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Fatty Acid Composition of the Maternal Diet Affects Egg and Larval Quality of Southern Flounder, *Paralichthys lethostigma*

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Fatty Acid Composition of the Maternal Diet Affects Egg and Larval Quality of Southern Flounder, *Paralichthys lethostigma*

by

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Dedication

To my parents, Robert and Lois –As I grew up, you encouraged me to kiss every fish I reeled in, and now look at what I am doing with my life.

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Abstract

Fatty Acid Composition of the Maternal Diet Affects Egg and Larval Quality of Southern Flounder, *Paralichthys lethostigma*

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Yolk of marine fish eggs is highly concentrated in fatty acids (FAs) that are used for energy, hormone production, and membrane structure. Essential fatty acids (EFAs) are fatty acids that cannot be biosynthesized in physiologically sufficient amounts and must be obtained from the diet. Since EFAs in yolk must originate in the maternal diet, a study was conducted to determine whether changes in maternal dietary docosahexaenoic acid (DHA, an important EFA for proper larval development) during a spawning season had an effect on the proportion of DHA in eggs of Southern flounder, *Paralichthys lethostigma.* Adult flounder were conditioned on a common diet and then switched to a high DHA diet, low DHA diet, or no change (control) after the first spawn. Spawns were produced weekly and DHA content of the eggs was measured by gas chromatography. Females fed a high DHA diet produced eggs with a significantly higher proportion of DHA after 3 weeks on the experimental diet. DHA in eggs from females fed a low DHA

diet decreased for 5 weeks, then increased, suggesting that those females first used dietary DHA to make yolk then shifted to DHA stored in liver or white muscle.

In a second study, spawns from the first study were incubated and larvae were reared to 15 and 35 days post-hatching (dph) to determine whether there was a relationship between FA composition of the eggs, FA composition of the larval body, and responsiveness to a visual predatory stimulus. Ratios of DHA to other EFAs (DHA:ARA and DHA:EPA) were positively correlated with larval responsiveness to a simulated predator. The amount of omega-3 docosapentaenoic acid (DPA)in the egg was strongly correlated with the amount of DHA in the larval body at 15 dph, and was significantly correlated with the amount of DHA in the larval body at 35 dph. This research suggests that southern flounder follow a mixed breeding strategy to supply developing eggs with DHA during vitellogenesis, and variability in FA composition of eggs, specifically (n-3) DPA, may influence larval mortality from predation in the wild.

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Chapter 1: Introduction

Yolk is the only source of nutrition for developing embryos and newly hatched larvae of oviparous fishes (Sargent 1995). This nutrition provides the energy and materials to support the fast cell differentiation, organ development, and growth that characterizes the embryonic and early larval stages. If an individual is able to develop normally as an embryo and hatch, then it must evade predation and forage for food to meet the energetic costs of a high larval metabolism. Nutrients in the yolk originate from the maternal diet, but whether they are quickly transferred from the diet into the yolk or stored in somatic reserves before later being moved into the yolk during vitellogenesis, is species specific (Jönsson 1997, McBride et al. 2015). Research has begun to investigate maternal effects of nutritional provisioning in fishes, that is, the effects of the quality and quantity of nutrients invested by the mother into the yolk on the survival and fitness of her offspring (Furuita et al. 2000, 2002, Fuiman and Ojanguren 2011, Perez and Fuiman 2015). Although the duration of the endogenous feeding period in fishes in relatively brief compared to the entire lifespan, there is evidence in one marine species, red drum (*Sciaenops ocellatus*), that the nutrition provided to an individual during this brief window of time influences metabolic processes later in life (Perez and Fuiman 2015). Therefore, it is important to understand the dynamics of nutrients in the egg that are important for early development, and how these changes in early larval nutrition affect larval survival, for both ecology and aquaculture.

Fatty acids are highly concentrated in fish yolk, and are utilized by the embryo for various purposes such as energy, hormone synthesis, and membrane structure (Sargent

1995). A subset of fatty acids, known as essential fatty acids (EFAs), must be obtained from the diet since they cannot be biosynthesized in quantities that are sufficient for biological processes. Each species has its own EFA requirements, which vary through ontogeny (Sargent et al. 1993a, Sargent 1997). Larval fish acquire EFAs from the yolk while feeding endogenously and from the larval diet while feeding exogenously. Three essential fatty acids are especially important to developing marine fishes: docosahexaenoic acid (DHA, 22:6(n-3)), arachidonic acid (ARA, 20:4(n-6)), and eicosapentaenoic acid (EPA, 20:5(n-3)) (Sargent 1997).

All EFAs deposited into the yolk are derived from the maternal diet, but the timing of transfer from maternal diet into the egg yolk varies between species based on reproductive strategies. Fishes that build yolk from nutrients previously stored in somatic tissues are known as "capital breeders" (Jönsson 1997). Use of stored nutrients for reproduction may buffer and maintain EFA concentrations in eggs when a species inhabits a variable nutritional environment (Jönsson 1997). Fish species known as "income breeders" transfer ingested nutrients relatively quickly from their diet to the yolk, causing nutrient composition of eggs to vary in response to a change in diet (Jönsson 1997). Red drum (*Sciaenops oceallatus*) is an example of an income breeder, because it has been shown that the ARA content of its eggs begins to change within 2 days of a change in the ARA content of its diet, and the rate of change in the egg is proportional to the magnitude of the dietary diet shift (Fuiman and Faulk 2013). Most marine fishes, and ectotherms in general, follow capital breeding strategies (Bonnet et al. 1998, Stephens et al. 2009, McBride et al. 2015). Evidence of mixed breeding, in which a

species uses a combination of recently ingested nutrients and stored reserves, has been documented in mammals (Wheatley et al. 2008), birds (Gauthier et al. 2003), reptiles (Winne et al. 2006), as well as marine fishes (Hirschfield 1980, Tricas 1989, Kawaguchi et al. 1990, Luo and Musick 1991, Harel et al. 1994).

Predation is the largest cause of mortality in larval fishes (Bailey and Houde 1989). If an individual possesses characteristics that make it better able to evade predation than its peers, it has a better chance of survival to recruitment (Fuiman and Magurran 1994). Larval red drum at 21 days post-hatching (dph) with higher amounts of the essential fatty acid, DHA, in their body have a significantly greater response to a visual predatory stimulus than larvae with lower amounts of DHA in their body (Fuiman and Ojanguren 2011). The amount of DHA in the larval body is correlated with both the amount of DHA in the larval diet as well as the amount of maternally-derived DHA in the egg (Fuiman and Ojanguren 2011, Perez and Fuiman 2015). DHA is concentrated in the retina and neural pathways during visual sensory system development and is critical for proper eye development in larval fishes (Sargent 1993b). These studies demonstrate the importance of proper nutrition, in both the larval and maternal diets, to larval survival in a marine fish species. Larvae that fail to respond to an approaching predator would die, whereas larvae that respond to a predator have a better chance of surviving (Fuiman 1994).

Metabolic programming occurs “when variations in nutrition during a specific developmental window result in long-term metabolic effects” (Lucas 1991). Results from Perez and Fuiman (2015) indicate that there is a difference in lipid metabolism between

groups of larvae due to differences in DHA content of the egg. Moreover, maternal effects of nutrition in red drum influences larval physiology by altering lipid metabolism and fatty acid composition of the larval body and, ultimately, by altering behaviors critical for survival like predator evasion (Perez and Fuiman 2015). Currently, metabolic programming and the connection between behavior and fatty acid composition has only be documented in red drum but may occur in other marine fish species.

