdale-Helland, et al., 2008), channel catfish *Ictalurus punctatus* (Sink & Lochmann, 2008), yellowfin sea bream *Acanthopagrus latus* (Zakeri et al., 2009), and orange-spotted spinefoot *Siganus guttatus* (Duray et al., 1994). Various biochemical mechanisms may account for the increased fecundity of fishes fed higher lipid-level diets including more energy available for spawning, an increase in the abundance of saturated and unsaturated fatty acids for inclusion in vitellogenins, and a higher proportion of essential long chain unsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in instances where marine oils are used as the lipid component. Increasing lipid levels from 6.5 to 12.1% in broodfish diets fed to channel catfish led to improved fecundity, egg volume, hatching success, and fry survival (Sink & Lochmann,

2008). Vitellogenesis in channel catfish is a protracted seasonal process that occurs annually and leads to ovulation of a single large batch of eggs. In Gulf killifish, the process is much more rapid and culminates in multiple fractional spawning events that take place over a period of months. For this reason, nutritional manipulations enhancing the fecundity of channel catfish may not be applicable to Gulf killifish.

Reproductive performance

The present study found no effect of changes in quantitative inclusion of dietary lipid on fecundity at the protein levels tested and was thus unable to suggest an optimal level of dietary energy or lipid content for Gulf killifish broodfish. Lack of variation in reproductive performance among treatment groups in the current study may have been caused by inadequate dietary protein levels. Diets were isonitrogenous and contained only ~33% protein (Table 4.1), a relatively low level for broodfish. Many reproductive experiments in other species have used protein levels ranging from 40-60% (Schulz et al., 2008; Zakeri., et al 2009; Lupatsch et al., 2010). Protein is vital as the other major component of egg yolk along with lipid and has important reproductive roles in hormones and enzymes (Brooks et al., 1997). Studies examining the effects of dietary protein content on reproduction have often determined that higher protein levels are optimal. For example, green swordtails produced significantly more fry when fed diets containing 50-60% protein while 30-40% protein significantly reduced fry production and 20% protein produced the lowest fry numbers (Chong et al., 2004). El-Sayed et al. (2003) tested Nile tilapia *Oreochromis niloticus*, a species regarded as having low requirements for dietary protein (Hafedh, 1999), and found optimum spawning performance occurred at 40% dietary protein, the highest experimental content. In the present experiment low protein levels may have acted as a limiting factor on reproductive capacity, especially in the high lipid content diets. Diet

consumption in fish is regulated by energy demand and the animal will not compensate for deficiencies of a particular dietary component by consuming past energetic requirements (Saravanan et al., 2012). Consumption data in the present study did not produce significant differences across treatments. Assuming similar levels of energy consumption, a higher percentage of energy consumed by fish fed at the lower lipid level was in the form of protein than fish fed the higher lipid content diets. This may have been a factor in the increased GSI observed in fish fed diet 1.

Larval morphometrics

While fecundity was unaffected by increased lipid content, high dietary lipid levels produced a statistically significant increase of as much as 10% in larval BCA at hatch when compared to the three lower lipid levels. In the present experiment, BCA was used rather than yolk or lipid droplet area to accurately reflect the presence of internal organs and other tissues within the area being measured and because measurement of a single discrete lipid droplet is not possible in Gulf killifish as the yolk contains numerous scattered smaller droplets. Previous studies examining Gulf killifish (Brown et al., 2011) and the California killifish *Fundulus parvipinnis* (Rao, 1974) have found that abiotic factors such as temperature and salinity can cause changes in yolk volume at hatch, with the differences attributed to incubation duration and efficiency of development. In such cases an inverse relationship between yolk volume at hatch and larval size was seen, suggesting that nutritional resources were allocated for growth and development in those larvae hatching with less yolk. No such inverse relationship existed in the present study, indicating that in females fed diets 4 and 5 some proportion of increased available lipids were mobilized for yolk deposition and would be available as substrates for catabolism or structural molecules for development until exogenous feeding began. Larval standard length at

hatch was significantly greater in the group of broodfish fed diet 2. This treatment level also produced eggs with the greatest mean volume (Table 4.3). Egg volume and length at hatch are highly positively correlated with female broodfish body size in Gulf killifish (Patterson et al., 2013) and differences in length at hatch observed in the present experiment are likely an artifact of the random event that the two largest females in the experiment were stocked into separate replicates fed diet 2. Considering previously discussed dominance behaviors and based on GSI data at the conclusion of the experiment, these females likely spawned a large proportion of the eggs representing their dietary lipid level and are thus responsible for the significant larval SL difference at hatch, rather than a treatment effect of consumed lipids.

Body composition indices

Fish fed the lowest lipid content diet had a significantly higher mean GSI value than other groups. Increased GSI in this group may be indicative of targeting nutritional resources for reproduction over somatic growth in fish fed at lower energy levels or the increased percentage of dietary energy from protein as discussed previously. Because increased dietary energy did not lead to significant gains in reproductive performance, its bioenergetic fate was probably assimilation into somatic tissues. In support of this assertion, fish fed higher lipid diets tend to incorporate those lipids as a higher proportion of total body mass relative to fish fed lower lipid levels (Lee et al.,2000; Grisdale-Helland et al., 2008; Hansen et al., 2008; López et al., 2009). Storage of additional lipid reserves in somatic tissues may have made gonad mass a smaller proportion of overall body mass and served as another factor contributing to depressed GSI values in fish fed diets 2-5. However, somatic lipid reserves are an important source for gonadal deposition and provide energy for spawning (Wiegand, 1996). Because the liver is the central management organ for lipid dynamics in fishes, Bolla et al. (2011) suggested utilizing liver

examinations such as TL concentration and quantitative histological analyses as biomarkers in the study of broodfish nutrition. Gulf killifish livers showed no significant differences in lipid content across dietary treatment levels in the present experiment. Both statistical analysis of TL content and visual analysis of liver sections across all dietary treatments supported this conclusion. High levels of within-replicate variation may have been due to the dominant feeding behavior of certain individuals. While across-treatment means for liver TL content and HSI did not vary, IP fat was significantly increased in broodfish fed the two highest lipid diets. In addition to the liver, the IP area is a common location for storage of excess energy in some fishes (Gao et al., 2010) and the present study demonstrates preferential IP lipid storage in Gulf killifish.

Fertilization rates

The low embryo viability rates observed in the current study are of particular concern as they reduce the quantity of viable larvae and exacerbate the low fecundity already present in this species. Culture techniques applied to Gulf killifish spawning in outdoor pools have yielded embryo viability rates of up to 70% during periods of the spawning season with optimal water temperatures (Gothreaux & Green, 2012). The 20-25% rates observed in the present study were not due to improper water temperature because 27°C was designed to mimic ideal seasonal spawning temperatures. Progressive sperm motility in males was not different among treatment levels and within the expected range; effectively eliminating physiologically deficient spermatozoa as a cause of poor fertilization. We hypothesize that low rates of embryo viability in the present study were due to animal behavior issues caused by tank design which resulted in an inability for males to effectively fertilize spawned eggs. The spawning units utilized in this study were relatively small (30 L), cylindrical, light colored, and had limited bottom surface

area. It is our opinion that some combination of these factors lead to stressful conditions for broodfish. Subsequent studies with Gulf killifish in the same system have also revealed low fertilization rates in spawning adults and high levels of cannibalism in juveniles. Indoor Gulf killifish fertilization rates have been improved by spawning in recirculation systems with larger (70 L) rectangular glass aquaria backed with opaque black plastic to minimize the contrast between animal and background (Patterson et al., 2013).

Conclusion

The present study did not duplicate promising results from other species. An increase in FO levels in broodfish diets for Gulf killifish was found to significantly increase only endogenous nutritional resources in newly hatched larvae while fecundity, GSI, and egg volume were not improved. Increased available energy appeared to be allocated to somatic tissues, specifically IP fat, rather than utilized for egg production. These data allow analysis of the balance between costs of additional dietary lipid inclusion and the limited reproductive gains observed. However other nutritional aspects of the experimental diets, such as a low protein level, may have negatively affected vitellogenesis and subsequent egg production.

CHAPTER 5
DIET INDUCED FATTY ACID VARIATION IN CRITICAL TISSUES OF A SPAWNING ESTUARINE FISH AND CONSEQUENCES FOR LARVAL FITNESS

Introduction

In fishes, non-genetic maternal factors including stress, age, pollution exposure, and nutritional status produce significant effects on reproductive performance and larval physiology (Berkeley et al., 2004; McCormick, 2006; Fuiman & Ojanguren, 2011; Chen et al., 2013). Factors that affect early life history can create magnified effects in terms of recruitment and population dynamics (Murphy et al., 2013). In addition, many fish populations rely on larval stages for connectivity and dispersal (Cowen & Sponaugle, 2009). Examinations of maternal nutritional status have frequently identified quantity and composition of dietary lipids as a factor in reproductive performance (Izquierdo et al., 2001; Lane & Kohler, 2006; Zeng et al., 2012; Norambuena et al., 2013).

Lipids serve diverse and critical functions in vertebrates as a storage form of energy, substrates for catabolic production of ATP, components of cellular and intracellular membranes, and signaling molecules (Zechner et al., 2009; Shevchenko & Simons, 2010). Fatty acids (FA) are a moiety that account for a large percentage of total lipid (Wheatley et al., 2008) and are responsible for fundamental biochemical and physiological properties. Specifically, organisms alter FA chains through modification of the number of carbon atoms or number and position of carbon-to-carbon double bonds (Jump, 2002). This *de novo* synthesis occurs by a series of desaturation and elongation reactions which insert double bonds or add pairs of carbon atoms to the chain, respectively. All vertebrates lack the desaturases Δ15 and Δ12 required to synthesize ω-3 and ω-6 unsaturated FA from C_{18} precursors. Because these desaturases are lacking, α-linolenic acid (ALA; 18:3ω-3) and linoleic acid (LA; 18:2ω-6) must be ingested in vertebrate

diets (Lands, 1992). FAs that must be acquired exogenously have been termed essential fatty acids (EFAs) (Rivers et al., 1975), with ω-3 and ω-6 representing distinct metabolic families as they cannot be interconverted *in vivo* (Mead, 1981; Jump, 2002).

In addition to requirements for ALA and LA, some animals lack the ability to efficiently biosynthesize longer chain C_{20} and C_{22} polyunsaturated fatty acids (LC-PUFAs). Figure 5.1 copied from Tocher (2003) details pathways of FA biosynthesis. Arachidonic acid (ARA;

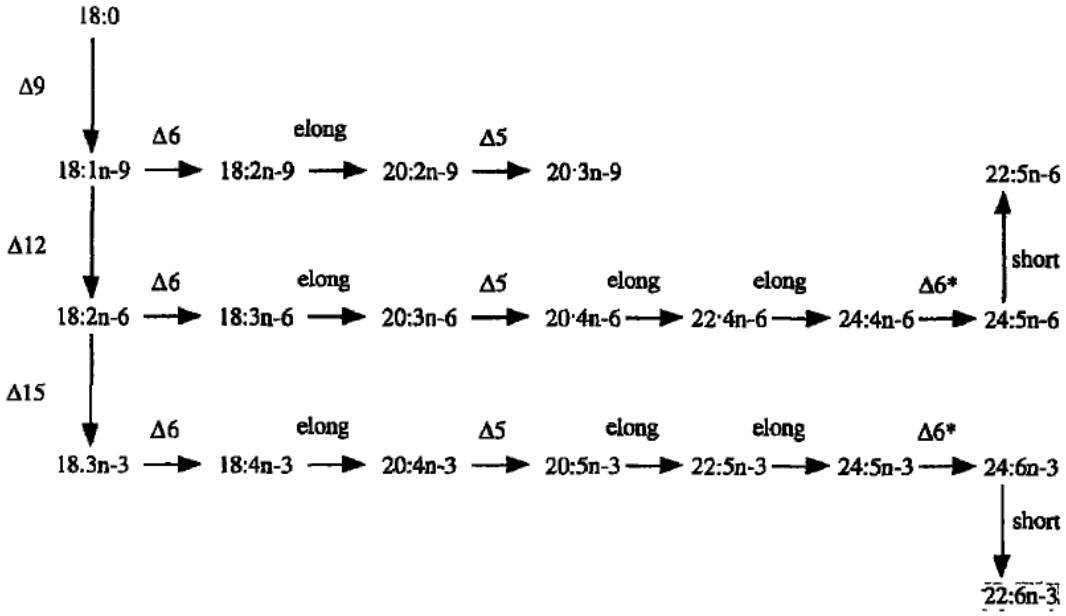

Figure 5.1. [Figure 6 in Tocher et al., (2003)]. Pathways of biosynthesis of C_{20} and C_{22} HUFA from n-3, n-6, and n-9 C_{18} precursors. Δ5, Δ6, Δ6*, Δ9, Δ12, Δ15, Fatty acyl desaturatses; Elong, Fatty acyl elongases; Short, chain shortening. Δ9 desaturase is found in all animals and plants whereas Δ12 and Δ15 desaturases are generally only found in plants and so 18:2n-6 and 18:3n-3 are "essential" fatty acids (EFA) for many animals including many species of freshwater fish. Carnivores and many marine fish generally have only limited ability to carry out the above conversions due to specific deficiencies in desaturases and/or elongases and so 20:5n-3 and 22:6n-3 are their EFA. The Δ6* enzyme acting on C_{24} fatty acids may or may not be the same enzyme (Δ6) that acts on C_{18} fatty acids.

20:4ω-6), eicosapentaenoic acid (EPA; 20:5ω-3) and docosahexaenoic acid (DHA; 22:6ω-3) are three LC-PUFAs that play vital roles in vertebrate physiology. For humans (Jump, 2002; Riediger et al., 2009), mammalian carnivores such as cats (Rivers et al., 1975; MacDonald et al., 1984), and marine fishes (Tocher, 2010), these LC-PUFAs are effectively EFAs. As fish lipids

are the primary source of ω-3 LC-PUFAs in the human diet, extensive work has elucidated the mechanisms of FA metabolism and function in fishes (Bell et al., 2002; Chatelier et al., 2006; González-Rovira et al., 2009; Tocher, 2010; Lu et al., 2013; Norambuena et al., 2013). Through this research, a significant difference in LC-PUFA biosynthesis has emerged between marine and freshwater fishes. Tocher (2003) published a comprehensive review which detailed this dichotomy. Briefly, marine diatoms and flagellates are rich in EPA and DHA, respectively. These FAs are transferred through the food web intact, such that marine fishes have not faced selective pressure to convert C_{18} precursors to LC-PUFAs. Conversely, freshwater food webs are deficient in LC-PUFAs, especially DHA, so fishes inhabiting these ecosystems have developed the ability to desaturate and elongate C_{18} precursors. Genes encoding the requisite elongase and desaturase enzymes are present in marine fish genomes, however, one or more of these genes are not sufficiently expressed and/or enzyme activity is reduced such that efficient completion of the biosynthetic pathway is not possible (González-Rovira et al., 2009).

Fishes of the genus *Fundulus* are utilized as important model organisms in studies of teleost biology (Burnett et al., 2007) but FA dynamics during reproduction have not been examined in this genus. Gulf killifish *Fundulus grandis* is characterized by high levels of physiological plasticity (Griffith, 1974; Martínez et al., 2006; Brown et al., 2012; Patterson et al., 2012) and is ecologically significant in estuaries it inhabits (Subrahmanyam & Drake, 1975). Established hypotheses regarding a dietary requirement for LC-PUFAs in fishes may not be applicable for *F. grandis* as this euryhaline species occupies a unique margin between freshwater and marine systems. Natural diets of *F. grandis* indicate a generalist feeding strategy with food sources including benthic invertebrates, small fishes, plants, algae, and terrestrial insects (Rozas & LaSalle, 1990; Ley et al., 1994). The plant material and terrestrial insects that account for a

significant portion of *F. grandis* diets contain lipids deficient in FAs with chain lengths greater than C_{18} (Hitchcock & Nichols. 1971; Stanley-Samuelson et al.. 1988). Rates of lipid mobilization for vitellogenesis are assumed to be high during reproduction in *F. grandis*. Fractional spawning events periodically produce small groups of relatively large eggs (Patterson et al., 2013) during a protracted spawning season (Green et al., 2010), with vitellogenic periods as short as two days for individual oocytes (Hsiao & Meier, 1988). As a consequence, variations in lipid composition of food consumed directly before and during spawning have the potential to greatly influence physiology of prefeeding larvae.

The current study utilized a controlled laboratory environment to construct a multi-tissue evaluation of diet-induced FA variations in actively spawning *F. grandis*. Physiological consequences of these variations for spawning performance and prefeeding yolk-sac larvae were quantified. We hypothesized that spawning *F. grandis* denied adequate levels of dietary LC-PUFAs would compensate by some combination of three potential means: mobilization of somatic FA reserves, biosynthesis of LC-PUFAs from C_{18} precursor FAs, or a reduction in reproductive output and offspring fitness. This study was designed to provide information on fatty acid partitioning during reproduction in an important model teleost occupying unique phylogenetic and ecological classifications whose lipid dynamics have not been widely examined.

Methods

Diets and Animal Care

Four experimental diets (Table 5.1) formulated to be isonitrogenous and isocaloric were analyzed for proximate composition (Table 5.2). Relative proportions of lipid as corn oil (CO) or menhaden fish oil (FO) were adjusted across the four diets to produce a gradient in FA

Table 5.1. Formulation of experimental diets fed to *Fundulus grandis* throughout the experiment.

Ingredient (g 100 g^{-1})	Diet 1	Diet 2	Diet 3	Diet 4
Soybean Meal	47.07	47.07	47.07	47.07
Corn Grain	20.00	20.00	20.00	20.00
Corn Gluten	15.00	15.00	15.00	15.00
Menhaden Fish Oil	--	2.00	4.00	6.00
Corn Oil	6.00	4.00	2.00	--
Wheat Flour	5.41	5.41	5.41	5.41
Dicalcium Phosphate	3.00	3.00	3.00	3.00
CMC	2.00	2.00	2.00	2.00
Vitamin Pre-Mix	0.50	0.50	0.50	0.50
L-methionine	0.40	0.40	0.40	0.40
Mineral Pre-Mix	0.25	0.25	0.25	0.25
L-taurine	0.10	0.10	0.10	0.10
L-lysine-HCl	0.10	0.10	0.10	0.10
Choline Chloride	0.09	0.09	0.09	0.09
Vitamin C (Stay-C)	0.06	0.06	0.06	0.06
Ethoxyquin	0.02	0.02	0.02	0.02

profiles. Fish meal was not utilized as a protein source because it contains significant amounts of FO, and the present experiment was designed to test a diet deficient in LC-PUFAs. Fatty acid content of the experimental diets (Table 5.2) was measured using methods identical to those described below for eggs and animal tissue.

Animals were handled using methods approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee under protocol #AE2009-22. Experimental diets were tested in quadruplicate by randomly assigning each of the four treatment levels to four replicated spawning units. Each of sixteen replicate spawning units were stocked with six females and three males selected at random from populations of mean body mass 16.02 ± 0.15 g and 15.87 ± 0.21 g (mean ± SEM), respectively. A single recirculating system serviced all spawning units. Individual spawning units were 100-L glass aquaria containing 75 L of water maintained at 12‰ using Crystal Sea Marinemix (Marine Enterprises International Inc.,

Table 5.2. Proximate analysis and fatty acid composition of diets fed to *F. grandis* throughout the experiment.

Analyzed Composition	Diet 1	Diet 2	Diet 3	Diet 4
Protein (% DM)	35.58	36.09	35.68	35.99
Fat (% DM)	8.85	9.04	9.09	8.77
NFE (% DM)	36.17	35.36	35.71	35.55
Fiber (% DM)	2.1	1.9	2.0	2.3
Ash (% DM)	6.31	6.4	6.44	6.47
Moisture (%)	11.05	9.04	9.09	8.77
Gross Energy (KJ/g)	18.46	18.50	18.46	18.46
Fatty Acid[#]				
12:0	0.41	0.00	0.00	0.00
14:0	0.00	2.33	4.88	8.45
16:0	16.35	17.81	19.71	23.55
18:0	2.95	3.25	3.56	4.03
22:0	0.00	0.00	0.10	0.31
∑SFA[†]	19.71	23.39	28.25	36.34
16:1n-7	0.15	3.58	7.65	13.39
18:1n-9	35.77	29.76	22.66	14.61
20:1n-7	4.21	4.62	5.42	6.17
20:1n-9	0.00	0.00	0.00	0.00
24:1n-9	0.00	0.47	0.68	1.17
∑MUFA[‡]	40.13	38.43	36.41	35.34
16:3n-4	0.00	0.27	0.48	0.70
16:4n-1	0.00	0.51	0.91	1.84
18:2n-6 LA	85.76	65.63	49.37	33.36
18:3n-3 ALA	0.39	0.41	0.43	0.46
20:2n-6	0.00	0.00	0.11	0.31
20:3n-6	0.12	0.35	0.13	0.31
20:4n-6 ARA	0.14	0.75	1.31	1.97
20:5n-3 EPA	0.10	5.99	12.89	22.54
22:4n-6	0.24	0.40	0.47	0.85
22:5n-3	0.00	1.07	2.44	3.76
22:6n-3 DHA	0.14	5.11	10.77	18.07
∑PUFA[*]	86.89	80.49	79.31	84.17
∑n-3	0.63	12.58	26.53	44.83
∑n-6	86.26	67.13	51.39	36.80
n-3:n-6	0.01	0.19	0.52	1.22

[#]µg mg dry mass^{-1}; †Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Baltimore, MD, USA) with dechlorinated municipal water added periodically to compensate for evaporation. Each tank was wrapped in opaque polyethylene sheeting to block visual interaction among spawning units.

Salinity, dissolved oxygen, total ammonia nitrogen, pH, and alkalinity (as $CaCO_3$) were measured weekly and remained within acceptable ranges for *F. grandis* (Tatum et al., 1982). Fish were fed respective twice daily at a rate of 2.5% body mass per day for an eight-week inclusion and conditioning period before spawning was initiated. Feeding was monitored to ensure that all or the vast majority of diet was consumed in each replicate tank. Induction of spawning was achieved using photoperiod and temperature manipulations over a two-week period. Water temperature was increased at a rate of 0.5 °C per day from 19 °C to a spawning temperature of 26 °C. Simultaneously, day length was increased by 15 min per day from an initial photoperiod of 11.5 h light:12.5 h dark to a spawning photoperiod of 13 h light:11 h dark. Water temperature and photoperiod remained constant throughout spawning. Eggs were collected weekly throughout a 10-week period and air incubated using techniques developed previously (Coulon et al. 2012). Feeding regime throughout spawning was maintained as during the inclusion period.

Fecundity and Embryo Viability

Each week replicate tanks received a single spawning substrate for 24 h before eggs were collected. After weekly collections, fecundity data were generated by counting eggs from each replicate and calculating a mean across all four replicates from each treatment level. Eggs collected from each replicate were maintained in separate incubation chambers for 48 h before neurulation was checked by light microscopy. Neurulated embryos were considered viable and utilized to calculate embryo viability rates. Viable embryos were immediately designated for

larval fitness examinations or preserved under nitrogen at -80°C for FA analysis. All larval fitness examinations were conducted on animals collected from weeks 3 through 7 of the spawning period while FA analysis was performed on embryos spawned in weeks 8 through 10.

Larval Indices

Larval fitness experiments were performed on newly hatched prefeeding yolk-sac larvae in order to quantify the physiological limits of acute thermal tolerance, endogenous nutritional resources, and hyposmoregulatory ability. Embryos remained associated with their respective spawning units so that critical thermal maximum (Ct_{max}) and survival activity index (SAI) experiments could be performed in quadruplicate. Embryos were pooled by treatment for salinity tolerance experiments due to the greater numbers of larvae required. Water used for larval fitness experiments was prepared from deionized water utilizing Crystal Sea Marinemix to regulate water chemistry.

Methodology of thermal tolerance trials followed the recommendations of Beitinger et al. (2000) for determination of CT_{max}. Three larvae from each of four replicates were transferred to 25 mL Erlenmeyer flasks containing 15 mL of 12‰ water. Thermal exposure was assessed using a temperature probe inserted into an additional flask containing 15 mL of 12‰ water. Flasks were anchored to a programmable orbital shaker water bath rotating at 50 rpm to provide a homogenous thermal environment. Larvae hatched at 25°C and acclimated for 2 h before a 0.3°C min^{-1} temperature increase was initiated. Water temperature at loss of equilibrium was recorded for each animal. Following loss of equilibrium, larvae were immediately transferred back to acclimation temperature and individuals which did not regain equilibrium and survive were excluded from analysis. Mean temperatures at loss of equilibrium for the three larvae from each replicate were used in statistical analysis.

Endogenous nutritional resources were assessed by calculation of SAI following the methods of Matsuo et al. (2006). Six larvae from each of four replicates were placed in separate tissue culture wells containing 25 mL of 12‰ water which was renewed in 48 h intervals to prevent accumulation of metabolites. Temperature was maintained at 24°C and larvae were monitored for mortality every 12 h. Observed mortality was recorded in half-day increments and SAI was calculated for each replicate using the formula:

$$SAI = \frac{1}{N}\sum_{i=1}^{k} N - h_i * i \quad (10)$$

where N was the total number of hatched larvae, h_i was the cumulative mortality by the ith day, and k was the number of days elapsed when the last mortality occured.

Hyposmoregulatory ability of newly hatched larvae at elevated salinities was quantified across treatment levels by generation of a median lethal concentration (LC_{50}) for salinity. Preliminary range finding tests were conducted at 30-80‰ to determine salinity intervals for 48 h-LC_{50} experiments. At each of the experimental exposure salinities, 30 larvae per treatment level were distributed among three replicate well plates containing 10 wells each. A 12‰ control was utilized with experimental salinities of 50, 60, 70, and 80‰. All larvae were hatched at 12‰, rinsed with water of their designated salinity, and distributed individually to wells containing 10 mL of exposure water. An acute 48 h static renewal design was utilized, whereby exposure water was renewed to prevent accumulation of metabolites and larvae were checked for mortality every 12 h.

Egg and Tissue Fatty Acid Analyses

At the conclusion of the 10-week spawning period, two females were selected at random from each replicate and euthanized with an overdose of tricaine methanesulfonate buffered to pH 7.0 with sodium bicarbonate. Dissections were performed immediately to remove liver, muscle,

and ovary. Post-vitellogenic oocytes were removed from dissected ovaries so that FA analyses of the gonad would not include significant amounts of lipoprotein yolk. These tissues, along with previously collected egg samples, were lyophilized prior to storage under ultra-pure nitrogen at -80°C. Sample dry mass was determined gravimetrically before analysis. Embryos were pooled across the four replicates within each treatment and analyzed in duplicate because number of embryos required for statistical analyses were not available after allocation for other experimental examinations.

Extraction and transesterification of FA utilized methods similar to those of Timmins et al. (2009). Heneicosanoic acid (C21:0; Sigma-Aldrich, St. Louis, MO, USA) was added to each sample prior to transesterification as an internal standard. Following extraction and transesterification, 1 μL of hexane solvent was injected into an Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA). A 30 m Supelco 2380 column (Sigma-Aldrich, St. Louis, MO, USA) was used with an injector temperature of 175°C and 50:1 split. Helium carrier gas was used at 24 cm sec^{-1}. Oven temperature began at 50°C for 2 min and increased 4°C min^{-1} to 250°C, which was held for 10 min. A flame ionization detector was utilized at 300°C. Fatty acid methyl esters (FAME) were individually quantified by comparing the ion current area of each FAME to that of the internal standard. FA were quantified on a concentration basis (μg FAME mg sample dry $mass^{-1}$) rather than mass percentage basis (% total FA mass) to clarify interpretation when comparing tissues with varying total lipid content (Schwertner & Mosser, 1993; Mocking et al., 2012). FAMEs were identified by retention time comparison to standards.

Statistical Analyses

Statistical tests were performed using Statistical Analysis Software version 9.3 (SAS Institute Inc., Cary, NC, USA). Values are presented as means ± SEM. Acceptance criteria for

statistical significance were $\alpha = 0.05$ for all tests. Fecundity count data were transformed as square root(y+1) prior to analysis by mixed model repeated measures ANOVA and the Tukey HSD *a priori* test. Embryo viability data were analyzed as binomial response variables by logistic regression. Salinity LC_{50} of larvae from each treatment level was calculated by probit analysis rather than the trimmed Spearman-Karber method because more than two concentrations experienced partial mortality and full mortality was not achieved at the highest concentration in all cases. Mean values for larval CT_{max}, SAI, and key FA across dietary treatment levels within each tissue type were compared across treatment levels using general linear model ANOVA with the Ryan-Einot-Gabriel-Welch *a priori* test. For individual FAs, sequential Bonferroni corrections were applied to probability values to maintain familywise $\alpha = 0.05$ for each tissue type.

Results

Reproductive Performance and Larval Indices

Periodic fecundity (Figure 5.2) was not significantly affected by lipid composition of broodfish diets ($F_{3,12} = 0.37$, $p = 0.7730$). Figure 5.2 also illustrates the characteristic cyclic nature of fractional spawning in *F. grandis*, which did not appear to be affected by dietary lipid composition. Embryo viability rates (Table 5.3) were significantly different across treatment levels (Wald $X^2 = 12.3009$, $p = 0.0005$) with fish fed diet 1 experiencing the lowest value for this parameter. Indices of larval tolerance to physiological extremes varied significantly among treatment levels. Figure 5.3 presents data for CT_{max} and SAI, which were significantly affected by lipid composition of broodfish diets ($F_{3, 12} = 4.07$, $p = 0.0328$) and ($F_{3,12} = 3.35$, $p = 0.0485$), respectively. 48 hour-LC_{50} for salinity in newly hatched larvae (Table 5.3) was high in all treatment levels, with the highest values occurring in diets 3 and 4.

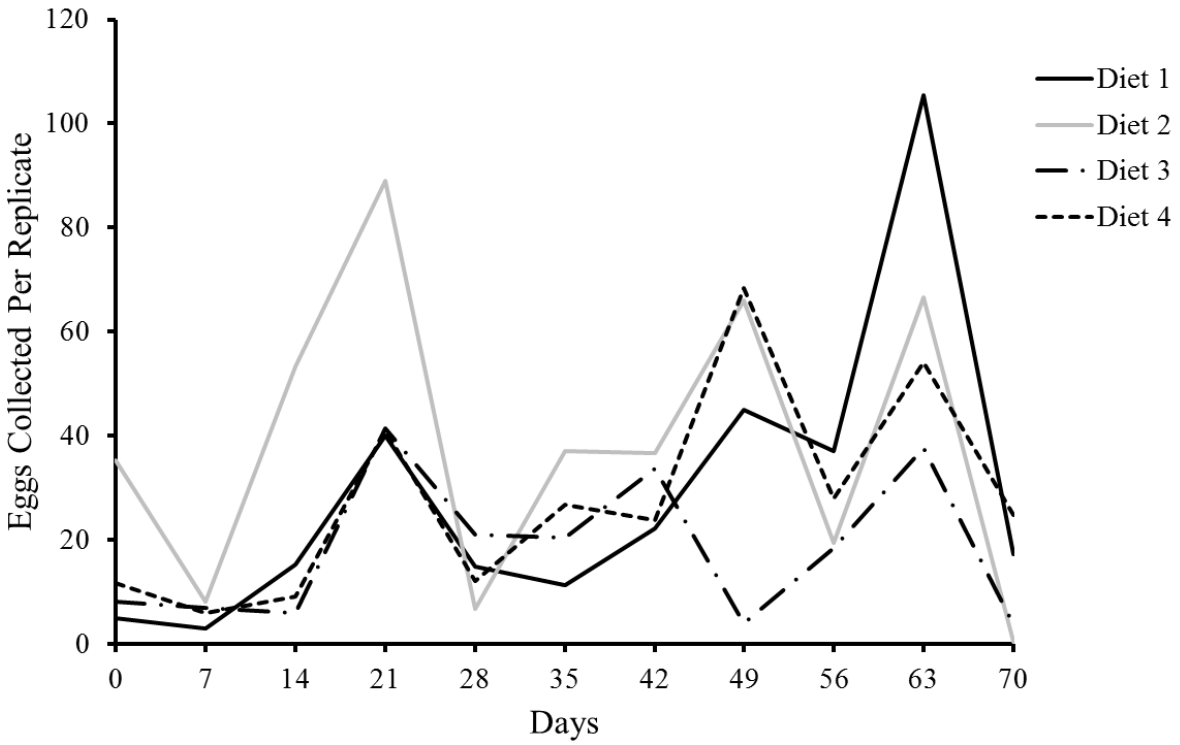

Figure 5.2. Periodic fecundity of four dietary treatment groups (see Tables 5.1 and 5.2) of *Fundulus grandis* throughout a 10-week spawning period. Repeated measures ANOVA showed no statistically significant difference in reproductive output.

Table 5.3. Embryo viability rates and salinity lethal concentration$_{50}$ for newly hatched larvae across dietary treatment levels (see Table 5.1 and 5.2).

Diet	% Viable Embryos	Lower 95% C.I.	Upper 95% C.I.	48 h-LC_{50} (‰)	Lower 95% C.I.	Upper 95% C.I.
1	70.4	66.7	74.0	72.4	69.5	76.1
2	80.7	78.2	83.3	71.2	67.9	76.5
3	76.0	71.7	80.0	79.0	74.0	88.9
4	84.3	81.2	87.1	75.8	72.3	80.9

Egg and Tissue Fatty Acid Composition.

Twenty FAs were identified above trace levels and quantified in egg, tissue, and diet samples. Total FA content was highest in liver tissue followed by ovary and then muscle.