Southern flounder, *Paralichthys lethostigma*, are the third most recreationally fished species along the Texas coast and the largest flatfish in the Gulf of Mexico, making them an economically important species within the region (GSMFC 2000). This species exhibits sexual dimorphism, with females growing much faster and larger than males. Each fall/early-winter, male southern flounder migrate out of coastal estuaries, followed closely by large females, into open gulf waters where females produce multiple batches of eggs until early spring when they return to estuarine waters (Stokes 1977). Peak spawning activity occurs from November to January (Gunter 1945). This spawning migration creates a bottleneck of individuals at the narrow passes that connect the estuaries to the gulf, which makes large individuals, the reproductive females, easy targets for recreational fisherman. Overfishing of southern flounder in Texas caused a steady decrease in the population beginning in the 1980s and continuing until the early 2000s (TPWD 2003, Froeschke et al. 2011). Changes in the management of southern flounder fisheries, such as gigging bans and reduced catch limits, were implemented in order to stabilize the population (Riechers 2008). Nevertheless, fishing pressure on

southern flounder populations have created a need for knowledge of basic biology and culturing methods of the species to aid in enhancement efforts.

Many experiments have been done on EFA requirements of larval flatfishes, including southern flounder. Larvae fed diets high in EFAs concentrate DHA in the head probably because DHA is concentrated in neural tissues (i.e., the brain and eyes; Oberg and Fuiman 2015). Larvae with higher whole body levels of DHA demonstrated increased responsiveness to a visual predatory stimulus (Oberg and Fuiman 2015). Other flatfishes, such as Atlantic halibut (*Hippoglossus hippoglossus*), fed DHA depleted diets as larvae exhibited increased abnormal pigmentation compared to halibut fed naturally occurring diets. This defect may be due to the halibut's inability to accurately process visual signals in the eye or the brain, or abnormal melanocyte production (Sargent et al. 1999). The olive flounder, *Paralichthys olivaceus*, shows a parabolic relationship between the amount of n-3 EFAs in the adult diet, the amount of n-3 EFAs in the eggs, and larval quality (Furuita et al. 2000). Increases in adult dietary intake of n-3 EFAs, up to a threshold, caused increased amounts of n-3 EFA in the egg and increased larval survival. Above the threshold level of dietary n-3 EFA, larval quality and survival decreased with increasing egg n-3 EFAs (Furuita et al. 2002). In olive flounder, a closely related Paralichthyidae species, maternal diet influences fatty acid composition of the egg.

The objectives of this research on southern flounder are: (1) to determine if maternal dietary DHA shifts influence DHA amounts in eggs within a spawning season and; (2) to determine the effects of fatty acids in the egg on the predator evasion ability of

larvae. Because of their spawning migration, female southern flounder may feed on different prey along the migration route, resulting in different qualities and quantities of fatty acids being consumed and deposited into the egg yolk during vitellogenesis. The first study investigates the transfer of one highly important EFA, DHA, from the diet of adult southern flounder into the eggs over an 8-week experimental period, simulating the peak natural spawning season (Stokes 1977). Further, while the effects of EFA concentrations in the larval diet have been investigated in southern flounder (Oberg and Fuiman 2015), the effects of maternally derived EFAs have not. Using spawns from multiple individuals fed different diets, the second study assesses the effects of egg fatty acid concentrations on anti-predator behaviors of larvae at two points during the larval period by measuring: (1) responsiveness of larvae at each age to a simulated rapidly approaching predator; (2) which fatty acids in the larval body are correlated with predator evasion performance; and (3) if there is a correlation between fatty acids in the egg and those fatty acids in the larval body that are influential in predator evasion behavior.

Chapter 2: Maternal diet affects egg DHA content and reproductive energy allocation strategy of southern flounder, *Paralichthys lethostigma*, within a spawning season

INTRODUCTION

Yolk is the sole source of nutrition for developing embryos and recently hatched larvae of oviparous fishes, and all yolk constituents ultimately derive from the maternal diet (Sargent 1995). Capital breeders store ingested nutrients in somatic reserves and draw upon those stores at a later date to build yolk (Jönsson 1997). Use of stored nutrients may provide a buffer against a variable nutritional environment so that a constant egg composition can be maintained. In contrast, income breeders quickly transfer recently ingested nutrients to the egg (Jönsson 1997). The shore lag between ingestion and deposition in eggs could result in highly variable egg composition.

Yolk of marine fish eggs is highly concentrated in lipids and fatty acids. Fatty acids are used for energy, hormone production, and membrane structure. Essential fatty acids (EFAs) are those fatty acids that cannot be biosynthesized in physiologically sufficient amounts and so must be obtained from the diet. Three EFAs have received much research attention, because of their nutritional importance to marine fishes: docosahexaenoic acid (DHA, 22:6(n-3)), arachidonic acid (ARA, 20:4(n-6)), and eicosapentaenoic acid (EPA, 20:5(n-3)) (Sargent 1997). DHA is concentrated in the retina and other neural tissues and is critical for proper visual development and function in larval fishes (Sargent 1993b, 1997).

Some subtropical fish species allocate energy to reproduction as income breeders (Luo et al. 1991, Harel et al. 1994, Furuita et al. 2000). For example, DHA content (mg DHA g^{-1} DW) in the eggs of red drum (*Sciaenops ocellatus*) changes quickly after a change in DHA content of the maternal diet (Fuiman and Faulk 2013). Importantly, the

DHA content of the egg of red drum is correlated with larval performance in behaviors critical for survival (Fuiman and Ojanguren 2011, Perez and Fuiman 2015). Therefore, changes in the EFA composition of the maternal diet in red drum can affect quality and survival of offspring.

Southern flounder is an important recreationally fished species along the coast of the Gulf of Mexico, but declines in local populations created interest in aquaculture of southern flounder to support stock-enhancement efforts. While protocols for rearing larval southern flounder have been established, little is known about how or when dietary nutrients are allocated to yolk production (Daniels et al. 1996, Benetti et al. 2001, Moustakas et al. 2004). Southern flounder are batch spawners (Fischer 1995) and migrate from estuaries to offshore waters of the Gulf of Mexico in the winter to spawn, with peak spawn activity occurring between November and January (Gunter 1945). Southern flounder are likely to consume different quantities, proportions, and species of prey in estuaries than they do offshore, resulting in large changes in fatty acid ingestion. Therefore, their spawning migration may impact the EFA content of eggs. The olive flounder, *Paralichthys olivaceus*, a closely related species, show that increased proportions of n-3 highly unsaturated fatty acids (HUFAs) in the maternal diet increase the proportion of n-3 HUFAs in the eggs, resulting in decreased larval quality (Furuita 2000, 2002).

This study aims to determine whether maternal dietary shifts in DHA near the start of the spawning season influence the proportion of DHA in eggs. Ecologically, if southern flounder allocate nutrients to yolk as income breeders, then temporal and spatial variability in the prey field of adult flounder will alter egg composition and possibly affect larval performance and survival. Depending on the speed of nutrient transfer to yolk, egg and larval quality could vary among females within a spawning season, and

perhaps between multiple batches produced by one female. Alternatively, if maternal somatic reserves are used to build yolk, then the proportion of DHA in the egg will be less influenced by variations in the adult prey field during the spawning season.

METHODS

Two experiments were conducted to examine the effect of a change in maternal diet on the proportion of DHA in southern flounder eggs. The first experiment, conducted in 2016 and 2017, assessed the first batch of eggs produced by each female during the spawning season. The second experiment, in 2017, assessed the proportion of DHA in successive batches of eggs from individual females. The proportion of DHA refers to the percentage of DHA relative to the total amount of fatty acids in the egg, tissue, or food item being discussed.

A southern flounder broodstock, comprised of 144 females and 40 males, was maintained at the Fisheries and Mariculture Laboratory of the University of Texas Marine Science Institute in Port Aransas, TX, USA, in 2016 and 2017. During experiments (mid-December through early April), broodstock were kept at a controlled temperature (18-20°C), salinity (33-35 ppt), and photoperiod (10 h: 14 h light:dark). Broodstock were held in 6, 36-kL recirculating tanks with 24 females per tank. Broodstock were fed a conditioning diet of equal parts (by weight) of Spanish sardines (*Sardinella aurita*) and brown shrimp (*Farfantepenaeus aztecus*) per tank three times a week for 3 months prior to spawning (October - January) in 2016 in order to establish an intermediate baseline for DHA content in maternal tissues. At the end of the first set of experiments (May 2016), the broodstock were placed on the conditioning diet until the start of the second set of experiments (8 months). Broodstock were fed to satiation at all feedings. Each tank was assigned to one of three treatment groups (high DHA diet, low DHA diet, and control [no

change] diet), with each treatment group having two replicate tanks each year. The control diet group was fed equal parts Spanish sardines (mean ± standard deviation [SD]; DHA 19.9 ± 3.3% of total fatty acids; 15.9 ± 4.5 mg DHA g^{-1} dry weight [DW]) and brown shrimp (10.4 ± 1.2% DHA; 2.8 ± 0.4 mg DHA g^{-1} DW), resulting in a diet containing a mean 15.2% DHA (9.4 mg g^{-1} DW) (Table 2.1). The high DHA diet group was fed only sardines, and the low DHA diet group was fed only shrimp. To restrict the study to maternal dietary effects, all males were kept in separate tanks that shared water with the control tanks, and were fed the control diet. Samples of the diet components were collected throughout the 9-week study period and frozen (-80°C) for subsequent fatty acid composition analysis (Table 2.1).

Experiment 1 – First Spawns (FT)

At the beginning of each spawning season (end of January 2016 and 2017), one female from each tank (two per treatment) was injected with Ovaprim (Western Chemical Inc.), a synthetic hormone that stimulates ovulation. After 48 h, each of these six females was anesthetized and eggs were manually stripped and immediately fertilized using milt from multiple broodstock males. These eggs were used to establish the common initial level (week 0) of DHA each year. Stripped females were tagged for identification and returned to their tank.

Diet changes began immediately after week 0 egg collections. Each week thereafter, one visibly ripe female that had not previously been strip spawned that season was removed from each tank, injected with Ovaprim, isolated for 48 h, anesthetized, and strip spawned. A sample of eggs from each spawn was rinsed with distilled water and frozen (-80°C) for subsequent fatty acid composition analysis. A different female from each tank was strip spawned each week for 8 weeks after the diet changes. After the

experiment was repeated in the second year, there were four replicates (n = 4) for each week for each of the three diet treatments.

Experiment 2 – Repeated Spawns (RS)

At the beginning of the second spawning season, two females from each tank (four per treatment) were stripped at week 0 and tagged for identification. Each of these females was injected with Ovaprim and manually stripped every 2 weeks throughout the spawning season, for a total of five spawns from each individual. At the end of the experiment, females were euthanized and ovary, liver, white muscle, and fatty tissue in the epaxial musculature (further referred to as dorsal fatty tissue) were collected, frozen, and analyzed for fatty acid composition.

Analyses

Fatty acid composition of all frozen tissues (diet components, eggs, maternal tissues) was measured using the method described by Faulk and Holt (2005). Briefly, samples were freeze dried and homogenized in a chloroform/methanol solution for lipid extraction. Fatty acid methyl esters were collected after transesterification of total lipids using boron triflouride. DHA content of eggs is expressed as a percentage of total fatty acids.

Two-way analysis of variance (ANOVA) was used to determine whether diet had an effect on the proportion of DHA in the eggs in first time spawns (FT). For repeated spawns (RS), two way repeated measures ANOVA was used to determine whether diet had an effect on the proportion of DHA in eggs from individual females over time. For both experiments, post-hoc multiple comparison tests comparing proportions of DHA from each experimental week to the mean week 0 value were computed to determine at which week(s) there was a significant change in DHA proportions of the egg for each diet

treatment. ANOVA was used to determine whether DHA proportions of ovary, liver, white muscle, and dorsal fatty tissues differed between the diet treatments.

RESULTS

Experiment 1 – First Spawns (FT)

Eggs were obtained from 42 females in 2016 and 58 females in 2017. Towards the end of the 8-week experiment, some tanks in all treatments had no ripe females, which resulted in unequal sample sizes in the treatments and sampling time points.

Differences in the mean proportion of DHA in eggs at week 0 was 21.7% $\pm$ 2.2% in 2016 (mean $\pm$ SD; n = 6 females) and 21.2% $\pm$ 1.3% in 2017 (n = 12 females) were not significant (P = 0.64). This indicated that the difference in time on the conditioning diet (3 months vs. 8 months) did not influence the proportion of DHA in the eggs, so data from 2016 and 2017 were pooled. The overall mean proportion of DHA in the eggs at week 0 (n = 18 females) was 21.4% $\pm$ 1.6%, and this value was used as the baseline for all three treatments.

There was a significant interaction between dietary treatment and weeks on diet in the FT experiment (P = 0.001). There was a significant effect of weeks on diet in each of the treatments: control diet (P= 0.007), high DHA diet (P = 0.002), and low DHA diet (P = 0.024) (Figure 2.1). In the control treatment, the proportion of DHA in the eggs did not differ from week 0 values until week 7 (P = 0.006) and week 8 (P = 0.016), when the proportion of DHA increased to 24.2% $\pm$ 0.9% and 24.0% $\pm$ 1.9% respectively. Females fed the high DHA diet produced eggs with significantly higher proportions of DHA starting at week 3 (24.2% $\pm$ 0.9%, P = 0.006) and remaining significantly higher than the mean week 0 value through the remainder of the experiment, with the exception of week 5 (23.2% $\pm$ 1.4%, P = 0.065; Figure 2.1). Eggs collected from females on the high DHA

diet showed the lowest amount of variability of DHA proportions of eggs between females at the same time period (Figure 2.1). Females fed the low DHA diet produced eggs with significantly lower proportions of DHA than the week 0 mean in week 4 (17.5% $\pm$ 5.5, P = 0.020) and week 5 (15.8% $\pm$ 4.5, P = 0.002), after which DHA levels increased through the remainder of the experiment. Variability in DHA proportions of eggs was highest in the low DHA diet treatment, especially in weeks 4 and 5 (Figure 2.1).

Experiment 2 – Repeated Spawns (RT)

The mean proportion of DHA in eggs at week 0 from successively spawned females was 21.2% $\pm$ 1.3%. Week 0 values were not significantly different between treatments. All 12 females produced eggs over the first 4 weeks, but some females in the control diet treatment stopped producing eggs after that. Eleven females produced eggs through week 6, and 9 females produced eggs throughout the entire 8 weeks (Figure 2.2).

There was a significant effect of diet on the proportion of DHA in eggs in the RS experiment (P < 0.001). In the control diet treatment, there was no difference in DHA proportion in eggs between successive spawns (P = 0.582). There was, however, a difference in DHA proportions in eggs in the high DHA treatment (P = 0.003), in which values were greater than the week 0 mean from week 4 (24.2% $\pm$ 1.2%, P = 0.003) onward (Figure 2.3). There was also a significant difference in DHA proportions in eggs in the low DHA treatment (P < 0.001), where the proportion of DHA of the last two spawns (weeks 6 and 8) was significantly lower than the week 0 mean (Figure 2.3).

The ovary was the only body tissue of females to show an effect of diet treatment on the proportion of DHA at the end of the experiment (P < 0.001; Figure 2.4, Table 2.2). Ovaries from females fed the high DHA diet contained significantly higher proportions of DHA than females on the control diet (P < 0.001). There was no significant difference in

the proportions of DHA in ovarian tissue between the low DHA diet and the control diet (P = 0.252). There was no effect of diet treatment on proportions of DHA in liver (P = 0.104), white muscle (P = 0.082), and dorsal fatty tissues (P = 0.607) (Table 2.2).