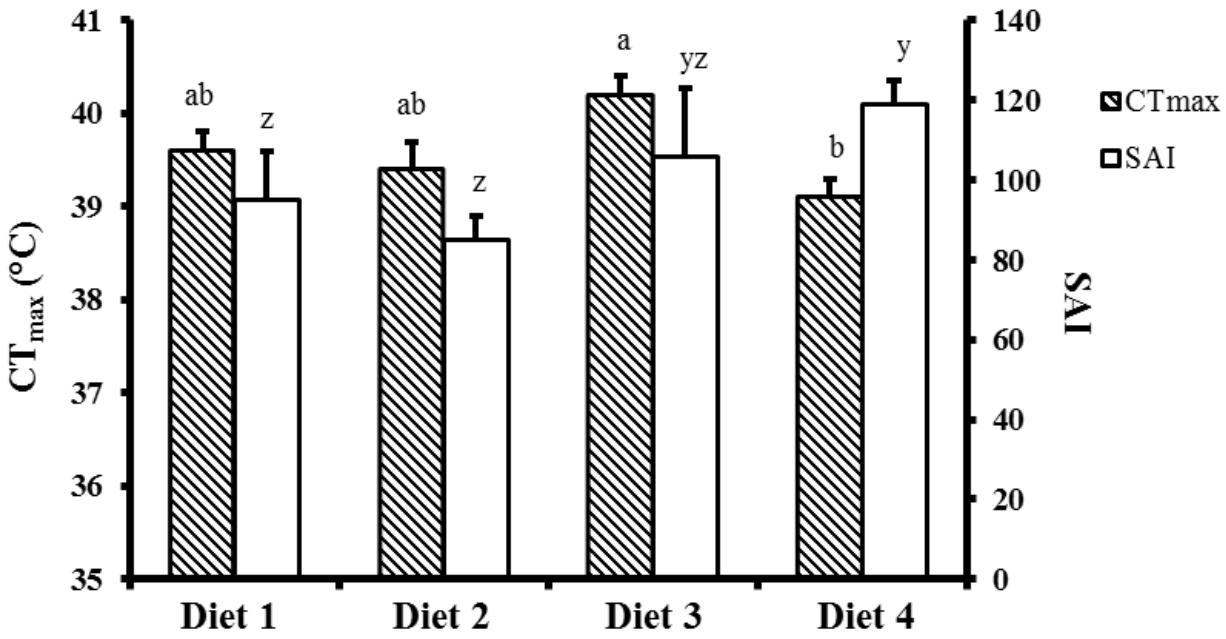

Figure 5.3. Mean critical thermal maximum (CT_{max}) and survival activity index (SAI) values for larvae produced by females fed four dietary treatment levels (see Table 5.1 and 5.2) differing in fatty acid composition. Letters denotes statistical significance within each parameter across treatments levels (REGWQ; $p<0.05$).

Across dietary treatment levels, total FA content of each tissue was statistically similar. FA profiles for liver, muscle, ovary, and eggs are contained in Appendix B. Analysis of variance results for C_{18} ω-3 and ω-6 precursors and primary LC-PUFA end products [ALA, LA, EPA, DHA, and ARA; (Tocher, 2003)] are contained in Appendix B with *a priori* designations appearing in tables for corresponding tissues. Figure 5.4 contains plots of tissue:diet ratios, or biocencentration factors, for ALA, LA, EPA, DHA, and ARA.

Discussion

The importance of lipids in animal reproduction has been well documented. Lipids are especially critical in the physiology of fishes because this macronutrient class is utilized as the main source of energy for all biological processes (Black & Skinner, 1986). Investigations of FA dynamics are central to understanding how lipids are utilized because FAs have important

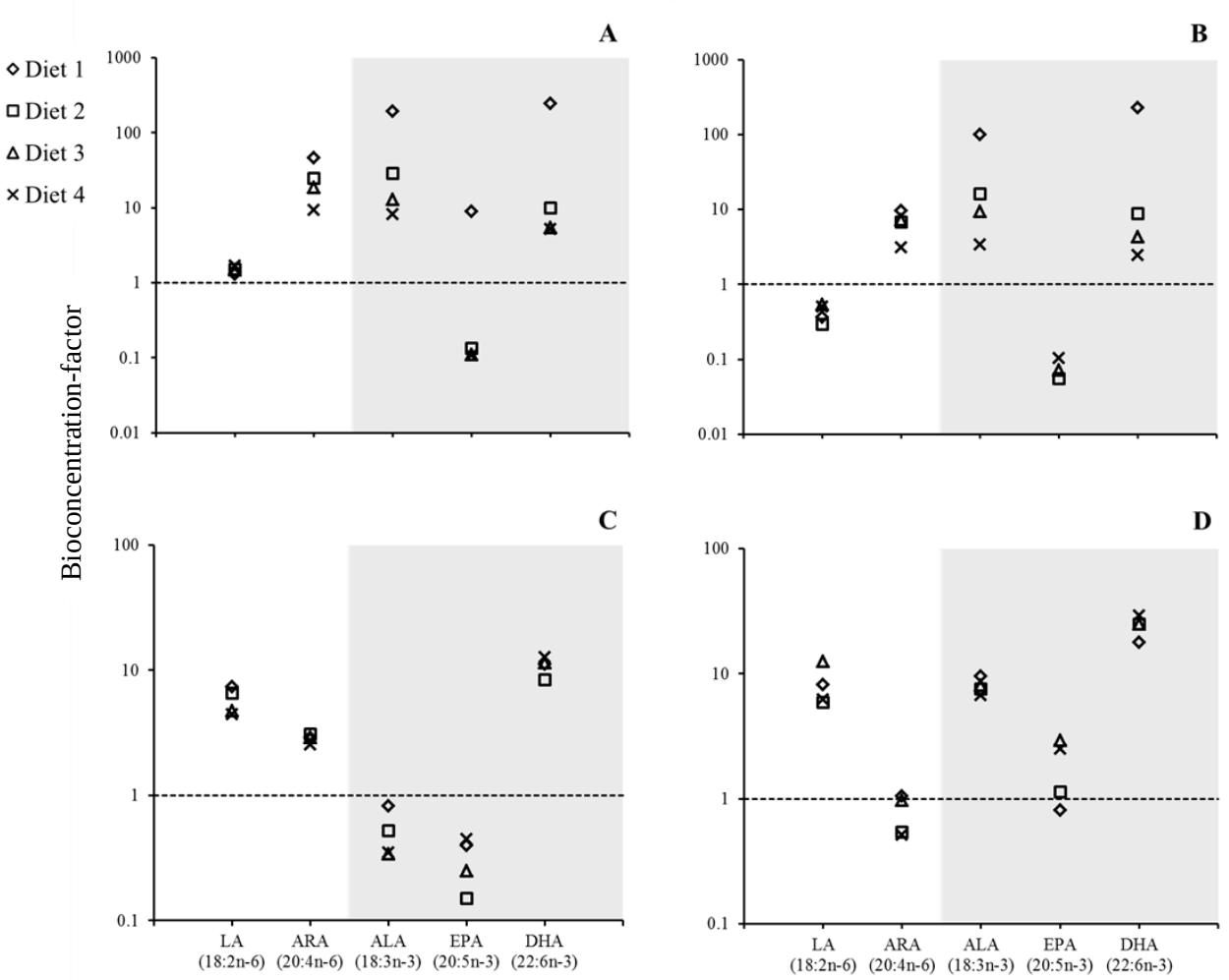

Figure 5.4. Bioconcentration factors for critical FAs in in (A) liver, (B), eggs, (C) muscle, and (D) ovary from females in the present study. Shaded plot area denotes ω-3 metabolic family while white area denotes ω-6. Dashed line represents equality between sample and dietary fatty acid concentration. Refer to Tables 5.1 and 5.2 for diets.

biochemical properties (Falk-Petersen et al., 2008). Reproduction in *F. grandis* typifies an income-spawning strategy (McBride et al., 2013) with rapid vitellogenesis (Hsiao & Meier, 1988) and thus high rates of lipid throughput due to continuous allocation for oocyte production over a protracted spawning season (Green et al., 2010). *F. grandis* also produces eggs containing discrete oil droplets in addition to lipoprotein yolk, an indication that Gulf killifish eggs are relatively high in lipid content compared with fishes whose eggs do not contain oil droplets (Tocher, 2003). Other income-spawning fishes have been shown to produce rapid

reproductive modifications in response to changes in nutritional status. Mummichog *F. heteroclitus*, a closely related congener of *F. grandis*, discontinued oocyte maturation within three days following experimentally induced starvation but was able to restore production of mature oocytes within three additional days following resumption of feeding (Wallace & Selman, 1978; Wallace, 1980). Continuous vitellogenesis and high per-oocyte lipid demand in *F. grandis* allowed reproductive effects of ingested FA to be captured within the timeframe of the present study.

Periodic fecundity of *F. grandis* was unaffected by the dietary FA compositions evaluated in this study (Figure 5.1). Of particular note was the reproductive output of fish fed diet 1 at the latter stages of the 10-week spawning period. This represents maintenance of normal fecundity after nearly 18 weeks of consuming a diet deficient in LC-PUFAs. In contrast to *F. grandis*, teleosts including white bass *Morone chrysops* (Lane & Kohler, 2006), Nile tilapia *Oreochromis niloticus* (El-Sayed et al., 2005), Catla *Catla catla* (Nandi et al., 2007), and European sea bass *Dicentrarchus labrax* (Cerda et al., 1995) have demonstrated significant positive correlations between fecundity and dietary ω-3 content. The ability of *F. grandis* to maintain reproductive output despite low dietary ω-3 FA content may be indicative of a low dietary requirement or high rates of somatic storage in this metabolic family of FAs. Although fecundity was unaffected by dietary FA composition, the lower embryo viability rates of fish fed diet 1 may have been caused by reduced ω-3 content and ω-3:ω-6 ratio in the eggs. Individual FAs have been shown to affect fertilization success. Fernández-Palacios et al. (1995) found significant increases in fertilization rate of gilthead sea bream *Sparus aurata* eggs when dietary EPA levels were increased.

Results of larval stress experiments may provide insight on physiological effects of FA composition for prefeeding larvae. FA composition of polar-lipid-rich yolk and the developing embryo can be modified during embryogenesis in fishes. Oil droplets are generally thought to serve as energy reserves because they contain high proportions of neutral lipids with monounsaturated FAs which are catabolized for energy after hatch (Tocher, 2010). The dynamics of FA provision for catabolism during embryogenesis and post-hatch are highly variable in fishes (Desvilettes et al., 1997; Cejas et al., 2004; Zengin & Akpinar, 2006; Palacios et al., 2007; Sewall & Rodgveller, 2009) and have not been described in *F. grandis*. However, egg FA profiles contained in Appendix B provide an early embryogenesis baseline of the FA available throughout development for larvae in the present study. Hatchling *F. grandis* larvae possess fully functional eyes and mouthparts (Patterson et al., 2013) but larvae in the present study were denied access to potential exogenous nutritional sources to ensure that maternal lipids contained in the yolk and oil droplet were the only available source.

While CT_{max} data from the present study were statistically different, the only *a priori* difference was a decrease in mean CT_{max} of 1.1°C between larvae of fish fed diets 3 and 4. All treatment level CT_{max} means were above 39.0°C, which is relatively high for fishes acclimated at 25°C (Beitinger et al., 2000). As a member of the order Cyprinodontiformes, high CT_{max} values in *F. grandis* larvae were not unexpected. A group of nine Cyprinodontiform fishes have produced CT_{max} values in adults between 42.4 and 45.4°C which possibly establish an upper thermal limit for biokinetic function in ectothermic aquatic vertebrates (Brock, 1985; Beitinger et al., 2000). Included in this group is the *F. grandis* congener *F. heteroclitus*, which recorded a CT_{max} of 44.1°C when acclimated to 27.0°C (Bulger, 1984; Bulger & Tremaine, 1985). Acclimation temperature for CT_{max} experiments is positively correlated to endpoint value

(Beitinger et al., 2000) and larvae in the present study would likely have produced CT_{max} means over 40.0°C if acclimated at higher temperatures. While most studies of CT_{max} have been conducted on adults, Galleher et al. (2010) acclimated 9 day old *F. grandis* and *F. heteroclitus* larvae at 29°C and observed CT_{max} ranging from 43.0 to 43.6°C with no significant differences between the species. It is evident that CT_{max} data for all treatment levels in the present study fall within expected ranges for *F. grandis* acclimated at 25°C and that ω-3 deficiencies in maternal diets did not produce any debilitating negative impacts on acute thermal stress tolerance.

48-h salinity LC_{50} data indicate a general trend towards increased resilience to hyperosmotic stress in larvae produced by fish consuming higher levels of ω-3 FAs. Studies examining effects of increasing dietary ω-3 levels in larval fishes have found a positive correlation between salinity tolerance and ω-3 consumption in Japanese flounder *Paralichthys olivaceus* (Furuita et al., 1999) and zander *Sander lucioperca* (Lund et al., 2012). Both studies noted a greater importance of DHA relative to EPA as the primary LC-PUFAs associated with osmoregulatory performance. In contrast, Noori et al. (2011) tested dietary enrichment with highly unsaturated EFA in larval Persian sturgeon *Acipenser persicus* and beluga sturgeon *Huso huso* and found no effect of dietary EFAs on tolerance for acute exposure to hyperosmotic media. While endogenous FA availability does appear to affect extreme hyposmoregulatory ability, it is important to note that larvae in the present study all had 48 h LC_{50} values for salinity >71‰. This level of salinity tolerance upon direct transfer for larvae hatched in 12‰ indicates that the characteristic euryhalinity of *F. grandis* was maintained at a level highly unlikely to adversely affect animals under environmentally relevant conditions.

Starvation tolerance as quantified by SAI was also greater in larvae from broodfish fed the highest level of ω-3 FA. The relationship between endogenous FA content and starvation

tolerance in fish larvae has not been previously examined, providing no basis for comparison to the present study. Egg content of monounsaturated FAs, which are preferentially catabolized for energy (Tocher, 2010), did not produce an interpretable gradient relative to SAI. Concentration data for other classes of FA also provided little explanation for the observed increase in starvation tolerance. Similar to differences in salinity tolerance, the statistically significant reduction in SAI is unlikely to represent an adverse effect under natural conditions. For example, larvae from fish fed diet 2 had the lowest SAI value and lived a mean of 13.9±0.1 days (n=24) while larvae from fish fed diet 4 had the highest SAI value and lived a mean of 16.4±0.4 (n= 24) days. Under culture or natural conditions this 2.5 day difference, while significant, is not likely to manifest in decreased survival for a species with precocial larvae which hatch with functional eyes and mouthparts. Cumulatively, the results of stress physiology experiments on *F. grandis* larvae suggest that variations in endogenous FA composition were associated with differences in fitness at physiological extremes but no debilitating complications under standard biological conditions for the species.

Comparison of tissue and dietary FA profiles generated in this study may provide insight into coping strategies when LC-PUFAs are deficient in *F. grandis* diets. Stanley-Samuelson et al. (1988) noted that FA analyses should be careful to acknowledge that samples used to generate FA composition data represent "an instantaneous observation of a suite of continuing dynamic processes". Recognizing this consideration, methodology of the present study was designed to create an endpoint observation of actively spawning *F. grandis* consuming diets of differing FA content. Tissue FA samples were collected following a lengthy pre-spawn feeding period and a spawning period encompassing numerous vitellogenic cycles. This methodology was designed to maximize influence of exogenous FA and reduce persistence of endogenous FA in test fish.

Tissue and egg FA profile data strongly indicate a significant effect of exogenous lipids on FA dynamics during spawning in the present study. In fishes, the liver serves as a storage organ for lipids in addition to its crucial role in their metabolism and trafficking. This organ is also where dietary FAs are transferred to endogenous loops including vitellogenesis (Sheridan 1988). Income-spawning fishes such as *F. grandis* must transfer lipids rapidly to facilitate prolonged periods of vitellogenic activity (McBride et al., 2013). Relatively high (~44% of dry mass) total FA content of livers which did not vary significantly across treatment levels indicated that the lipid storage function of this organ was not affected by dietary FA composition. However, dietary FA composition did produce significant effects on liver FA profiles. Important ω-3 and ω-6 C_{18} precursors and LC-PUFA end products all varied significantly across dietary treatment levels in liver tissue.

Biosynthesis of LC-PUFAs from C_{18} precursors had potential support from matching gradients of ARA and its precursor LA across diets. Levels of ARA were lowest in diet 1 and highest in diet 4, as FO became a greater proportion of the lipid component while the LA precursor was highest in diet 1 and lowest in diet 4. However, in liver tissue, ARA concentration was highest in fish fed diet 1 and lowest in fish fed diet 4. The gradient of ARA in the liver mirrored that of its C_{18} precursor while contrasting what was available in the diet. Because the same suite of enzymes desaturate and elongate ω-6 and ω-3 FAs (Tocher, 2003), the ability of *F. grandis* to biosynthesize ARA from LA, as evidenced by the present study, is indicative of a concurrent ability to synthesize ω-3 LC-PUFAs from precursors. Euryhaline members of the genus *Fundulus* including *F. grandis* have been documented as establishing successfully reproducing populations in landlocked freshwater lakes and streams (Simpson & Gunter, 1956; Denoncourt et al., 1978; Samaritan & Schmidt, 1982). This occurrence would be greatly aided by

an ability to biosynthesize LC-PUFAs from C_{18} precursors and selectively retain ω-3 FAs. Despite evidence suggesting its possible occurrence, methods utilized in this study were not suitable for direct measurement of FA elongation and desaturation rates in *F. grandis* and this determination of any potential biological significance is not possibly.