Different results were obtained by comparing DHA and total fatty acid content (expressed as mg g-1 DW) of tissues among diet treatments. There was a significant effect of diet treatment on the DHA content of both the liver (P = 0.023) and white muscle (P = 0.031) but not the ovary (P = 0.070) or dorsal fatty tissue (P = 0.395). White muscle tissue from females in the low DHA diet treatment had lower DHA content (mg g^{-1} DW) than the same tissue from females in control treatment (P = 0.026). There were also significant differences between tissues from the high and low DHA diet treatment. Comparing total fatty acid content of tissues, there was a significant effect of diet treatment on white muscle (P = 0.033). White muscle from females fed the control diet had significantly more total fatty acids than white muscle from females fed the high DHA diet (P = 0.041). There was no effect of diet treatment on the total amount of fatty acids in the ovary, liver, or dorsal fatty tissues (P = 0.338, 0.280, and 0.491, respectively).

DISCUSSION

The fact that the proportion of DHA in eggs changed within a few weeks of a diet change indicates that southern flounder generally use an income breeding reproductive strategy but are able to use stored resources (capital breeding) under some conditions. Mixed breeding allocation strategies have been seen in other fishes such as European anchovy (*Engraulis encrasicolus*), Japanese anchovy *(Engraulis japonicas*), and medaka (*Oryzias latipes*) (Hirshfield 1980, Kawaguchi et al. 1990, Somarkis et al. 2004, McBride et al. 2015). Under income breeding, there would be no change in the proportion of DHA in eggs from females held on the control diet if that diet contained sufficient DHA.

Results from the RS experiment supported this expectation, but DHA proportions in FT eggs increased in weeks 7 and 8 (Figure 2.5a). A possible explanation for these results is that the control diet had more DHA than required and the surplus appeared in eggs when females delayed spawning by 7 weeks. RS females may have used the dietary surplus to maintain DHA levels in many more spawns.

Under income breeding, proportions of DHA in eggs from females switched to a higher DHA diet should increase over time. Results from both experiments supported this hypothesis. DHA content of eggs from the RS experiment should increase more gradually or at a later time than the FT spawns, because surplus dietary DHA would be used to produce more spawns. This prediction does not appear to be supported, as the trends of increasing DHA proportions were almost identical for first time spawns and repeated spawns (Figure 2.5b). This suggests that the high DHA diet provided surplus DHA even greater than could be used by fish that spawned every 2 weeks. Interestingly, tissue fatty acid analyses suggest that females in the RS experiment fed the high DHA diet mobilized fatty acids from somatic reserves, since total amounts of DHA and total fatty acids in the white muscle were significantly less than those from females held on the control diet. One explanation for these results is that a diet of only Spanish sardines provides females with enough DHA for multiple batches of eggs, but does not provide females with adequate amounts of other important essential fatty acids, such as ARA or EPA, which they have to mobilize from tissues such as liver and white muscle. The proportion of arachidonic acid in eggs of *P. olivaceus* has been shown to increase egg quality and early larval survival (Furuita 2002). If arachidonic acid is important for offspring quality in Paralichthyidae species, southern flounder may mobilize arachidonic acid from stores to buffer eggs when dietary amounts are low.

Income breeding females fed a low DHA diet should produce eggs containing decreasing proportions of DHA over time. Further, repeated spawning should elevate the demand for DHA, thereby reducing DHA proportions in eggs sooner or more quickly than in the FT experiment. Results from the two experiments showed different trends (Figure 2.5c). As expected, FT spawners on the low DHA diet produced eggs with decreasing proportions of DHA for the first 5 weeks. At week 6, this trend reversed and the proportion of DHA in FT eggs increased for the next 3 weeks. DHA proportions in eggs from the RS experiment did not decrease until weeks 6 and 8. The reversing trend and the unusually high variability in egg DHA proportions around weeks 4 and 5 in the FT experiment suggest the onset of a physiological change in response to the low dietary intake of DHA. Those changes suggest that DHA mobilized from somatic tissues appeared in eggs in the latter part of the FT experiment. The lack of a decrease in the proportions of DHA in eggs in the first 4 weeks of the RS experiment could be the result of earlier mobilization of somatic stores of DHA under the high-demand conditions. Insufficient somatic stores could have resulted in the decrease in egg DHA composition toward the end of the RS experiment. Mobilization of stored lipids is supported by significantly lower amounts of DHA measured in liver, and lower DHA content in white muscle and dorsal fatty tissues from females fed the low DHA diet than the control diet, although those differences were not significant.

While capital and income breeding are extremes of a continuum, mixed breeding strategies allow a species to use stored energy when needed to improve reproductive and offspring success (Drent 2006). Evidence of mixed breeding strategies has been documented in many taxa, including mammals, birds, and reptiles, (Gauthier 2003, Winne et al. 2006, Wheatley et al. 2008) as well as marine fishes such as Japanese anchovy (*Engraulis japonicus*), medaka (*Oryzias lapites*), and pebbled butterflyfish

(*Chaetodon multicinctus*) (Kawaguchi et al. 1990, Hirshfield 1980, Tricas 1986). Resource allocation to reproduction within a spawning season or between years may be a plastic trait in southern flounder.

This study demonstrates that differences in dietary DHA content can affect the proportion of DHA in eggs. This could be relevant to natural populations of southern flounder since they migrate from estuaries to an offshore habitat to spawn. The effect of the offshore diet on the proportion of DHA in the eggs depends on the DHA composition of the diet as well as on how long a female feeds offshore before spawning. Results of this study indicate that a significant change in DHA proportion in the eggs can occur within 3 to 4 weeks of a diet change, which is well within the 8-week peak spawning season period for this species (Gunter 1945). Interannual variation in prey populations in estuaries and offshore will likely alter egg composition. Since southern flounder have a wide geographic range, it is also likely that egg composition will vary on large spatial scales as a result of differences in estuarine and marine prey that are available to adult flounder. When enough DHA is available in the spawning environment, southern flounder females may quickly transfer DHA (and possibly other essential fatty acids) from the maternal diet into the eggs throughout the spawning season. When DHA in the spawning environment is limited, southern flounder females may transfer DHA that was accumulated from the non-spawning ecosystem (estuaries) and stored in somatic tissues, into eggs. This could be important to larval survival and recruitment if DHA content of eggs is related to behavioral performance of southern flounder larvae (Burns, Chapter 3), as has been shown for red drum. Variations in fatty acid composition of eggs is also important because mass spawning of marine animals represents an important pathway for distributing energy and nutrients through marine food webs (Fuiman et al. 2015).

Fatty acid	Broodstock diet	
	Spanish sardine	Brown shrimp
12:0	0.1 ± 0.1	0.1 ± 0.1
14:0	4.9 ± 0.9	2.0 ± 0.7
15:0	1.4 ± 0.2	1.3 ± 0.2
16:0	24.7 ± 1.4	13.2 ± 0.6
16:1(n-7)	4.3 ± 1.9	4.1 ± 0.9
16:2(n-4)	1.2 ± 0.1	0.5 ± 0.1
17:0	1.6 ± 0.1	2.1 ± 0.2
16:3(n-4)	0.3 ± 0.3	1.7 ± 0.3
18:0	7.0 ± 0.7	9.5 ± 1.3
18:1(n-9)	8.1 ± 2.1	6.3 ± 1.0
18:1(n-7)	2.8 ± 0.4	3.7 ± 1.0
18:2(n-6)	1.5 ± 0.1	1.6 ± 0.5
18:3(n-6)	0.7 ± 0.1	0.4 ± 0.1
18:3(n-4)	0.1 ± 0.1	0.1 ± 0.1
18:3(n-3)	0.6 ± 0.1	0.5 ± 0.3
18:4(n-3)	0.6 ± 0.2	0.1 ± 0.1
20:1(n-9)	0.3 ± 0.1	0.3 ± 0.1
20:2(n-6)	0.2 ± 0.1	1.0 ± 0.2
20:3(n-6)	0.4 ± 0.1	0.4 ± 0.1
20:4(n-6)	2.8 ± 0.4	7.8 ± 1.5
20:3(n-3)	0.1 ± 0.1	0.2 ± 0.1
20:4(n-3)	0.4 ± 0.1	0.3 ± 0.1
20:5(n-3)	5.9 ± 0.9	13.6 ± 1.7
22:5(n-6)	1.2 ± 0.2	1.0 ± 0.2
22:5(n-3)	0.8 ± 0.4	2.1 ± 0.2
22:6(n-3)	19.9 ± 3.3	10.4 ± 1.2

Table 2.1. Fatty acid composition (% of total fatty acids) of broodstock diet components.

	DHA (%)			DHA (mg DHA g^{-1} DW)			Fatty acid (mg g^{-1} DW)		
Tissue	control	high	low	control	high	low	control	high	low
ovary	20.4 ± 0.8	27.6 ± 2.4 *	18.6 ± 2.4	25.2 ± 23.9	55.9 ± 5.0	33.1 ± 15.7	110.4 ± 104.8	190.5 ± 18.2	158.1 ± 67.8
liver	14.4 ± 3.8	20.6 ± 2.8	13.2 ± 6.6	37.7 ± 3.7	39.9 ± 8.4	25.4 ± 6.5 *	254.1 ± 68.1	187.3 ± 63.9	193.4 ± 48.9
white muscle	26.2 ± 2.0	30.5 ± 2.5	25.3 ± 4.2	7.2 ± 0.9	5.9 ± 0.9	5.1 ± 1.1 *	25.3 ± 5.0	17.3 ± 1.1 *	18.2 ± 4.3
dorsal fatty tissue	22.9 ± 3.5	24.8 ± 3.3	22.7 ± 2.8	17.8 ± 15.8	23.8 ± 24.5	6.9 ± 0.7	80.9 ± 84.4	98.9 ± 118.9	27.8 ± 4.5

Table 2.2. DHA and total fatty acid content (mean ± SD) of tissues collected from repeatedly spawned females (n = 4 females per diet treatment; * denotes a significant difference from the control treatment.

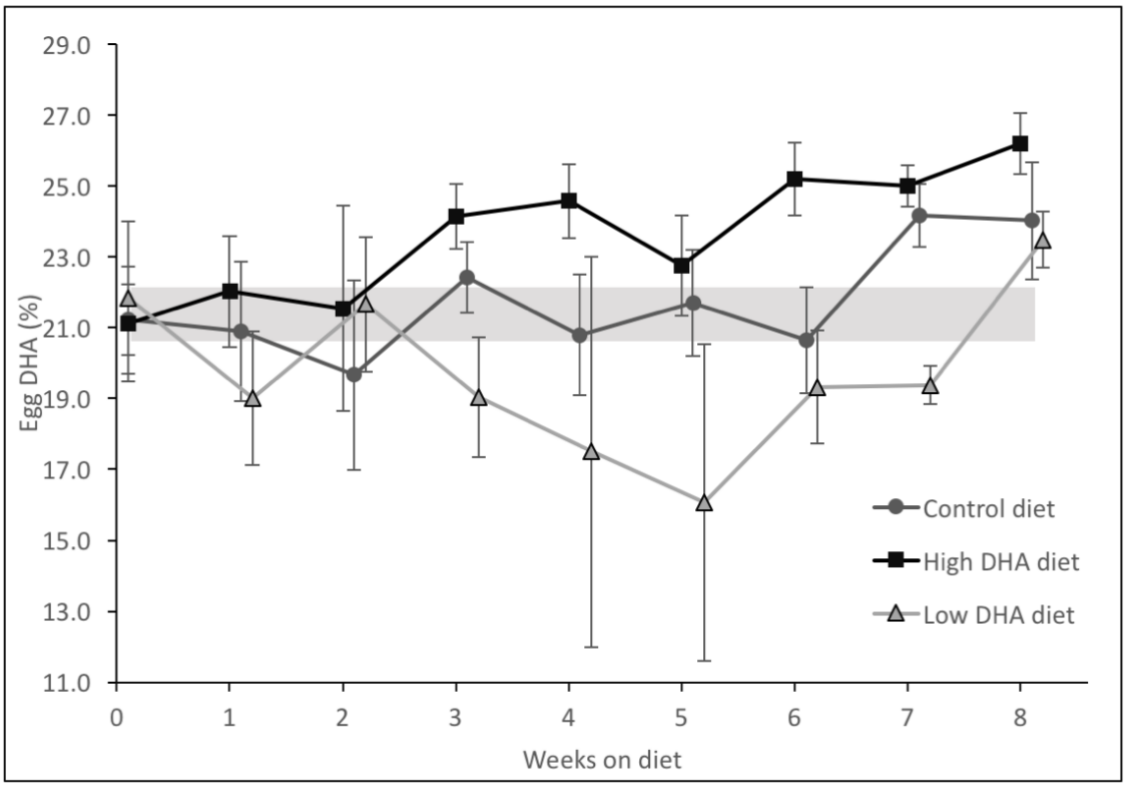

Figure 2.1. Change in proportion of DHA in eggs (%, mean ± SEM) of first time spawns over time. Shaded area indicates 95% CI around week 0 mean.

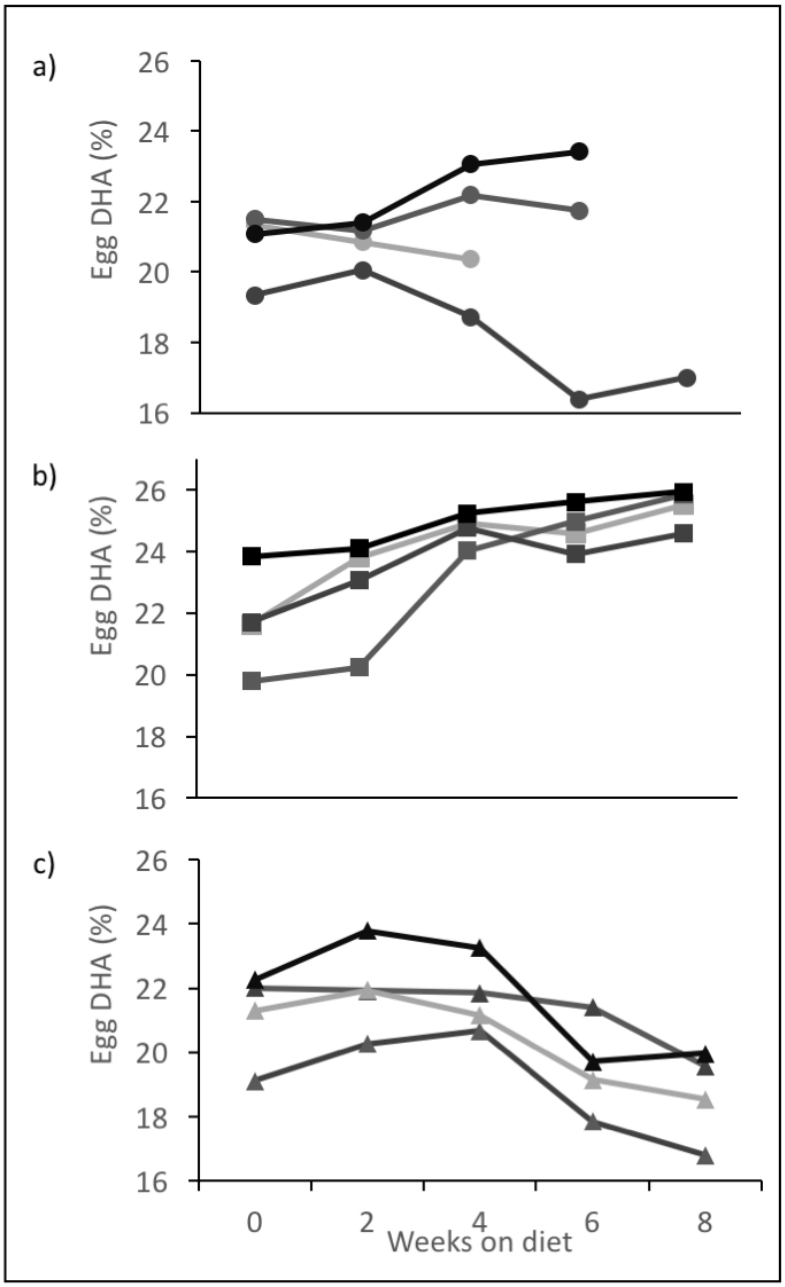

Figure 2.2. Change in proportion of DHA of eggs produced by each female through repeated spawning spanning 8 weeks. a) control diet; b) high DHA diet; c) low DHA diet.