Content of C_{18} precursor FAs and end product LC-PUFAs did not vary significantly across dietary treatment levels in muscle and ovaries. These statistical similarities coupled with the variation observed in the liver indicate that wide discrepancies in dietary lipid FA composition are managed in the liver of *F. grandis*, allowing peripheral tissues to maintain normal function. Plots of tissue:diet FA ratios demonstrate diet dependence or independence in the five critical FAs. Diet dependent FAs, which vary in proportion to dietary content across the four treatment levels, appear as closely grouped points within each tissue type. Ratios of the ω-3 metabolic family in liver and eggs for females fed diet 1 were strongly diet independent. Diet independence was indicative of a physiological mechanism being utilized to compensate for a lack of dietary availability in a FA. Of note is the absence of detectable levels of EPA in eggs of fish fed diet 1. This FA was concentrated in liver tissue relative to the diet and thus should have been available for vitellogenesis. Fernández-Palacios et al. (1995) noted that EPA concentration was more diet dependent than DHA in eggs of *S. aurata* and other authors have noted that DHA is the most highly conserved ω-3 LC-PUFA in fishes (Zengin & Akpinar, 2006; Sewall & Rodgveller, 2009; Tocher, 2010). The present study is in agreement with these previous observations, and suggests that high levels of EPA may not be essential for early development in *F. grandis* larvae.

FA dynamics and the management of LC-PUFA deficiency in spawning *F. grandis* indicate a high level of physiological plasticity. Results of the present study addressed the

hypothesis that spawning animals consuming diets deficient in LC-PUFA would compensate by mobilization of somatic FA reserves, biosynthesis of some LC-PUFA from C_{18} precursor FA, or a reduction in reproductive output and offspring fitness. Of the three potential means of physiological compensation, a reduction in reproductive output or offspring fitness has the least support from our data. The more important compensatory mechanisms in *F. grandis* appear to be some combination of EFA mobilization from somatic reserves and biosynthesis of LC-PUFAs from C_{18} precursors. The very-long-term ability of *F. grandis* to utilize elongation and desaturation of C_{18} precursor FA when faced with LC-PUFA deficiency was neither confirmed nor rejected by data from this study.

CHAPTER 6
PHYSIOLOGICAL MANAGEMENT OF ω-3 LONG CHAIN-POLYUNSATURATED FATTY ACID DEFICIENT DIETS BY SPAWNING GULF KILLIFISH, *FUNDULUS GRANDIS*

Introduction

Lipid and energy homeostasis in vertebrates results from the interaction of numerous genes and signaling compounds, regulating communication between various tissues and organs including the liver, gastrointestinal tract, adipose tissue (if present), pancreas, and nervous system (Zechner et al., 2009). In fishes, lipids are utilized for buoyancy in many species, as well as the primary metabolic fuel for ATP production through the process of β-oxidation (Tocher, 2003; Metcalf & Gemmell, 2005). These features increase the importance of lipids in the biology of fishes relative to those vertebrates which primarily catabolize carbohydrates for energy. Like other vertebrates, fishes possess exogenous and endogenous loops for lipid trafficking (Tocher, 2003). In addition to acting as the transfer point from exogenous to endogenous trafficking loops, the liver of fishes also serves as a storage organ for lipids (Sheridan, 1988). Many fish species do not possess persistent discrete adipose tissue (Metcalf & Gemmell, 2005), and instead utilize transient deposits of intraperitoneal fat (IPF) to store excess energy as lipid (Craig et al., 1999; Piedecausa et al., 2007).

Fatty acids (FAs) are components which constitute the overwhelming proportion of total lipid mass (Wheatley et al., 2008) and impart many fundamental physiological and biochemical properties. In addition to storing energy and acting as substrates for catabolic production of ATP, FAs are required in biosynthesis of signaling molecules and also serve as essential components of cellular and intracellular membranes (Zechner et al., 2009; Shevchenko & Simons, 2010). Because of the importance of FAs in fish physiology, the mechanisms of FA acquisition, metabolism, and function have received much attention from scholars (Bell et al.,

2002; Chatelier et al., 2006; González-Rovira et al., 2009; Tocher, 2010; Lu et al., 2013; Norambuena et al., 2013). Maintenance of lipid homeostasis is a constant process, and Stanley-Samuelson et al. (1988) observed that samples used to generate FA composition data are "an instantaneous observation of a suite of continuing dynamic processes" and analyses of FA dynamics should be careful to acknowledge this limitation. Previous studies characterizing FA dynamics in fish, including those from the present authors, have often utilized an observation from a single time point to represent FA composition of a given species or experimental treatment. For this reason, studies which have the ability to examine changes in FA composition across multiple time points may be able to provide a different type of insight.

Euryhaline members of the genus *Fundulus* have been extensively utilized as model species (Burnett et al., 2007) and are thought to be recently derived from freshwater ancestors (Whitehead, 2010). The Gulf killifish *Fundulus grandis*, along with its closely related congener the mummichog *F. heteroclitus*, are recognized as having extremely high levels of physiological plasticity (Griffith, 1974; Beitinger et al., 2000; Martínez et al., 2006; Brown et al., 2012; Patterson et al., 2012) and ecological importance in the estuaries which they inhabit (Subrahmanyam & Drake, 1975). Successfully reproducing populations of *F. grandis*, generally considered a brackish water species, have been documented in landlocked freshwater systems (Simpson & Gunter, 1956; Campbell et al., 1980), but this occurrence is not widespread or common. *F. grandis* spawn multiple times across a protracted spawning season (Green et al., 2010), producing lipid-rich ova containing multiple oil droplets. Vitellogenic periods as short as 2 days (Hsiao & Meier, 1988) indicate high rates of lipid mobilization during reproduction in this species. Because of these characteristics, the lipid composition of food consumed before and during spawning has the potential to influence FA dynamics of critical organs and tissues as well

as ova. Diet induced FA differences in spawning *F. grandis* have been documented (Patterson & Green, *Revision in Preparation*), but it is unclear how these differences occur across time from a common diet origin.

The current study utilized a controlled laboratory environment, common baseline diet, and two experimental diets differing in FA composition to examine lipid dynamics in spawning *F. grandis*. An ovum and multi-tissue evaluation of FA profiles was generated at several time points across an 8-week spawning period. Previous research indicated the ability in *F. grandis* to biosynthesize long chain polyunsaturated FAs (LC-PUFAs) from shorter chain precursors (Patterson & Green, *Revision in Preparation*), a trait generally ascribed to marine but not freshwater fishes (Tocher, 2003). Accordingly, we hypothesized that spawning *F. grandis* fed diets deficient in LC-PUFAs but containing C_{18} ω-3 precursor FAs would retain higher levels of ω-3 LC-PUFAs across time than fish consuming a diet deficient in LC-PUFAs and C_{18} ω-3 precursors. This study was designed to provide a time-delineated account of FA dynamics in spawning *F. grandis* as the composition of FAs available in the diet changed.

Methods

Diet formulation and proximate analyses

Two experimental diets (Table 6.1) formulated to be isonitrogenous and isocaloric were analyzed for proximate composition (Table 6.2). FA content of experimental diets (Table 6.2) was determined by methods identical to those described below for ova and fish tissues. Supplemental lipid added to each diet was either soy oil (SO) or cottonseed oil (CO) to produce two treatments which varied principally in the availability of C_{18} ω-3 precursor FAs. Marine source lipids such as fish oil (FO) were excluded to create diets deficient in LC-PUFAs.

Table 6.1. Formulation of experimental diets fed to *Fundulus grandis* during an 8 week spawning period. Fat source was either cottonseed oil or soy oil.

Ingredient	(g kg^{-1})
Soy Protein Concentrate	488.1
Corn Grain	150.0
Soybean Meal	150.0
Fat	130.0
Dicalcium Phosphate	30.0
Carboxymethylcellulose	20.0
Menhaden Fish Meal	15.9
Vitamin C (Stay-C)	6.0
Vitamin Pre-Mix	5.0
Mineral Pre-Mix	2.5
Choline Chloride	0.9
Methionine 88%	0.8
L-lysine 95%	0.8

Table 6.2. Proximate analysis and fatty acid composition of cottonseed oil (CO) and soy oil (CO) based experimental diets fed to *Fundulus grandis* during an 8-week spawning period.

Analyzed Composition	CO	SO
Protein (% DM)	41.57	41.36
Fat (% DM)	12.50	12.60
NFE (% DM)	25.67	25.94
Fiber (% DM)	1.96	1.96
Ash (% DM)	7.57	7.58
Moisture (%)	10.75	10.57
Fatty Acid[#]		
14:0	0.86	0
16:0	22.55	11.9
18:0	3.02	4.64
18:1n-9	19.30	24.98
18:2n-6 LA	63.33	62.56
18:3n-3 ALA	0.82	8.66

[#]µg mg dry $mass^{-1}$

Animal care

Animals were handled using methods approved by the Louisiana State University Agricultural Center's Institutional Animal Care and Use Committee under protocol #AE2009-22. Prior to stocking for the experiment, a large group of animals were fed a high quality commercial diet containing 40% protein and 10% lipid as FO (Aquafeed 4010 Transition [FK], Burris Aquaculture, Cargill Animal Nutrition, Franklinton, LA, USA) for 16 weeks to create a common baseline. Experimental diets were fed in triplicate by randomly assigning each of the two treatment levels to three replicated spawning units. Each of six spawning units were stocked with ten females and five males randomly selected from populations of mean body mass 17.4 ± 0.7 and 14.2 ± 0.6 (mean ± SEM), respectively. All spawning units were serviced by a single recirculating system. Salinity was maintained using Crystal Sea Marinemix (Marine Enterprises International Inc., Baltimore, MD, USA) and water chemistry was monitored weekly with (mean ± SEM) for all parameters as follows: salinity ‰ (9.1 ± 0.1), pH (8.41 ± 0.01), total ammonia nitrogen mg/L NH_3-N (0.03 ± 0.01), total alkalinity mg/L HCO_3^- + CO_3^{-2} (428 ± 25). All of these parameters remained within acceptable *F. grandis* culture limits (Tatum et al. 1982) for the duration of the experiment.

Ova and tissue sampling

Before feeding of experimental diets was initiated, spawned ova with neurulated embryos were collected from four separate spawning units. Concurrently, one female randomly selected from each of four spawning units was euthanized with an overdose of tricaine methanesulfonate buffered to pH 7.0 with sodium bicarbonate. Dissections were performed immediately to remove ovary, liver, and IPF. Whole body and organ or tissue mass was obtained in order to calculate gonadosomatic index (GSI), hepatosomatic index (HSI), and IPF %, respectively.

Vitellogenic and post-vitellogenic oocytes were removed from dissected ovaries such that FA analyses would not include large proportions of lipoprotein yolk. Ova and body tissues were lyophilized prior to storage under ultra-pure nitrogen at -80°C. Spawning materials were placed in tanks once weekly with ova removed following each spawning event. Similar sampling protocols occurred after one, four, and eight weeks of ingesting experimental diets. At these times, two females were sampled at random from each of the three replicate spawning units with neurulated ova from each spawning unit being pooled into a single FA sample per tank. All collected ova and tissues were handled and preserved in the same manner as baseline samples.

Fatty acid analyses

Samples were stored until the eight-week spawning period was complete and subsequent FA analyses occurred within a single contiguous time period. Mass of each tissue sample to be used was determined prior to processing for gas chromatography. Extraction and transesterification of FA followed methods comparable to those of Timmins et al. (2009). 50 ng heneicosanoic acid (C21:0; Sigma-Aldrich, St. Louis, MO, USA) was added to each sample prior to transesterification as an internal standard. Following extraction and transesterification, 1 μL of hexane solvent containing fatty acid methyl esters (FAMEs) was injected into an Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA). A 30 m Supelco 2380 column (Sigma-Aldrich, St. Louis, MO, USA) was used with an injector temperature of 175°C and 30:1 split. Helium carrier gas flow rate was 24 cm sec^{-1}. Oven temperature began at 50°C for 2 min and increased 4°C min^{-1} to 250°C, which was held for 15 min. A flame ionization detector was utilized at 300°C. FAMEs were identified by retention time comparison to standards, and individually quantified by comparing the ion current area of each FAME to that of the internal standard. FA were quantified on a concentration basis (μg FAME mg sample dry $mass^{-1}$) rather

than a mass percentage basis (% total FA mass) to clarify interpretation when comparing tissues with varying total lipid content (Schwertner and Mosser 1993; Mocking et al. 2012).

Statistical analyses

Statistical tests were performed using Statistical Analysis Software Version 9.1 (SAS Institute Inc., Cary, NC, USA). Means are presented as mean ± SEM. Body composition indices (HSI, IPF ratio, GSI) were tested for the assumption of normality with the Shapiro-Wilk test and percentage data were transformed as arc-sin(square root *y*) before analysis. The folded F test was used to determine equality of variance before an independent sample T-test was conducted to establish significant difference between dietary treatments. A paired sample T-test was conducted to determine if dietary treatments caused a change in fatty acid profiles from the baseline. In cases were equality of variance was not met, Satterthwaite's approximation was used to determine degrees of freedom. Differences in body composition indices throughout the study were assessed with general linear model analysis of variance. All statistical tests were performed at a significance level of $\alpha = 0.05$.

Results

Body composition indices

Table 6.3 contains data on body composition indices. Broodfish body composition indices did not vary significantly between dietary lipid source treatments, but significant variation did occur across time in GSI ($F_{3, 36} = 8.61$, $P < 0.0002$), while HSI and GSI remained statistically unchanged from baseline values throughout the experiment ($F_{3,36} = 1.86$, $P < 0.1536$) and ($F_{3,36} = 1.36$, $P < 0.2720$), respectively.

Table 6.3. Mean gonadosomatic index (GSI), hepatosomatic index (HSI) and intraperitoneal fat percentage (IPF %) of female *F. grandis* at all sampled time points. Dietary treatments utilized cottonseed oil (CO) or soy oil (SO) as the lipid component.

Time	Baseline		1 week	4 weeks	8 weeks
Index		Diet			
GSI	5.24 ± 2.76	CO	4.45 ± 0.94	6.53 ± 0.47	4.21 ± 0.86
		SO	3.97 ± 0.79	5.18 ± 0.53	4.41 ± 0.59
HSI	4.32 ± 0.79	CO	3.20 ± 0.22	3.07 ± 0.31	3.07 ± 0.34
		SO	3.60 ± 0.17	3.19 ± 0.32	3.56 ± 0.43
IPF %	3.12 ± 0.42	CO	1.97 ± 0.39	2.44 ± 0.55	1.78 ± 0.22
		SO	2.14 ± 0.35	2.18 ± 0.43	2.46 ± 0.55

Fatty acid dynamics

Dietary lipid source produced a significant effect on the FA content of eggs and certain tissues. Data ALA, EPA, and DHA across time are contained in Figure 6.1, Figure 6.2, Figure 6.3, and Figure 6.4 for liver, ovary, IPF, and eggs, respectively. Statistical significance is indicated in the figures. No significant differences in the ω-6 family of FAs occurred between dietary lipid source treatments and these data are not presented. Complete FA profiles for all three tissue types and eggs at each sampling time point are contained in appendix C.