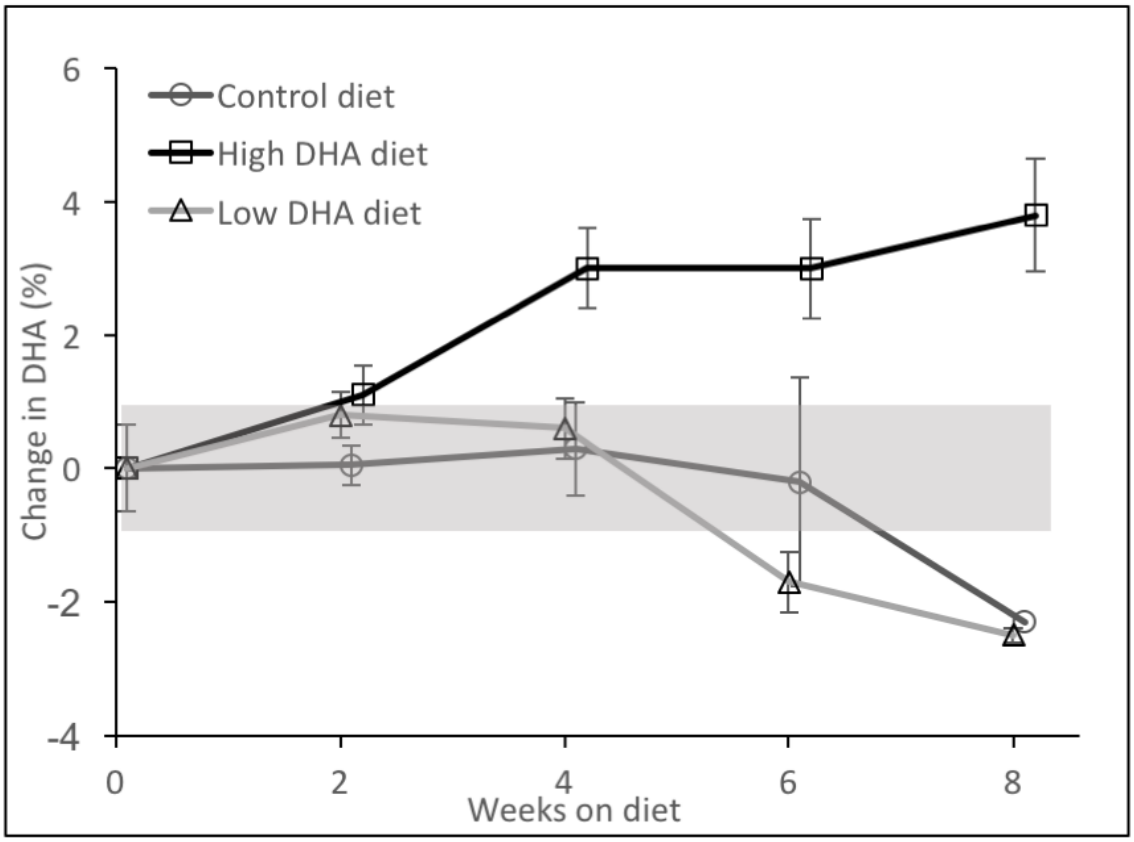

Figure 2.3. Mean change in proportion of DHA (mean ± S.E.) in eggs from week 0 value through repeated spawning (RS) spanning 8 weeks. Shaded area indicates 95% CI around week 0 mean proportion of DHA in eggs.

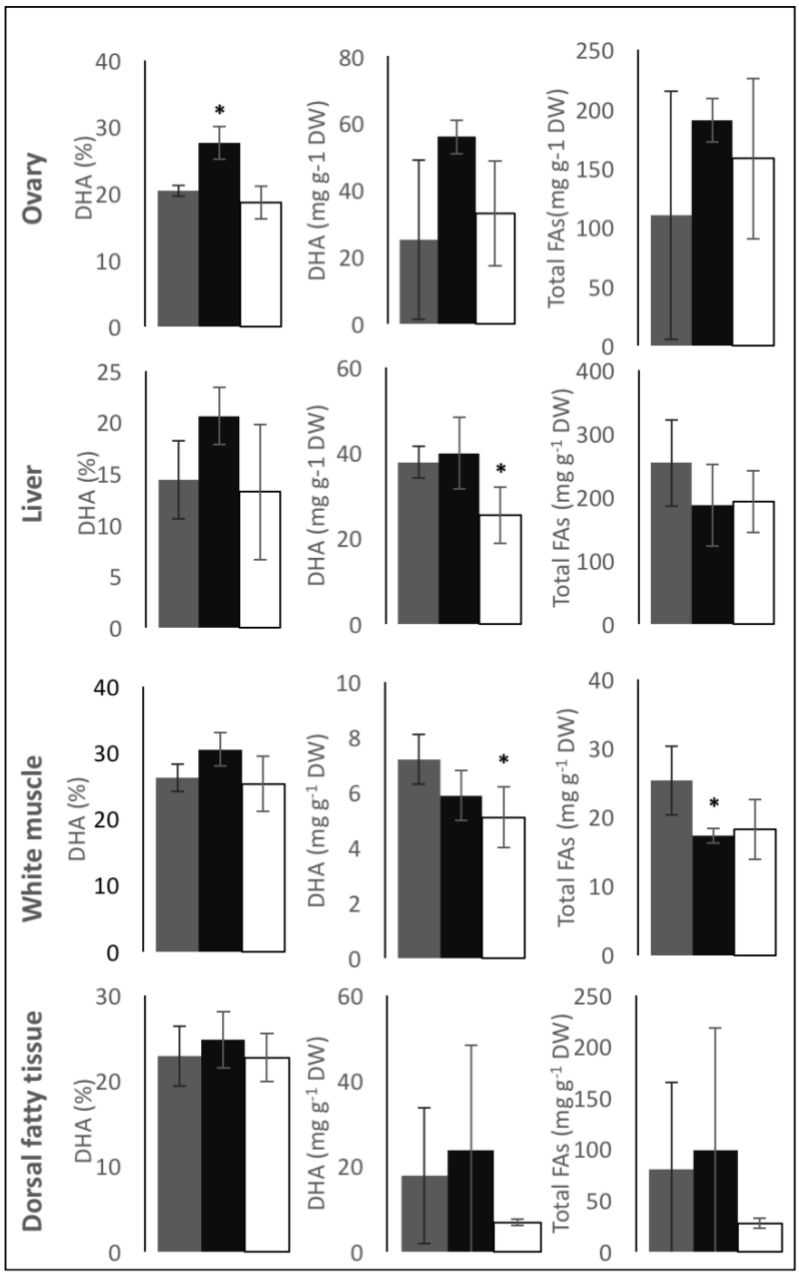

Figure 2.4. Fatty acid compositions (mean ± SD; n = 4 females per treatment) of ovary, liver, white muscle, and dorsal fatty tissue collected from repeatedly spawned females fed the ■ control, ■ high DHA diets, and □ low DHA diets. * denotes significant difference ($P < 0.05$) from the control diet treatment.

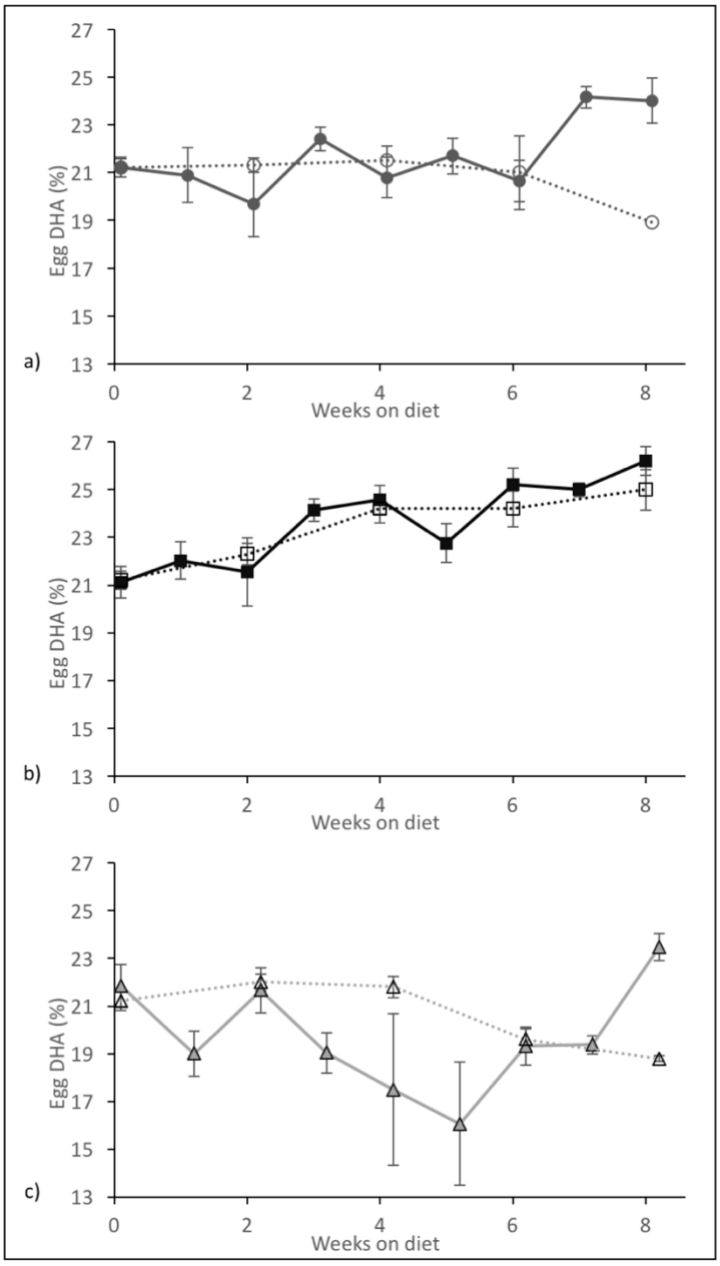

Figure 2.5. Comparison of trends in DHA content of eggs from first time (FT) and repeated spawning (RS) experiments. a) control diet; b) high DHA diet; and c) low DHA diet. Solid symbols and lines are for data from FT experiment; open symbols and broken lines are for data from RS experiment applied to the week 0 mean from the FT experiment.

Chapter 3: Maternally derived nutrients influence fatty acid composition and predator evasion behavior of larval southern flounder, *Paralichthys lethostigma*

INTRODUCTION

Marine fish eggs contain a nutrient-rich yolk that provides the developing embryo and newly-hatched larva all of its lipids, proteins, and carbohydrates before it begins to feed exogenously (Sargent 1995). These nutrients, or their precursors, ultimately derive from the maternal diet, which can differ substantially from the exogenous larval diet. Among the predominant nutrients in yolk are fatty acids, which are used by developing embryos and larvae for energy, hormone synthesis, and membrane structure. Some fatty acids, known as essential fatty acids (EFAs), must be obtained from the diet, because they cannot be biosynthesized within the body in physiologically relevant quantities. Three EFAs are especially important to embryos and larvae of marine fishes, because they are critical for proper larval development: docosahexaenoic acid (DHA, 22:6(n-3)), eicosapentaenoic acid (EPA, 20:5(n-3)), and arachidonic acid (ARA, 20:4(n-6)) (Sargent 1997).

Predation is the main source of mortality during the larval stage of marine fishes. In order to evade predation, a larva must be able to detect the predator and initiate an escape response (Fuiman and Magurran 1994). Previous research has shown correlations between performance related to predator evasion and EFAs in larval marine fishes. For example, at 21 days post-hatching (dph), larval red drum (*Sciaenops ocellatus*) that contained higher amounts of DHA in their body tissues were better able to detect and respond to a simulated looming predator than larvae that contained lower amounts of DHA (Perez and Fuiman 2015). There was also a correlation between the amount of DHA in the larval body of red drum at 21 dph and the amount of DHA in the egg,

suggesting a connection between embryonic nutrition and later larval performance (Perez and Fuiman 2015). In southern flounder, *Paralichthys lethostigma*, responsiveness of larvae to a visual predatory stimulus was positively correlated with the DHA:EPA ratio of the head as well as the amount of DHA in the larval diet (Oberg and Fuiman 2015). However, the influence of maternally-derived EFAs in the egg (embryonic nutrition) on larval performance in this species has not been explicitly measured.

Southern flounder are batch spawners and migrate from estuarine to offshore habitats to spawn. It is likely that during this migration southern flounder encounter different prey, resulting in a change in diet just before and during the spawning period. A previous study (Burns, Chapter 2) has shown that increases or decreases in the amount of DHA in the maternal diet at the beginning of a spawning season result in significantly different amounts of DHA in eggs and this change can occur within 3 weeks of a diet change. Therefore, changes in prey encountered by adult flounder during their spawning migration are likely to alter egg composition. Such variations in egg composition may affect performance and survival of larvae from those spawns, and larval quality may fluctuate within one spawning season.

The objective of this study was to determine whether fatty acid composition of eggs influences fatty acid composition of the larval body and predator evasion behavior in larval southern flounder. This objective was addressed by determining: (1) which fatty acids in the larval body correlate with predator evasion behavior; (2) whether there is a correlation between fatty acids in the egg and larval predator evasion behavior; and (3) if there is a correlation between fatty acids in the egg that affect predator evasion behavior and fatty acids in the larval body that affect predator evasion behavior.

METHODS

Animal care

Southern flounder broodstock were held at the Fisheries and Mariculture Laboratory of the University of Texas Marine Science Institute in Port Aransas, TX, USA in six recirculating tanks with controlled temperature (17.9 $\pm$ 0.7 C), salinity (31.2 $\pm$ 1.8 ppt), and photoperiod (10 h:14 h light:dark). Broodstock tanks were fed one of three dietary treatments to satiation in order to produce eggs with different fatty acid compositions – Spanish sardines (*Sardinella aurita*), brown shrimp (*Farfantepenaeus aztecus*), or an equal weight of both sardines and shrimp – over an 8-week experimental period starting at the end of January in 2016 and 2017 (Table 3.1). Naturally developed females were injected with Ovaprim (0.5 mL kg^{-1} wet weight of fish; Chemical, Inc., Ferndale, WA, USA), a synthetic hormone that induces ovulation, and strip spawned 48 h after injection. Eggs were immediately fertilized using freshly stripped milt. All spawns used for experiments were assumed to be the first spawns of the season by each female, because no eggs were observed in the tanks. Each female was tagged after being stripped for future identification.