Discussion

Measurement of body composition indices was intended to track gross partitioning of lipids into the different organ compartments of broodfish. Fishes utilize both IPF and the liver for storage of excess lipid (Gao et al., 2010), a feature which may represent an evolutionary strategy for dealing with periods of starvation (Kjær et al., 2009). Lipid storage in the liver is so prevalent that some species may have in excess of 80% of total body lipid content deposited in the liver (Hansen et al., 2008). Stored body lipids could serve not only as an energy reserve but

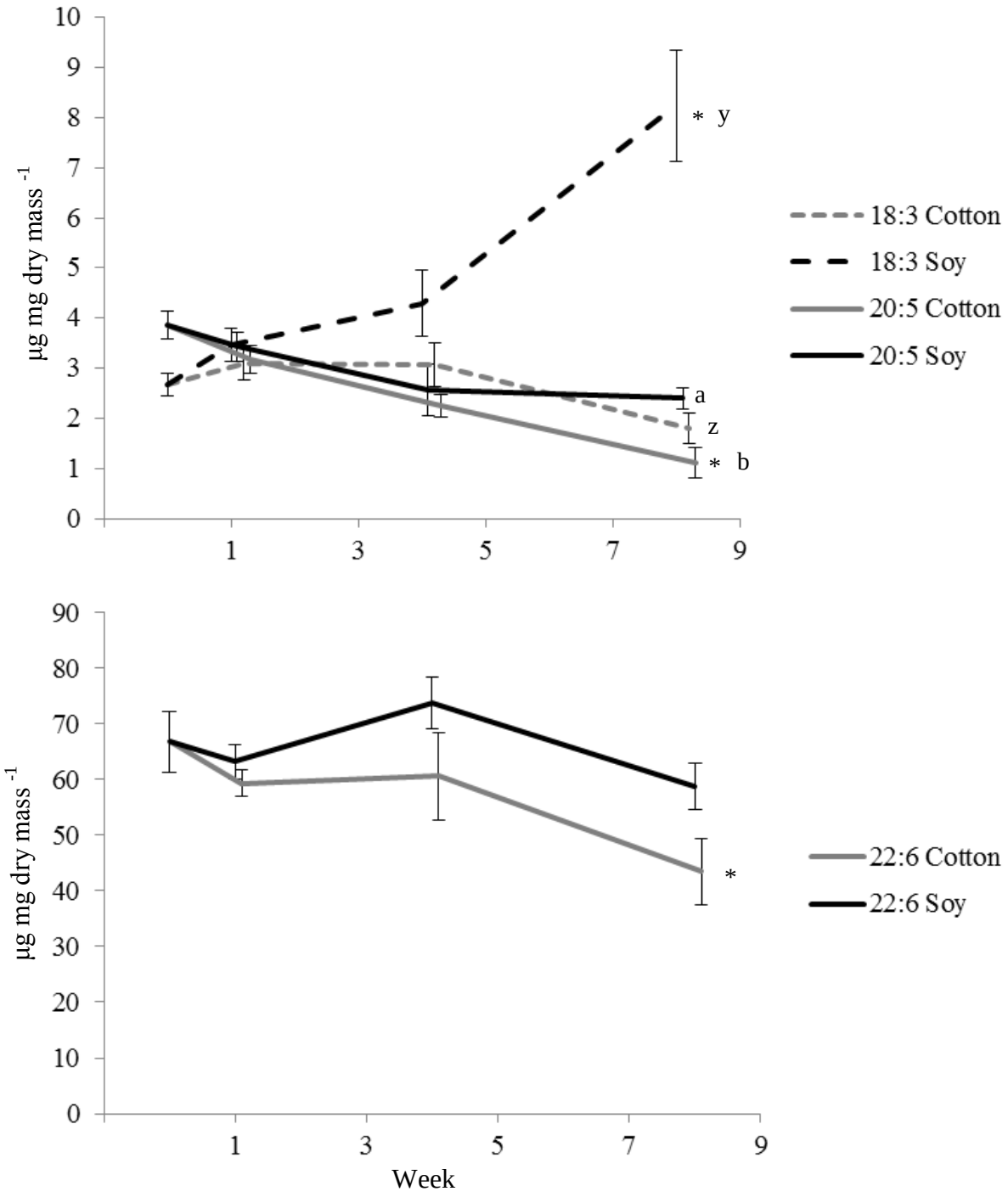

Figure 6.1. Liver concentrations of ω-3 C_{18} precursor and LC-PUFA end products in spawning *F. grandis* females fed diets with cottonseedoil or soy oil as the lipid component (n = 6 at each point). Letters denote significance across diets at the same time point while an asterisk (*) denotes deviation from baseline values across time within diets (REGWQ; P<0.05).

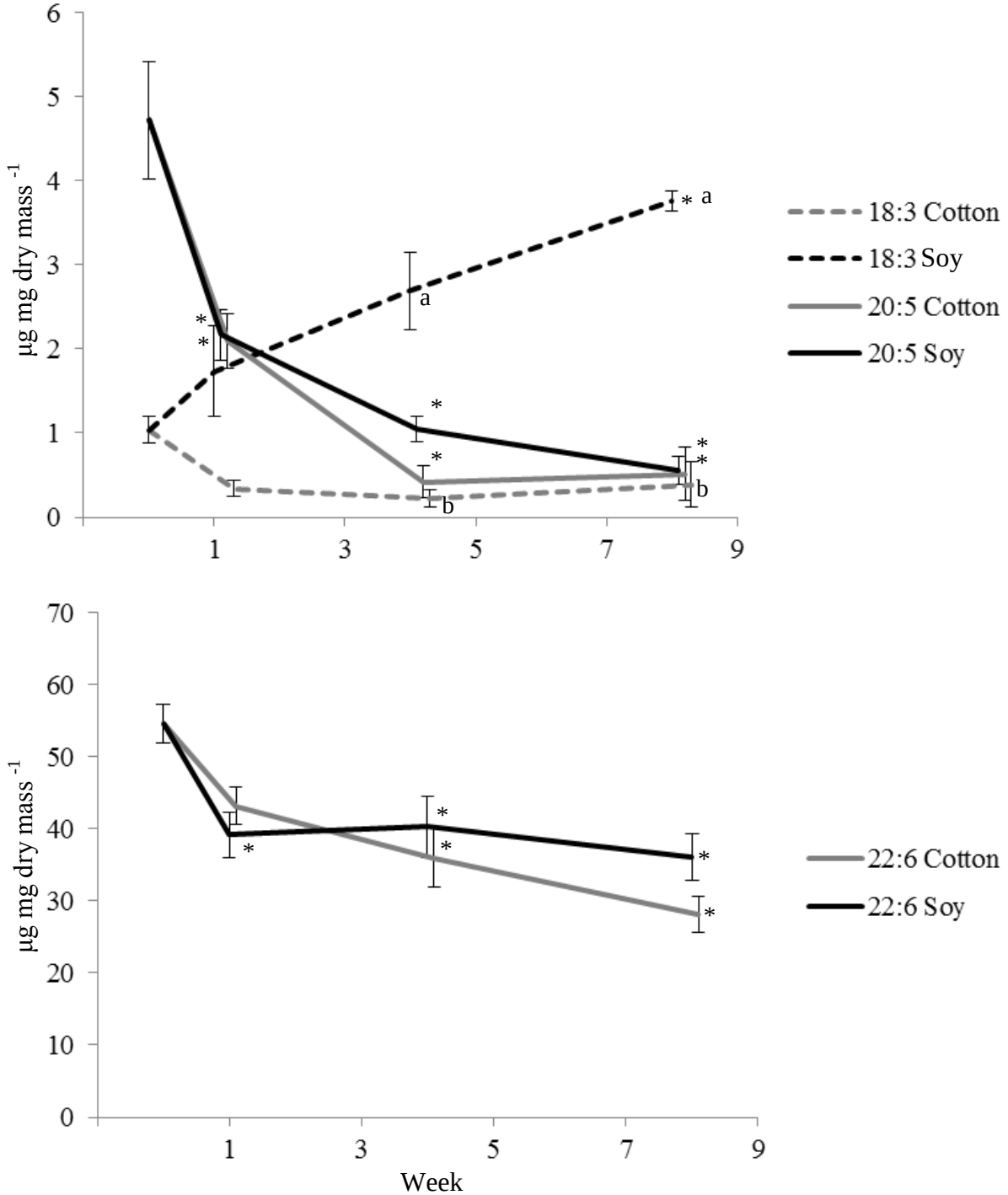

Figure 6.2. Ovary concentrations of ω-3 C_{18} precursor and LC-PUFA end products in spawning *F. grandis* females fed diets with cottonseed oil or soy oil as the lipid component (n = 6 at each point). Letters denote significance across diets at the same time point while an asterisk (*) denotes deviation from baseline values across time within diets (REGWQ; P<0.05).

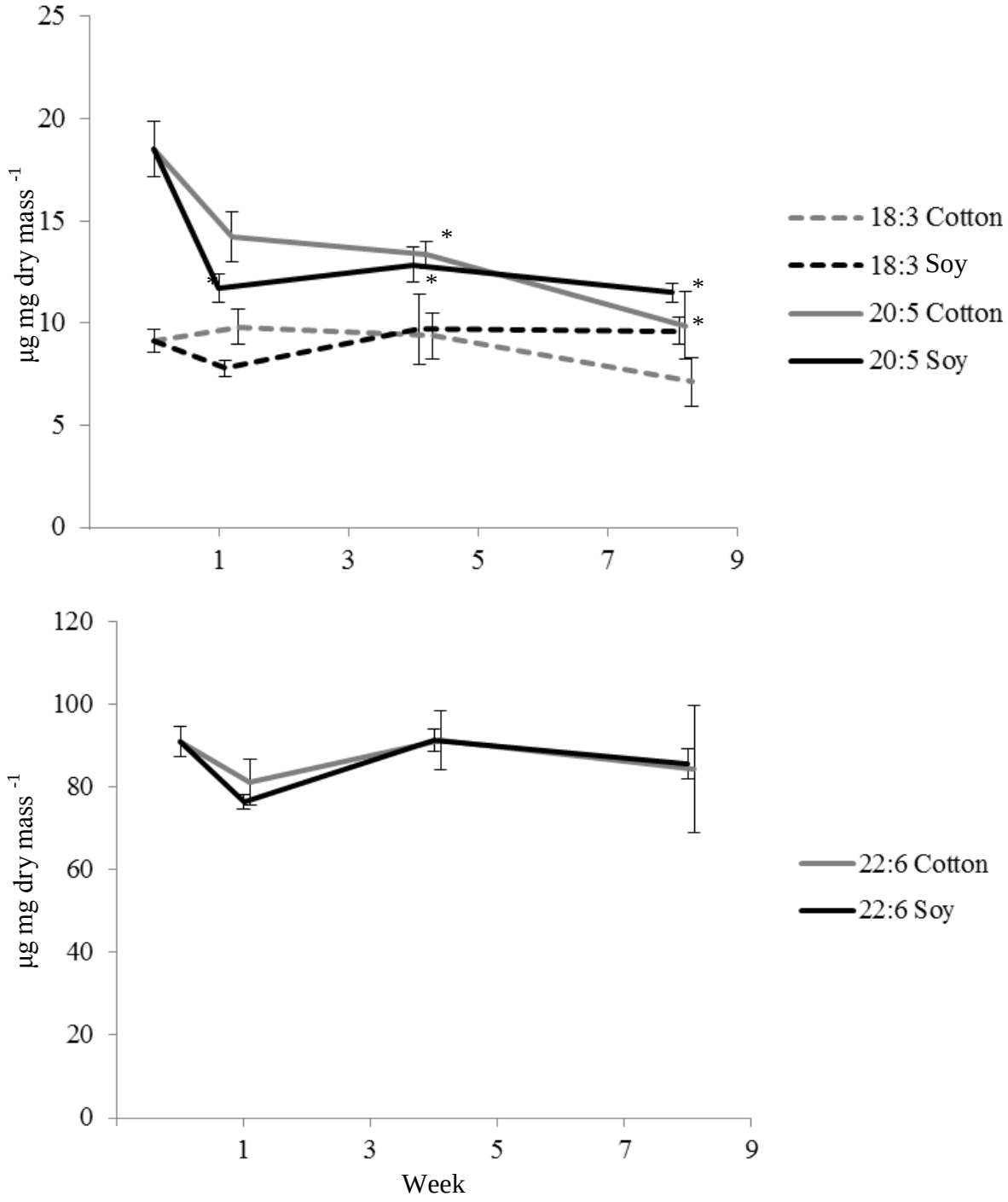

Figure 6.3. Intraperitoneal fat concentrations of ω-3 C_{18} precursor and LC-PUFA end products in spawning *F. grandis* females fed diets with cottonseed oil or soy oil as the lipid component (n = 6 at each point). An asterisk (*) denotes deviation from baseline values across time within diets (REGWQ; P<0.05). No differences were detected between the diets.

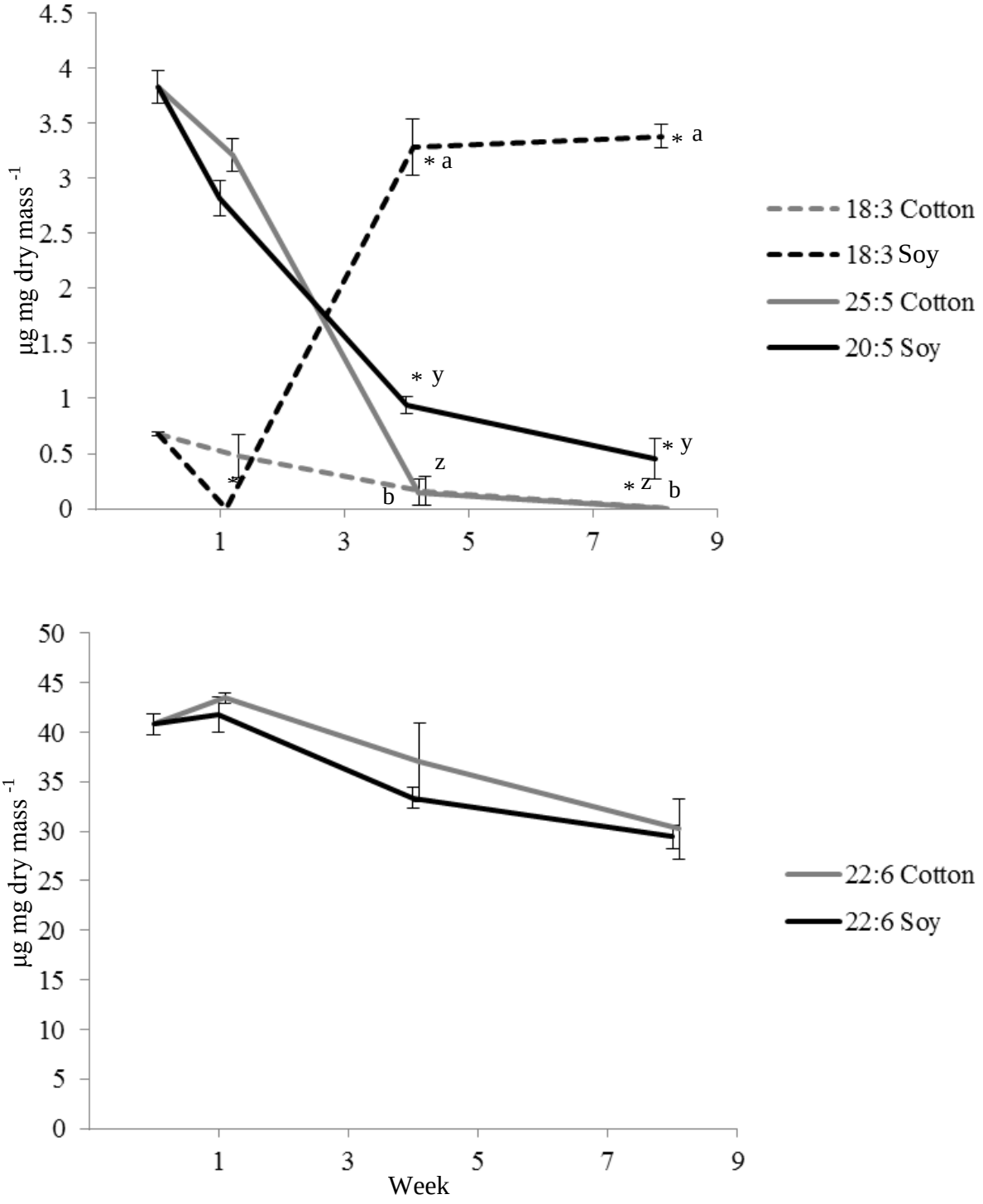

Figure 6.4. Egg concentrations of ω-3 C_{18} precursor and LC-PUFA end products in spawning *F. grandis* females fed diets with cottonseed oil or soy oil as the supplemental lipid component (n = 3 for each point). Letters denote significance across diets at the same time point while an asterisk (*) denotes deviation from baseline values across time within diets (REGWQ; P<0.05).

also as a source of specific FAs when dietary availability decreases. Previous research in *F. grandis* has indicated that this species preferentially stores excess energy as IPF rather than in the liver (Patterson & Green, 2014). In the present study, a low *n* of 4 for baseline values may have resulted in a lack of statistical significance for HSI and IPF %. However, both IPF % and HSI did show proportionally large decreases from baseline values in fish fed diets deficient in ω-3 LC-PUFAs. Selective mobilization of FAs from somatic reserves commonly occurs in fishes (Sheridan, 1988). The numerical decrease in HSI and IPF % in this study is indicative of mobilization of FAs which were biologically necessary but deficient in the diet. GSI values did vary significantly across time. This observation was expected because the euryhaline *Fundulus spp.* are fractional spawning animals that maintain semilunar spawning cycles even after extended periods in a laboratory environment (Hsiao & Meier, 1989). The significant increase of GSI from week 1 to week 4 represents a peak spawning period occurring during week 4. Spawning peaks for an income-breeding species are accompanied by an increase in lipid mobilization to the gonad (McBride et al., 2013), but this increase is dictated in the genome and not the result of a change in dietary availability of FAs. No difference was observed between dietary treatments in GSI or other body composition indices, so the increased availability of C_{18} ω-3 precursor FAs in the SO based diet did not produce a measureable effect on gross lipid trafficking.

Multi-tissue FA evaluation has the potential to clarify the interrelationships between selective mobilization and biosynthesis from shorter chain precursors. The primary site of FA biosynthesis in fishes is liver microsomes (Tocher, 2003), so the liver is the most critical organ for direct observations of changes in FA profile across time. Other potential somatic sites playing roles in FA transport and storage include IPF and muscle, both of which have been

implicated in energy homeostasis of various fish species (Black & Skinner, 1986; Chatelier et al., 2006). Previous work in *F. grandis* indicated that dietary discrepancies in FA availability did not manifest as dramatic differences in muscle (Patterson & Green, *Revision in Preparation*) and a functional level of all FAs were maintained in this tissue. Although we believe IPF to be the primary site of lipid storage in *F. grandis* (Patterson & Green, 2014), previous work had not examined the FA dynamics of this tissue. Thus, the present study included IPF in its examination while excluding muscle. Vitellogenins synthesized in the liver are transported to the ovary early in oogenesis, but taken into developing oocytes intact by receptor-mediated micropinocytosis (Wallace, 1985). Thus, FA profiles of ovarian tissue, when vitellogenic and post-vitellogenic oocytes are excluded, can bear a surprising resemblance to those somatic tissues which do not play an active role in FA biosynthesis or mobilization (Patterson & Green, *Revision in Preparation*). This means that enrichment of ω-3 and ω-6 LC-PUFAs observed in the liver and ova is not necessarily mirrored in the ovaries. However, because of its direct association with oocytes and critical role in reproduction, female gonad tissue was included in analysis of the present study.

One of the basic questions addressed in the present study is; when presented with a deficiency in ω-3 LC-PUFAs, can provision of ω-3 C_{18} precursor FAs in the diet of *F. grandis* lead to higher concentrations of LC-PUFA end products? If this question was answered with a yes, then we can be certain that some degree of *de novo* FA biosynthesis took place. Results of the present study indicate that physiological compensation for dietary deficiencies of ω-3 LC-PUFAs occurred by a combination of FA mobilization from somatic (probably IPF and liver) reserves in addition to desaturation and elongation activity in the liver. Over the eight week experimental period the treatment group fed a CO based diet, which was deficient in the C_{18}

precursor ALA, experienced a statistically significant decrease in EPA and DHA levels in the liver. In contrast, the treatment group fed SO, which contained higher levels of ALA, did not experience a significant decrease in EPA or DHA content of the liver. Surprisingly, work in *F. grandis* has indicated that high levels of EPA are not critical for developing embryos, and fish fed ω-3 deficient diets have produced healthy embryos and larvae which did not contain measureable levels of this FA (Patterson & Green, *Revision in Preparation*). The present study corroborated results in Chapter 5 because after eight weeks, CO fed animals produced ova with no measureable EPA. However, ova spawned by SO fed animals had levels of EPA which were significantly reduced from the initial baseline but still measurable. Significantly higher EPA levels in ova after eight weeks of spawning under experimental treatments also indicate potential ability of *F. grandis* to elongate and desaturate C_{18} precursor FAs.

The ability to elongate and desaturate precursor FA for LC-PUFA synthesis was suggested in Chapter 5 from observations of changes in hepatic levels of ω-6 C_{18} precursors and end products. The previous Chapter relied solely on an endpoint observation of FA concentration, analogous to the week 8 measurement in this study. Through measurement of changes in body composition indices and FA concentrations across time, we were able to expand on the findings of previous research and confirm that both mobilization from somatic stores and biosynthesis from C_{18} precursors are likely to be important to maintaining adequate levels of LC-PUFAs for reproduction. Neither study was able to confirm if FA biosynthesis occurs in *F. grandis* with enough efficiency to be biologically significant in sustaining very-long-term dietary LC-PUFA deficiency.

CHAPTER 7
GENERAL SUMMARY AND CONCLUSIONS

Gulf killifish *Fundulus grandis* and their congener the mummichog *F. heteroclitus* have been widely used as biological model species, especially in examinations of osmoregulation and toxicology. This dissertation investigated *F. grandis* as a potential marine baitfish aquaculture species. Research was designed to identify aspects of the reproductive and early life-history stages in *F. grandis* which might represent potential areas for improving a culture bottleneck of low *per capita* fecundity. In addition, previously undescribed aspects of the reproductive biology of the species, specifically related to lipid dynamics, were revealed.

Chapter 2 describes the effects of osmoregulation on growth, survival, and body condition at 0.5, 5.0, 8.0 and 12.0‰ salinities in *F. grandis* juveniles during a 12-week trial. Relative expression of genes encoding the ion transport proteins Na+/K+-ATPase (NKA), Na+/K+/2Cl^- cotransporter (NKCC1), and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel was analyzed. At 0.5‰, *F. grandis* showed depressed growth, body condition, and survival relative to higher salinities. NKA relative expression was elevated at 7 days post-transfer but decreased at later time points in fish held at 0.5‰ while other salinities produced no such increase. NKCC1, the isoform associated with expulsion of ions in saltwater, was downregulated from week 1 to week 3 at 0.5‰ while CFTR relative expression produced no significant results across time or salinity. These results suggest that *F. grandis* juveniles acclimated to higher salinities have physiological difficulties with osmoregulation at 0.5‰ and that this leads to reduced growth performance and survival while salinities in the 5.0-12.0‰ range are adequate for normal function.

One of the first studies to examine effects of maternal body size on reproduction in the genus *Fundulus*, and more generally in fractional spawning species with eggs capable of

development out of water is described in Chapter 3. This work assessed the effects of female body size on reproductive investment in laboratory held *F. grandis* as well as subsequent implications for larvae. Larger females had significantly higher fecundity and produced individual eggs of greater volume. Gonadosomatic index more than doubled as female body mass increased from 6.9 to 12.9 g, but did not increase significantly in females of body mass >12.9 g. Larger eggs required significantly longer incubation periods, and newly hatched larvae from larger eggs had more endogenous nutritional resources but shorter body length. These results describe maternal effects in a fractional spawning species producing eggs with the ability to incubate in air. Results also provide useful information on broodfish management for aquaculturists, as *F. grandis* is increasingly cultured commercially and in laboratory settings.

Broodfish nutrition is an expanding field of aquaculture and improved nutritional status has been shown to increase reproductive output in some cultured fishes. Thus, Chapter 4 examines quantitative inclusion of lipid as fish oil in complete diets fed to actively spawning *F. grandis,* and its effects on physiological and reproductive parameters and subsequent larval morphometrics. An increase in dietary lipid levels across a gradient from 4.0 to 13.8% did not affect periodic fecundity, egg size, embryo viability rate, sperm motility, hepatosomatic index, or liver total lipid content. Intraperitoneal fat increased significantly in fish fed higher lipid levels while gonadosomatic index was significantly higher in the lowest dietary lipid group. Larvae produced by fish fed higher lipid levels had significantly increased endogenous nutritional resources at hatch while standard length was unaffected. No strong reproductive benefits of increased lipid inclusion were found in Gulf killifish.

A dichotomy in ability to biosynthesize long chain polyunsaturated fatty acids (LC-PUFAs) from C_{18} precursors exists between marine and freshwater fishes. Because *F. grandis* is

a euryhaline cyprinodont, the details of their fatty acid (FA) dynamics may not fit neatly into one of the two categories. Chapter 5 describes experimental variations in exogenous FAs available to spawning *F. grandis* and a multi-tissue evaluation of FA allocation to quantify effects on reproductive output and offspring fitness. No significant decrease in fecundity occurred in animals consuming low levels of LC-PUFAs, although embryo viability rates were affected. Maternal dietary FA variation did produce differences in starvation tolerance, hypo-osmoregulatory ability, and acute thermal stress tolerance for larvae. These variations occurred at physiologically marginal conditions, and would likely have little effect in environmentally relevant conditions. FA composition of eggs and tissues from spawning females suggested that biosynthesis of LC-PUFAs from shorter chain precursors in *F. grandis* might occur, but the significance of such processes as a means to overcome dietary FA deficiency could not be determined. Results of Chapter 5 suggest that *F. grandis* possess physiological mechanisms which allow maintenance of reproductive function when subjected to dietary deficiencies in FAs that are generally considered essential for marine fishes.

Chapter 6 also presents results of manipulation of dietary FA profiles. However, in this case, FA content of broodfish and ova from a common baseline through an eight-week spawning period was measured to examine relative changes across time. An ova and multi-tissue evaluation of changes in FA concentrations across time after fish were switched from LC-PUFA rich to deficient diets was employed. Results indicate that a combination of mobilization and biosynthesis is utilized to maintain physiologically required FA levels in critical tissues and embryos. Mobilization of FA reserves was indicated by an observed decrease in intraperitoneal fat content and liver mass. Potential biosynthesis was suggested by maintentance of ω-3 LC-PUFAs in some tissues and ova of fish fed a diet containing the ω-3 C_{18} precursor ALA versus

those fed a diet with low ALA levels. The characteristic physiological plasticity of *F. grandis* is maintained by an ability to handle essential FA-deficient diets for the duration of all studies conducted in this dissertation.

Research contained in this dissertation provided valuable knowledge for scientists or entrepreneurs seeking to culture *F. grandis*. Practical considerations for optimized culture salinity, broodfish body size, and dietary lipid content and composition have been described, and new information on the biology of the species is presented. Below is an itemized inventory of *F. grandis* husbandry practices which were determined to enhance fitness and reproduction:

- Low salinity grow-out of Gulf killifish is feasible in the range 5-12‰.
- In the 5-12‰ range, growth rate increases with increasing salinity and is highest at 12‰.
- Gulf killifish survival is negatively affected at 0.5‰ salinity.
- Female broodfish should be approximately 13 g or 100 mm TL for best fecundity.
- Female broodfish less than 7 g in mass should not be utilized for spawning.
- Vegetable oil can be used to replace up to 66% of fish oil in Gulf killifish diets without negative impacts on fecundity or embryo viability.

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Title: Effects of low salinity media on growth, condition, and gill ion transporter expression in juvenile Gulf killifish,*Fundulus grandis*

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APPENDIX B
CHAPTER 5 SUPPLEMENTAL TABLES

Table B.1. Fatty acid composition of female *F. grandis* liver tissue at conclusion of an 18 week feeding and spawning period. Letters denote significance across dietary treatments levels in the five statistically analyzed fatty acids.

Fatty Acid (μg mg dry mass^{-1})	Diet 1	Diet 2	Diet 3	Diet 4
14:0	3.07 ± 0.58	2.87 ± 0.57	5.15 ± 0.57	4.78 ± 0.73
16:0	51.08 ± 5.75	37.13 ± 5.73	45.05 ± 3.08	42.85 ± 4.22
18:0	25.94 ± 1.36	24.53 ± 2.60	23.01 ± 1.13	24.13 ± 1.41
22:0	3.88 ± 0.33	4.27 ± 0.96	3.22 ± 0.65	2.94 ± 0.54
∑SFA	83.96 ± 6.75	68.79 ± 9.33	76.43 ± 4.19	74.70 ± 6.09
16:1n-7	13.48 ± 2.13	10.16 ± 2.77	21.78 ± 2.20	17.19 ± 3.93
18:1n-9	147.20 ± 12.65	127.09 ± 25.23	124.13 ± 10.55	105.47 ± 20.07
20:1n-7	1.76 ± 0.24	2.23 ± 0.49	2.61 ± 0.61	2.40 ± 0.47
20:1n-9	4.02 ± 0.56	4.03 ± 0.70	3.13 ± 0.30	2.33 ± 0.57
24:1n-9	7.46 ± 0.94	7.85 ± 1.08	5.90 ± 0.96	4.58 ± 0.75
∑MUFA	173.92 ± 14.62	151.37 ± 28.99	157.56 ± 12.81	131.97 ± 24.34
16:3n-4	1.97 ± 0.15	1.86 ± 0.29	2.48 ± 0.17	2.72 ± 0.26
16:4n-1	1.41 ± 0.37	1.50 ± 0.32	2.33 ± 0.32	2.60 ± 0.37
18:2n-6 LA	110.38 ± 14.37^{a}	98.67 ± 22.02^{ab}	72.13 ± 12.66^{bc}	56.32 ± 8.71^{c}
18:3n-3 ALA	17.88 ± 3.14^{a}	9.88 ± 2.43^{b}	7.90 ± 0.53^{b}	4.31 ± 0.67^{b}
20:2n-6	5.02 ± 0.55	5.22 ± 1.07	3.73 ± 0.75	2.77 ± 0.54
20:3n-6	2.06 ± 0.12	1.71 ± 0.27	1.03 ± 0.08	0.64 ± 0.07
20:4n-6 ARA	27.45 ± 2.13^{a}	21.53 ± 2.75^{ab}	16.98 ± 1.02^{b}	16.21 ± 1.78^{b}
20:5n-3 EPA	0.90 ± 0.36^{b}	0.80 ± 0.19^{b}	1.40 ± 0.32^{ab}	2.43 ± 0.41^{a}
22:4n-6	26.34 ± 3.32	22.26 ± 3.31	15.30 ± 2.19	12.31 ± 1.96
22:5n-3	2.53 ± 0.57	4.93 ± 0.85	5.64 ± 1.75	10.84 ± 2.13
22:6n-3 DHA	34.28 ± 5.61^{b}	50.32 ± 8.23^{b}	58.51 ± 12.48^{ab}	93.47 ± 13.67^{a}
∑PUFA	230.23 ± 14.94	218.69 ± 35.25	187.43 ± 22.33	204. 64 ± 25.11
∑n-3	55.59 ± 3.88	65.94 ± 9.80	73.45 ± 14.06	111.05 ± 16.37
∑n-6	171.25 ± 16.82	149.39 ± 27.84	109.17 ± 14.22	88.26 ± 9.85
n-3:n-6	0.32 ± 0.07	0.44 ± 0.08	0.67 ± 0.11	1.26 ± 0.11

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table B.2. Fatty acid composition of female *F. grandis* muscle tissue at conclusion of an 18 week feeding and spawning period. Letters denote significance across dietary treatments levels in the five statistically analyzed fatty acids.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Diet 1	Diet 2	Diet 3	Diet 4
14:0	0.43 ± 0.13	0.49 ± 0.10	0.41 ± 0.05	0.50 ± 0.09
16:0	6.50 ± 0.88	6.34 ± 0.52	6.20 ± 0.55	6.01 ± 0.55
18:0	2.69 ± 0.16	2.91 ± 0.13	2.88 ± 0.12	2.72 ± 0.12
22:0	0.41 ± 0.06	0.41 ± 0.03	0.32 ± 0.03	0.27 ± 0.06
∑SFA†	10.03 ± 1.06	10.15 ± 0.66	9.80 ± 0.52	9.50 ± 0.72
16:1n-7	1.52 ± 0.59	1.32 ± 0.30	0.96 ± 0.23	1.25 ± 0.30
18:1n-9	9.06 ± 2.28	8.34 ± 1.44	6.21 ± 0.79	6.93 ± 1.48
20:1n-7	0.20 ± 0.07	0.18 ± 0.02	0.18 ± 0.04	0.20 ± 0.07
20:1n-9	0.21 ± 0.06	0.25 ± 0.05	0.14 ± 0.03	0.11 ± 0.06
24:1n-9	1.54 ± 0.52	1.12 ± 0.07	0.87 ± 0.04	0.73 ± 0.07
∑MUFA‡	12.53 ± 3.11	11.21 ± 1.83	8.35 ± 1.00	9.23 ± 1.92
16:3n-4	0.14 ± 0.05	0.19 ± 0.01	0.17 ± 0.01	0.17 ± 0.01
16:4n-1	0.17 ± 0.08	0.15 ± 0.04	0.10 ± 0.04	0.16 ± 0.05
18:2n-6 LA	7.34 ± 1.46[a]	6.56 ± 0.84[a]	4.74 ± 0.57[a]	4.43 ± 0.92[a]
18:3n-3 ALA	0.82 ± 0.17[a]	0.52 ± 0.07[a]	0.34 ± 0.05[a]	0.35 ± 0.08[a]
20:2n-6	0.34 ± 0.03	0.40 ± 0.04	0.23 ± 0.02	0.13 ± 0.05
20:3n-6	0.09 ± 0.03	0.09 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
20:4n-6 ARA	2.90 ± 0.56[a]	3.09 ± 0.14[a]	2.88 ± 0.20[a]	2.54 ± 0.16[a]
20:5n-3 EPA	0.40 ± 0.19[a]	0.15 ± 0.05[a]	0.25 ± 0.07[a]	0.45 ± 0.07[a]
22:4n-6	2.85 ± 0.49	1.12 ± 0.07	1.83 ± 0.10	1.40 ± 0.13
22:5n-3	2.49 ± 1.44	1.25 ± 0.27	1.26 ± 0.17	1.59 ± 0.17
22:6n-3 DHA	11.14 ± 2.01[a]	8.40 ± 0.92[a]	11.53 ± 1.45[a]	12.78 ± 1.64[a]
∑PUFA*	28.69 ± 3.47	23.37 ± 1.42	23.33 ± 1.88	23.99 ± 2.66
∑n-3	14.84 ± 2.36	10.32 ± 0.82	13.38 ± 1.62	15.17 ± 1.64
∑n-6	13.53 ± 1.57	12.71 ± 0.81	9.68 ± 0.39	8.49 ± 1.10
n-3:n-6	1.10 ± 0.18	0.81 ± 0.06	1.38 ± 0.14	1.79 ± 0.14

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table B.3. Fatty acid composition of female *F. grandis* ovarian tissue at conclusion of an 18 week feeding and spawning period. Letters denote significance across dietary treatments levels in the five statistically analyzed fatty acids.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Diet 1	Diet 2	Diet 3	Diet 4
14:0	1.03 ± 0.30	0.73 ± 0.11	1.55 ± 0.33	1.17 ± 0.21
16:0	15.12 ± 2.05	12.14 ± 0.37	16.76 ± 1.75	14.24 ± 1.11
18:0	9.17 ± 1.10	6.92 ± 0.58	8.56 ± 0.50	7.90 ± 0.79
22:0	0.96 ± 0.10	0.74 ± 0.08	0.91 ± 0.14	0.72 ± 0.18
∑SFA†	26.28 ± 3.49	20.53 ± 0.98	27.77 ± 2.48	24.04 ± 2.09
16:1n-7	2.12 ± 0.76	1.28 ± 0.30	2.71 ± 0.97	2.40 ± 0.50
18:1n-9	12.16 ± 2.13	9.40 ± 1.42	16.88 ± 3.59	13.14 ± 1.45
20:1n-7	0.29 ± 0.04	0.13 ± 0.07	0.54 ± 0.18	0.21 ± 0.08
20:1n-9	0.44 ± 0.08	0.24 ± 0.07	0.58 ± 0.13	0.36 ± 0.08
24:1n-9	2.94 ± 0.54	1.81 ± 0.17	2.38 ± 0.23	1.61 ± 0.42
∑MUFA‡	17.95 ± 3.10	12.86 ± 1.93	23.09 ± 4.93	17.72 ± 2.14
16:3n-4	0.42 ± 0.08	0.33 ± 0.03	0.50 ± 0.07	0.46 ± 0.11
16:4n-1	0.24 ± 0.11	0.12 ± 0.06	0.35 ± 0.09	0.27 ± 0.07
18:2n-6 LA	8.14 ± 1.22	5.86 ± 0.49	12.55 ± 4.37	6.20 ± 0.41
18:3n-3 ALA	1.04 ± 0.16[a]	0.54 ± 0.09[a]	0.97 ± 0.26[a]	0.51 ± 0.13[a]
20:2n-6	0.95 ± 0.16[a]	0.67 ± 0.14[a]	0.80 ± 0.17[a]	0.63 ± 0.20[a]
20:3n-6	0.28 ± 0.03	0.06 ± 0.04	0.14 ± 0.04	0.05 ± 0.03
20:4n-6 ARA	9.47 ± 0.89[a]	7.56 ± 0.54[a]	8.04 ± 0.58[a]	6.69 ± 0.37[a]
20:5n-3 EPA	0.32 ± 0.20[a]	0.31 ± 0.12[a]	0.69 ± 0.21[a]	0.66 ± 0.20[a]
22:4n-6	6.77 ± 1.06	4.08 ± 0.19	3.68 ± 0.30	2.65 ± 0.26
22:5n-3	0.81 ± 0.14	1.13 ± 0.10	2.93 ± 0.84	2.51 ± 0.45
22:6n-3 DHA	17.67 ± 2.14[a]	24.86 ± 3.01[a]	24.82 ± 2.74[a]	29.20 ± 4.73[a]
∑PUFA*	46.09 ± 3.00	45.52 ± 2.56	55.46 ± 7.38	49.83 ± 5.33
∑n-3	19.84 ± 2.31	26.85 ± 3.06	29.40 ± 3.42	32.88 ± 4.75
∑n-6	25.60 ± 3.10	18.23 ± 2.99	25.21 ± 4.58	16.22 ± 0.92
n-3:n-6	0.78 ± 0.19	1.47 ± 0.27	1.17 ± 0.15	2.03 ± 0.27

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table B.4. Fatty acid composition of eggs spawned by *F. grandis* during weeks 16 through 18 of an 18 week feeding and spawning period.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Diet 1	Diet 2	Diet 3	Diet 4
14:0	0.75	1.11	1.87	2.30
16:0	23.69	20.31	27.05	21.02
18:0	13.84	10.27	14.89	10.56
22:0	1.74	1.28	1.91	1.09
∑SFA†	40.02	32.97	45.72	34.97
16:1n-7	3.15	2.90	6.61	5.89
18:1n-9	32.83	21.10	36.29	20.96
20:1n-7	0.63	0.62	0.93	1.19
20:1n-9	0.83	0.54	0.79	0.61
24:1n-9	3.23	2.58	2.83	1.47
∑MUFA‡	40.67	27.74	47.45	30.12
16:3n-4	0.63	0.56	0.96	0.80
16:4n-1	0.28	0.15	0.69	0.60
18:2n-6 LA	30.96	19.48	26.44	16.91
18:3n-3 ALA	3.76	2.78	3.08	1.43
20:2n-6	1.56	0.90	1.34	0.75
20:3n-6	0.84	0.48	0.55	0.26
20:4n-6 ARA	14.42	12.17	12.27	6.66
20:5n-3 EPA	0.00	0.33	0.93	2.35
22:4n-6	15.76	8.06	8.85	3.00
22:5n-3	0.81	1.87	3.76	6.80
22:6n-3 DHA	32.33	45.32	46.86	44.00
∑PUFA*	101.35	92.10	105.73	83.56
∑n-3	36.90	50.30	54.63	54.58
∑n-6	63.54	41.09	49.45	27.58
n-3:n-6	0.58	1.22	1.10	1.98

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table B.5. Analysis of variance (ANOVA) data for five critical fatty acids (FAs). Sequential Bonferroni corrections were applied to probability values across FAs within tissue type to maintain familywise acceptance criteria $\alpha = 0.05$. Ryan-Einot-Gabriel-Welch *a priori* results appear in tables B.1 – B.3 for corresponding tissue type.

Fatty Acid	Tissue	d.f.	F	*p*
LA	Liver	3, 20	6.20	0.0148
(18:2ω6)	Ovary	3, 20	1.51	0.5715
	Muscle	3, 20	1.98	0.5952
ARA	Liver	3, 20	5.47	0.0198
(20:4ω6)	Ovary	3, 20	2.85	0.3160
	Muscle	3, 20	0.53	0.7026
ALA	Liver	3, 20	6.67	0.0135
(18:3ω3)	Ovary	3, 20	2.13	0.5160
	Muscle	3, 20	4.75	0.0585
EPA	Liver	3, 20	4.27	0.0240
(20:5ω3)	Ovary	3, 20	1.06	0.5715
	Muscle	3, 20	1.54	0.7026
DHA	Liver	3, 20	4.72	0.0240
(22:6ω3)	Ovary	3, 20	1.74	0.5715
	Muscle	3, 20	1.41	0.7026

APPENDIX C
CHAPTER 6 SUPPLEMENTAL TABLES

Table C.1. Baseline fatty acid composition of female *F. grandis* livers at the beginning of an 8 week spawning period. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Baseline
14:0	15.84 ± 1.06
16:0	65.25 ± 3.00
18:0	20.00 ± 0.93
22:0	4.92 ± 0.59
∑SFA†	106.01 ± 4.29
16:1n-7	39.28 ± 2.73
18:1n-9	172.04 ± 5.99
24:1n-9	3.53 ± 0.20
∑MUFA‡	214.85 ± 7.01
16:2n-4	2.66 ± 0.22
16:4n-1	4.59 ± 0.23
18:2n-6 LA	48.99 ± 4.36
18:3n-3 ALA	2.68 ± 0.22
18:3n-6	4.52 ± 0.27
20:2n-6	3.26 ± 0.30
20:4n-6 ARA	9.58 ± 0.50
20:5n-3 EPA	3.87 ± 0.27
22:4n-6	8.06 ± 0.73
22:5n-3	19.84 ± 1.30
22:6n-3 DHA	66.83 ± 5.53
∑PUFA*	175.14 ± 13.53
∑n-3	93.21 ± 7.34
∑n-6	77.34 ± 6.08
n-3:n-6	1.21 ± 0.03

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.2. Fatty acid composition of female *F. grandis* livers after 1 week of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	14.39 ± 1.08	14.19 ± 0.86
16:0	61.20 ± 2.09	62.35 ± 2.80
18:0	18.99 ± 1.62	17.18 ± 0.46
22:0	5.41 ± 0.49	5.63 ± 0.61
∑SFA†	99.99 ± 4.62	99.36 ± 4.66
16:1n-7	38.07 ± 1.90	37.75 ± 1.89
18:1n-9	149.18 ± 6.14	156.54 ± 3.01
24:1n-9	3.41 ± 0.29	3.70 ± 0.25
∑MUFA‡	190.66 ± 8.03	197.99 ± 3.67
16:2n-4	2.57 ± 0.24	2.71 ± 0.19
16:4n-1	4.43 ± 0.64	3.76 ± 0.25
18:2n-6 LA	62.38 ± 4.37	62.99 ± 4.34
18:3n-3 ALA	3.11 ± 0.34	3.46 ± 0.34
18:3n-6	7.09 ± 1.00	8.52 ± 1.11
20:2n-6	2.78 ± 0.17	2.93 ± 0.18
20:4n-6 ARA	10.00 ± 0.42	12.49 ± 0.92
20:5n-3 EPA	3.18 ± 0.28	3.43 ± 0.28
22:4n-6	7.15 ± 0.48	9.84 ± 0.68
22:5n-3	16.41 ± 1.21	13.86 ± 1.10
22:6n-3 DHA	59.36 ± 2.45	63.31 ± 3.10
∑PUFA*	178.46 ± 4.43	187.30 ± 7.54
∑n-3	82.05 ± 3.70	84.06 ± 4.31
∑n-6	91.98 ± 6.27	99.48 ± 7.06
n-3:n-6	0.92 ± 0.10	0.87 ± 0.09

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.3. Fatty acid composition of female *F. grandis* livers after 4 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	8.50 ± 0.67	8.83 ± 0.68
16:0	59.53 ± 1.31	53.29 ± 2.90
18:0	17.10 ± 0.77	14.55 ± 0.55
22:0	9.23 ± 1.22	5.32 ± 0.73
∑SFA†	94.36 ± 3.37	81.99 ± 3.57
16:1n-7	28.10 ± 2.61	30.98 ± 2.07
18:1n-9	127.85 ± 9.54	139.04 ± 8.43
24:1n-9	4.42 ± 0.40	4.64 ± 0.32
∑MUFA‡	160.37 ± 13.05	174.66 ± 11.23
16:2n-4	1.94 ± 0.07	2.05 ± 0.14
16:4n-1	3.09 ± 0.28	3.15 ± 0.34
18:2n-6 LA	92.05 ± 8.56	85.54 ± 7.51
18:3n-3 ALA	3.06 ± 0.43	4.29 ± 0.66
18:3n-6	9.76 ± 1.48	11.22 ± 2.72
20:2n-6	4.56 ± 0.34	2.99 ± 0.22
20:4n-6 ARA	14.98 ± 0.93	14.49 ± 1.12
20:5n-3 EPA	2.25 ± 0.24	2.57 ± 0.52
22:4n-6	10.64 ± 0.67	11.67 ± 0.85
22:5n-3	11.32 ± 1.58	13.39 ± 0.82
22:6n-3 DHA	60.61 ± 7.88	73.77 ± 4.60
∑PUFA*	214.26 ± 6.95	225.12 ± 8.13
∑n-3	77.23 ± 10.12	94.02 ± 5.53
∑n-6	133.93 ± 11.04	127.95 ± 12.11
n-3:n-6	0.62 ± 0.14	0.80 ± 0.13

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.4. Fatty acid composition of female *F. grandis* livers after 8 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	5.89 ± 0.61	6.79 ± 0.57
16:0	60.98 ± 3.97	53.06 ± 2.96
18:0	15.94 ± 1.18	13.75 ± 0.32
22:0	13.60 ± 1.83	8.95 ± 1.16
∑SFA†	96.41 ± 5.80	82.55 ± 4.25
16:1n-7	20.20 ± 2.21	26.56 ± 1.85
18:1n-9	113.20 ± 9.74	141.84 ± 7.38
24:1n-9	4.75 ± 0.29	4.61 ± 0.59
∑MUFA‡	138.15 ± 13.02	173.01 ± 10.20
16:2n-4	1.89 ± 0.20	1.69 ± 0.33
16:4n-1	2.51 ± 0.29	2.17 ± 0.46
18:2n-6 LA	125.71 ± 10.97	122.98 ± 9.85
18:3n-3 ALA	1.80 ± 0.30	8.25 ± 1.11
18:3n-6	13.46 ± 1.15	14.43 ± 1.41
20:2n-6	5.51 ± 0.44	3.86 ± 0.31
20:4n-6 ARA	17.44 ± 1.57	18.73 ± 1.98
20:5n-3 EPA	1.11 ± 0.30	2.40 ± 0.21
22:4n-6	12.99 ± 0.63	11.61 ± 1.28
22:5n-3	6.27 ± 0.95	10.21 ± 0.80
22:6n-3 DHA	43.46 ± 5.93	58.76 ± 4.13
∑PUFA*	232.14 ± 17.11	255.10 ± 13.62
∑n-3	52.65 ± 7.72	79.63 ± 5.03
∑n-6	176.99 ± 12.55	173.30 ± 11.37
n-3:n-6	0.30 ± 0.04	0.47 ± 0.03

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.5. Baseline fatty acid composition of *F. grandis* ovaries at the beginning of an 8 week spawning period. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Baseline
14:0	6.23 ± 0.73
16:0	30.33 ± 1.97
18:0	11.59 ± 0.13
22:0	1.72 ± 0.19
∑SFA†	49.87 ± 3.24
16:1n-7	11.98 ± 1.45
18:1n-9	36.73 ± 5.86
24:1n-9	2.15 ± 0.24
∑MUFA‡	50.86 ± 8.65
16:2n-4	1.01 ± 0.08
16:4n-1	1.37 ± 0.15
18:2n-6 LA	16.80 ± 1.74
18:3n-3 ALA	1.04 ± 0.16
18:3n-6	1.29 ± 0.14
20:2n-6	1.03 ± 0.13
20:4n-6 ARA	8.77 ± 0.91
20:5n-3 EPA	4.72 ± 0.69
22:4n-6	3.87 ± 0.49
22:5n-3	12.69 ± 1.40
22:6n-3 DHA	54.63 ± 2.65
∑PUFA*	107.21 ± 9.41
∑n-3	73.08 ± 5.45
∑n-6	32.76 ± 3.84
n-3:n-6	2.27 ± 0.10

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.6. Fatty acid composition of *F. grandis* ovaries after 1 week of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	3.30 ± 0.43	3.24 ± 0.50
16:0	26.80 ± 1.26	23.26 ± 1.94
18:0	10.28 ± 0.57	9.16 ± 0.81
22:0	2.34 ± 0.42	2.25 ± 0.74
∑SFA†	42.72 ± 2.29	37.91 ± 3.68
16:1n-7	6.83 ± 0.82	6.67 ± 0.99
18:1n-9	29.27 ± 1.59	29.83 ± 2.25
24:1n-9	1.75 ± 0.09	1.15 ± 0.35
∑MUFA‡	37.85 ± 2.47	37.66 ± 3.77
16:2n-4	0.43 ± 0.13	0.54 ± 0.16
16:4n-1	0.81 ± 0.08	0.46 ± 0.15
18:2n-6 LA	27.40 ± 4.42	23.10 ± 2.92
18:3n-3 ALA	0.31 ± 0.10	1.74 ± 0.53
18:3n-6	2.38 ± 0.53	2.71 ± 0.58
20:2n-6	0.87 ± 0.27	0.57 ± 0.23
20:4n-6 ARA	7.53 ± 0.27	7.86 ± 0.57
20:5n-3 EPA	2.10 ± 0.32	2.17 ± 0.30
22:4n-6	2.88 ± 0.15	3.12 ± 0.30
22:5n-3	6.84 ± 0.83	5.52 ± 0.85
22:6n-3 DHA	43.19 ± 2.58	39.17 ± 3.17
∑PUFA*	94.77 ± 3.45	86.95 ± 5.75
∑n-3	52.47 ± 3.84	48.60 ± 4.28
∑n-6	41.49 ± 5.59	37.89 ± 4.61
n-3:n-6	1.44 ± 0.27	1.44 ± 0.31

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.7. Fatty acid composition of *F. grandis* ovaries after 4 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	1.90 ± 0.17	1.59 ± 0.13
16:0	26.02 ± 1.30	22.45 ± 0.53
18:0	11.32 ± 0.51	10.74 ± 0.55
22:0	3.56 ± 0.55	2.58 ± 0.47
∑SFA†	42.80 ± 1.97	37.36 ± 0.85
16:1n-7	4.03 ± 0.67	4.10 ± 0.38
18:1n-9	26.08 ± 1.72	30.13 ± 0.98
24:1n-9	2.10 ± 0.09	2.04 ± 0.09
∑MUFA‡	32.21 ± 2.58	36.27 ± 1.00
16:2n-4	0.28 ± 0.13	0.28 ± 0.12
16:4n-1	0.42 ± 0.15	0.27 ± 0.11
18:2n-6 LA	36.41 ± 3.09	35.28 ± 4.68
18:3n-3 ALA	0.22 ± 0.10	2.69 ± 0.47
18:3n-6	2.64 ± 0.40	4.11 ± 0.97
20:2n-6	1.76 ± 0.16	1.26 ± 0.14
20:4n-6 ARA	8.70 ± 0.41	9.96 ± 0.57
20:5n-3 EPA	0.42 ± 0.20	1.05 ± 0.15
22:4n-6	4.19 ± 0.32	4.07 ± 0.22
22:5n-3	3.24 ± 0.51	3.46 ± 0.38
22:6n-3 DHA	35.97 ± 4.10	40.33 ± 4.26
∑PUFA*	94.26 ± 3.97	102.76 ± 4.62
∑n-3	39.85 ± 5.10	47.52 ± 4.86
∑n-6	54.00 ± 3.56	54.97 ± 6.63
n-3:n-6	0.77 ± 0.17	1.03 ± 0.28

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.8. Fatty acid composition of *F. grandis* ovaries after 8 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	1.95 ± 0.66	1.14 ± 0.22
16:0	29.03 ± 2.87	23.12 ± 1.30
18:0	10.21 ± 0.66	11.20 ± 0.59
22:0	4.97 ± 0.62	2.94 ± 0.31
∑SFA†	46.16 ± 4.91	38.40 ± 2.20
16:1n-7	3.79 ± 1.28	3.23 ± 0.32
18:1n-9	29.20 ± 6.84	31.71 ± 2.06
24:1n-9	2.59 ± 0.43	2.09 ± 0.49
∑MUFA‡	35.58 ± 9.34	37.03 ± 3.12
16:2n-4	0.27 ± 0.17	0.29 ± 0.12
16:4n-1	0.21 ± 0.13	0.40 ± 0.13
18:2n-6 LA	45.00 ± 5.89	45.60 ± 1.32
18:3n-3 ALA	0.39 ± 0.28	3.76 ± 0.11
18:3n-6	4.71 ± 0.90	4.38 ± 0.44
20:2n-6	1.94 ± 0.22	1.56 ± 0.08
20:4n-6 ARA	9.15 ± 0.77	10.38 ± 0.98
20:5n-3 EPA	0.51 ± 0.32	0.56 ± 0.16
22:4n-6	5.50 ± 0.54	4.18 ± 0.58
22:5n-3	2.58 ± 1.12	2.66 ± 0.28
22:6n-3 DHA	28.17 ± 2.52	36.08 ± 3.18
∑PUFA*	98.43 ± 8.06	109.85 ± 5.52
∑n-3	31.65 ± 2.96	43.07 ± 3.82
∑n-6	66.56 ± 8.38	66.39 ± 2.13
n-3:n-6	0.53 ± 0.11	0.64 ± 0.04

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.9. Baseline fatty acid composition of female *F. grandis* intraperitoneal fat at the beginning of an 8 week spawning period. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Baseline
14:0	37.95 ± 1.79
16:0	144.98 ± 3.29
18:0	22.54 ± 1.13
22:0	9.65 ± 0.99
∑SFA†	215.12 ± 5.81
16:1n-7	82.77 ± 4.09
18:1n-9	270.56 ± 3.81
24:1n-9	8.45 ± 0.29
∑MUFA‡	361.77 ± 3.49
16:2n-4	4.90 ± 0.27
16:4n-1	9.26 ± 0.67
18:2n-6 LA	142.18 ± 6.51
18:3n-3 ALA	9.14 ± 0.56
18:3n-6	12.55 ± 1.31
20:2n-6	7.15 ± 0.32
20:4n-6 ARA	18.67 ± 0.20
20:5n-3 EPA	18.53 ± 1.35
22:4n-6	14.61 ± 0.73
22:5n-3	48.26 ± 2.73
22:6n-3 DHA	91.04 ± 3.72
∑PUFA*	376.29 ± 1.15
∑n-3	166.97 ± 7.26
∑n-6	200.06 ± 8.85
n-3:n-6	0.83 ± 0.08

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.10. Fatty acid composition of female *F. grandis* intraperitoneal fat after 1 week of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	33.82 ± 2.62	28.42 ± 1.85
16:0	139.49 ± 3.72	129.92 ± 4.82
18:0	26.45 ± 1.08	22.99 ± 1.21
22:0	8.45 ± 0.36	12.03 ± 2.04
∑SFA†	208.21 ± 7.41	193.36 ± 9.18
16:1n-7	70.09 ± 2.48	63.92 ± 2.61
18:1n-9	274.04 ± 11.74	284.27 ± 8.58
24:1n-9	9.58 ± 1.03	11.06 ± 0.71
∑MUFA‡	353.71 ± 11.69	359.25 ± 7.78
16:2n-4	4.24 ± 0.24	3.84 ± 0.22
16:4n-1	7.26 ± 0.70	6.58 ± 0.74
18:2n-6 LA	156.98 ± 10.42	174.51 ± 9.75
18:3n-3 ALA	9.82 ± 0.83	7.79 ± 0.39
18:3n-6	16.84 ± 1.58	19.20 ± 1.62
20:2n-6	7.47 ± 1.23	6.35 ± 0.18
20:4n-6 ARA	19.00 ± 0.89	20.90 ± 1.02
20:5n-3 EPA	14.22 ± 1.23	11.69 ± 0.69
22:4n-6	16.24 ± 0.96	20.60 ± 2.00
22:5n-3	44.15 ± 2.39	37.48 ± 2.04
22:6n-3 DHA	81.36 ± 5.54	76.56 ± 1.82
∑PUFA*	377.57 ± 8.67	385.52 ± 9.49
∑n-3	149.54 ± 9.00	133.52 ± 3.46
∑n-6	220.77 ± 12.83	245.42 ± 12.67
n-3:n-6	0.70 ± 0.07	0.56 ± 0.05

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.11. Fatty acid composition of female *F. grandis* intraperitoneal fat after 4 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	33.23 ± 1.28	30.30 ± 1.65
16:0	136.53 ± 1.40	134.97 ± 3.55
18:0	25.55 ± 1.24	26.05 ± 1.15
22:0	9.49 ± 1.66	12.15 ± 1.76
∑SFA†	204.82 ± 2.05	203.47 ± 6.09
16:1n-7	66.52 ± 2.01	66.02 ± 1.48
18:1n-9	265.12 ± 8.93	273.83 ± 8.07
24:1n-9	10.93 ± 0.50	11.84 ± 0.64
∑MUFA‡	342.57 ± 9.32	351.69 ± 8.71
16:2n-4	4.17 ± 0.26	4.47 ± 0.37
16:4n-1	6.63 ± 0.89	6.55 ± 0.58
18:2n-6 LA	166.31 ± 7.15	169.45 ± 6.11
18:3n-3 ALA	9.36 ± 1.12	9.69 ± 1.73
18:3n-6	15.17 ± 2.07	16.90 ± 1.08
20:2n-6	6.45 ± 0.22	6.73 ± 0.37
20:4n-6 ARA	20.79 ± 1.79	22.13 ± 1.95
20:5n-3 EPA	13.35 ± 0.66	12.84 ± 0.85
22:4n-6	18.28 ± 0.70	19.96 ± 1.36
22:5n-3	47.73 ± 2.43	43.37 ± 2.28
22:6n-3 DHA	91.40 ± 7.04	91.42 ± 2.72
∑PUFA*	399.64 ± 8.61	403.50 ± 2.65
∑n-3	161.84 ± 9.75	157.32 ± 5.38
∑n-6	231.17 ± 8.85	239.63 ± 6.37
n-3:n-6	0.71 ± 0.06	0.66 ± 0.04

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.12. Fatty acid composition of female *F. grandis* intraperitoneal fat after 8 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	27.97 ± 1.56	29.89 ± 0.93
16:0	135.09 ± 1.97	122.61 ± 3.39
18:0	27.45 ± 0.90	22.11 ± 0.74
22:0	12.62 ± 2.23	16.59 ± 2.48
∑SFA†	204.83 ± 3.02	191.20 ± 3.91
16:1n-7	58.59 ± 2.52	62.42 ± 1.11
18:1n-9	266.05 ± 11.73	267.78 ± 9.27
24:1n-9	12.27 ± 0.70	10.09 ± 1.10
∑MUFA‡	336.91 ± 13.12	336.27 ± 11.21
16:2n-4	4.89 ± 0.43	3.61 ± 0.11
16:4n-1	5.90 ± 0.68	7.04 ± 0.20
18:2n-6 LA	198.23 ± 6.76	180.42 ± 10.10
18:3n-3 ALA	7.08 ± 0.47	9.61 ± 0.68
18:3n-6	21.34 ± 1.62	18.49 ± 0.80
20:2n-6	7.69 ± 0.36	6.31 ± 0.41
20:4n-6 ARA	24.72 ± 3.68	22.66 ± 1.04
20:5n-3 EPA	9.91 ± 0.74	11.48 ± 0.48
22:4n-6	19.93 ± 1.11	17.28 ± 0.72
22:5n-3	41.60 ± 3.32	43.48 ± 1.37
22:6n-3 DHA	81.41 ± 8.81	85.63 ± 3.65
∑PUFA*	422.71 ± 11.37	406.01 ± 12.37
∑n-3	140.00 ± 13.89	150.20 ± 5.33
∑n-6	276.81 ± 9.35	248.77 ± 12.25
n-3:n-6	0.51 ± 0.06	0.61 ± 0.04

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.13. Baseline fatty acid composition of *F. grandis* ova at the beginning of an 8 week spawning period. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	Baseline
14:0	4.44 ± 0.06
16:0	22.94 ± 0.31
18:0	11.19 ± 0.19
22:0	1.70 ± 0.40
∑SFA†	40.27 ± 0.58
16:1n-7	8.25 ± 0.10
18:1n-9	24.28 ± 2.45
24:1n-9	1.28 ± 0.04
∑MUFA‡	33.81 ± 2.95
16:2n-4	0.76 ± 0.02
16:4n-1	0.98 ± 0.04
18:2n-6 LA	12.23 ± 0.13
18:3n-3 ALA	0.68 ± 0.01
18:3n-6	1.14 ± 0.06
20:2n-6	0.80 ± 0.15
20:4n-6 ARA	7.30 ± 0.23
20:5n-3 EPA	3.84 ± 0.15
22:4n-6	2.62 ± 0.08
22:5n-3	8.53 ± 0.10
22:6n-3 DHA	40.83 ± 1.07
∑PUFA*	79.70 ± 1.25
∑n-3	53.88 ± 1.20
∑n-6	24.84 ± 0.40
n-3:n-6	2.17 ± 0.06

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.14. Fatty acid composition of *F. grandis* ova after 1 week of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	4.24 ± 0.10	4.34 ± 0.02
16:0	23.80 ± 0.29	22.73 ± 0.51
18:0	10.74 ± 0.24	10.60 ± 0.02
22:0	1.02 ± 0.47	5.26 ± 2.37
∑SFA†	39.80 ± 0.25	42.93 ± 3.41
16:1n-7	8.24 ± 0.07	7.90 ± 0.16
18:1n-9	26.00 ± 0.82	24.96 ± 0.28
24:1n-9	0.87 ± 0.36	0.00 ± 0.00
∑MUFA‡	35.11 ± 1.38	32.86 ± 0.35
16:2n-4	0.51 ± 0.21	0.00 ± 0.00
16:4n-1	0.64 ± 0.26	0.00 ± 0.00
18:2n-6 LA	15.44 ± 0.46	13.37 ± 0.93
18:3n-3 ALA	0.48 ± 0.20	0.00 ± 0.00
18:3n-6	0.76 ± 0.31	0.00 ± 0.00
20:2n-6	0.53 ± 0.23	0.00 ± 0.00
20:4n-6 ARA	6.87 ± 0.38	7.60 ± 0.56
20:5n-3 EPA	7.61 ± 0.26	7.06 ± 0.18
22:4n-6	2.51 ± 0.11	2.56 ± 0.13
22:5n-3	7.61 ± 0.26	7.06 ± 0.18
22:6n-3 DHA	43.44 ± 0.47	41.79 ± 1.73
∑PUFA*	81.99 ± 2.19	75.19 ± 3.16
∑n-3	54.74 ± 0.98	51.67 ± 2.48
∑n-6	26.61 ± 0.89	23.53 ± 1.20
n-3:n-6	2.05 ± 0.03	2.20 ± 0.12

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.15. Fatty acid composition of *F. grandis* ova after 4 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid (μg mg dry mass^{-1})	CO Diet	SO Diet
14:0	1.89 ± 0.16	1.60 ± 0.11
16:0	25.98 ± 1.42	19.56 ± 0.37
18:0	12.07 ± 0.30	10.85 ± 0.23
22:0	6.04 ± 2.45	2.03 ± 0.17
∑SFA†	45.98 ± 1.92	34.04 ± 0.91
16:1n-7	3.21 ± 0.30	3.45 ± 0.10
18:1n-9	22.71 ± 1.34	26.80 ± 0.36
24:1n-9	0.63 ± 0.51	1.42 ± 0.08
∑MUFA‡	26.55 ± 1.45	31.66 ± 0.54
16:2n-4	0.13 ± 0.11	0.52 ± 0.02
16:4n-1	0.11 ± 0.09	0.32 ± 0.13
18:2n-6 LA	35.17 ± 2.57	35.13 ± 2.43
18:3n-3 ALA	0.16 ± 0.13	3.28 ± 0.26
18:3n-6	2.65 ± 0.28	3.73 ± 0.55
20:2n-6	0.58 ± 0.48	1.17 ± 0.07
20:4n-6 ARA	9.34 ± 1.19	9.12 ± 0.29
20:5n-3 EPA	0.15 ± 0.12	0.94 ± 0.08
22:4n-6	3.59 ± 0.17	3.07 ± 0.25
22:5n-3	1.55 ± 0.71	2.68 ± 0.06
22:6n-3 DHA	36.98 ± 4.00	33.39 ± 1.06
∑PUFA*	90.41 ± 3.51	93.35 ± 4.97
∑n-3	38.84 ± 4.54	40.29 ± 1.53
∑n-6	51.46 ± 5.25	52.74 ± 3.75
n-3:n-6	0.75 ± 0.16	0.77 ± 0.04

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

Table C.16. Fatty acid composition of *F. grandis* ova after 8 weeks of spawning while consuming cottonseed oil (CO) or soy oil (SO) based diets. Data are presented as mean ± SEM.

Fatty Acid ($\mu g\ mg\ dry\ mass^{-1}$)	CO Diet	SO Diet
14:0	0.84 ± 0.34	0.68 ± 0.28
16:0	26.50 ± 0.71	18.94 ± 0.20
18:0	11.71 ± 0.10	10.52 ± 0.14
22:0	5.01 ± 0.53	2.61 ± 0.26
∑SFA†	44.06 ± 0.97	32.75 ± 0.32
16:1n-7	1.41 ± 0.58	1.70 ± 0.71
18:1n-9	21.21 ± 0.71	24.78 ± 1.08
24:1n-9	0.59 ± 0.48	1.10 ± 0.46
∑MUFA‡	23.21 ± 0.82	27.58 ± 2.54
16:2n-4	0.16 ± 0.13	0.31 ± 0.13
16:4n-1	0.50 ± 0.41	0.30 ± 0.25
18:2n-6 LA	41.11 ± 1.87	40.75 ± 2.22
18:3n-3 ALA	0.00 ± 0.00	3.38 ± 0.11
18:3n-6	3.84 ± 0.37	4.89 ± 0.36
20:2n-6	1.07 ± 0.44	0.79 ± 0.32
20:4n-6 ARA	9.26 ± 0.43	9.20 ± 0.49
20:5n-3 EPA	0.00 ± 0.00	0.45 ± 0.18
22:4n-6	5.03 ± 0.18	3.45 ± 0.39
22:5n-3	0.43 ± 0.35	1.33 ± 0.55
22:6n-3 DHA	30.22 ± 3.06	29.46 ± 1.18
∑PUFA*	91.62 ± 1.82	94.31 ± 1.11
∑n-3	30.65 ± 4.10	34.62 ± 2.17
∑n-6	60.47 ± 2.83	59.39 ± 2.90
n-3:n-6	0.51 ± 0.09	0.58 ± 0.06

†Total saturated fatty acid content

‡Total monounsaturated fatty acid content

*Total polyunsaturated fatty acid content

VITA

Joshua T. Patterson was born in 1984 in Norfolk, Virginia. After graduating from Cave Spring High School in Roanoke, Virginia in 2002, Josh attended George Mason University in Fairfax, Virginia. Josh graduated from George Mason University in May 2006 with a Bachelor of Science degree in biology. In January 2007, Josh enrolled at Kentucky State University in Frankfort, Kentucky. Josh earned his Masters of Science degree in Aquaculture and Aquatic Sciences in May 2010, after continuing his education by enrolling in the doctoral program in the School of Renewable Natural Resources at Louisiana State University in January of 2010. Josh expects to receive his Doctor of Philosophy degree in May 2014.