Within 6 h of fertilization, 10 mL of eggs (approximately 14,000 eggs) from each spawn were placed into a 160-L conical tank. Larvae were reared under common garden conditions with constant temperature (17.8 $\pm$ 0.9 C), salinity (31.4 $\pm$ 1.8 ppt), and aeration for 35 days. Water changes were performed if ammonia levels exceeded 0.5 ppm. Larvae were fed once per day each morning, beginning at the onset of exogenous feeding (4 dph). Between 4 dph and 20 dph, larvae in each tank were fed live rotifers (*Brachionus plicatilis*) at a density of 5 ml^{-1}. Rotifers were enriched with a commercially available fatty acid emulsion, Algamac 3050 (Aqua-fauna Bio-Marine, Hawthorne, CA, USA), at the manufacturer's recommended concentration (0.2 g Algamac 3050 per

1,000,000 rotifers) for 45 min prior to feeding (Table 3.2). Between 20 dph and 25 dph, larvae were fed enriched rotifers and unenriched *Artemia* nauplii. At 25 dph, larvae were fed *Artemia* nauplii at a density of 0.01 ml^{-1} which had been enriched overnight with Algamac 3050 at a concentration of 0.3 g Algamac 3050 per 100,000 *Artemia* (Table 3.2). All larvae reared under these conditions differed only in the fatty acid composition of the eggs that produced them.

Larval quality

Behavioral performance was measured at two stages of development (15 dph and 35 dph) in two assays, routine swimming activity and visually-mediated escape response, following methods established by Fuiman and Ojanguren (2011) and Oberg and Fuiman (2015). For each tank of larvae at the designated ages, 15 individuals were transferred prior to daily feeding from the conical tank into individual acrylic testing chambers (4.1 x 4.1 x 5.6 cm for 15 dph larvae; 10.0 x 10.0 x 10.0 cm for 35 dph larvae) and allowed to acclimate in the chambers for at least 45 min. Individual chambers were then moved into a testing arena in the center of a square array of four liquid crystal displays (LCD) showing a white screen and allowed to acclimate for 3 min. The routine swimming assay was then conducted by recording the activity of the larva from above for 30 s at 30 frames per second (fps). Following this the escape response assay was conducted by triggering an animation of a black ellipse rapidly increasing in size on one of the LCD displays. The animation was intended to simulate the cross-section of an approaching predator. The response of the larva to the visual stimulus was recorded from above on high speed video at 240 fps. Frame-by-frame analyses of both assays were conducted using Image analysis software (ImageJ).

These two behavioral assays produced six measured traits for each larva. Using X-Y coordinates for the position of the fish's eyes at 1-s intervals, mean routine swimming speed (mm s^{-1}) and net:gross displacement ratio (net distance travelled divided by total distance travelled) were measured from the routine swimming assay. Net:gross displacement ratio is a measure of the linearity of the larva's swimming path, with values close to 1 indicating straighter paths. For the visual response assay, visual responsiveness was the proportion of larvae out of the 15 larvae tested from each spawn that responded to the visual stimulus. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin.

All individual larvae used in behavioral performance assays were pooled within spawns and analyzed for total body fatty acid composition using methods established by Faulk and Holt (2005). In 2017, 35-dph larvae used in behavioral performance assays were analyzed individually for individual fatty acid composition. Larvae were frozen at -80 C immediately after the behavioral assays. Later, they were freeze dried and homogenized in a chloroform/methanol solution for lipid extraction. Fatty acid methyl esters were collected by transesterification of total lipids using boron triflouride.

Responsiveness was recorded as the proportion of larvae that responded and an angular transformation (arcsin of the square root) was applied to establish normality. Principal component analyses (PCA) were computed separately for fatty acid composition of eggs, 15-dph larvae, and 35-dph larvae to summarize the major patterns of variation in each of these groups of samples. Pearson product-moment correlation coefficients (r) were calculated between mean behavioral trait values for each spawn and principal component factor scores. When a significant correlation was obtained, individual fatty acids that loaded heavily on that principal component ($|\text{loading}| \geq 0.70$)

were identified and correlations between individual fatty acids and behavioral traits were determined.

RESULTS

Larval quality and larval fatty acid composition

Behavioral traits of 15-dph (4.2 mm ± 0.03 standard length (SL); mean ± standard error [SE]) larvae from 15 spawns were measured. Principal component analysis of fatty acid composition of whole 15-dph larvae produced 6 principal components with eigenvalues > 1.0. The first 4 principal components explained 81.6% of the total variance, and these 4 principal components were used for the rest of the analyses (Table 3.3). There was a significant correlation between responsiveness to the visual stimulus and scores on principal component 1 ($r = -0.52$, $P = 0.047$, Figure 3.1a). Seven fatty acids and one fatty acid ratio loaded heavily on PC1: 18:1(n-7), DHA:ARA, Σ(n-3): Σ (n-6), 16:0, 18:1(n-9), 22:5(n-6), 18:2(n-6), and 20:3(n-6) (Table 4) Of these seven fatty acids, two were significantly correlated with larval responsiveness: DHA:ARA ($r = 0.65$, $P = 0.008$) and 18:2(n-6) ($r = -0.55$, $P = 0.034$) (Figure 3.1b, 3.1c).

Mean routine swimming speed was significantly correlated with scores on PC2 ($r = 0.57$, $P = 0.027$) in 15 dph larvae (Table 3.3, Figure 3.2). Only one fatty acid loaded heavily on PC2, 20:5(n-3) (EPA) but the relationship between mean swimming speed and EPA content of larvae was not significant ($r = 0.39$, $P = 0.151$ (Figure 3.1d). Net:gross displacement was not significantly correlated with any of the principal components that described fatty acid composition of 15 dph larvae.

Behavioral traits of 35-dph larvae (7.19 mm ± 0.07 SL) from 12 spawns were measured. Principal component analysis of fatty acid composition of whole 35-dph larvae produced 4 principal components with eigenvalues > 1.0. The first 3 principal

components explained 85.4% of the total variance, and these 3 principal components were used for the rest of the analyses (Table 3.4). There were no significant correlations between responsiveness of larvae to the visual stimulus and principal component scores. The strongest relationship was between responsiveness and PC1 ($r = -0.56$, $P = 0.058$) (Figure 3.2a). Eleven fatty acids and two fatty acid ratios loaded heavily on PC1, among them were DHA:EPA, and DHA (Table 3.5). Two fatty acids were significantly correlated with responsiveness at 35 dph: 18:3(n-4) ($r = -0.62$; $P = 0.031$) and 20:1(n-9) ($r = -0.59$; $P = 0.043$) (Figure 3.2b, 3.2c). Both of these fatty acids are minor components of the fatty acid profiles of 35-dph larvae (< 0.2% and < 0.8% of total fatty acids, respectively) for which analytical error could exaggerate their loadings in the principal component analysis. The strongest correlation between responsiveness and fatty acids that were present in larval tissues at > 1.0% of total fatty acids was with DHA:EPA ($r = 0.57$, $P = 0.053$) (Figure 3.2d). There were no significant correlations between mean routine swimming speed or net:gross displacement and any of the principal components that described fatty acid composition of larvae at 35 dph.

Larval quality and egg fatty acid composition

For both stages of larvae (15 and 35 dph), the amount of DHA in whole larvae, relative to two other essential fatty acids (ARA and EPA), was correlated with responsiveness, even though responsiveness at these ages was not significantly correlated with larval DHA alone ($r = 0.39$, $P = 0.151$ at 15 dph; $r = 0.53$, $P = 0.076$ at 35 dph). Analyses were conducted to determine whether DHA content of larvae was related to fatty acids in the egg. At 15 dph, mean larval DHA content was significantly correlated with concentrations of five fatty acids in the eggs that produced those larvae: 14:0 ($r = -0.68$, $P = 0.005$), 16:2(n-4) ($r = 0.53$, $P = 0.042$), 18:1(n-9) ($r = =0.58$, $P = 0.023$), 18:1(n-

7) ($r = -0.64$, $P = 0.010$), and 22:5(n-6) ($r = 0.71$, $P = 0.003$). Of these, 16:2(n-4) occurred at very low levels (< 1.0%) in eggs. In 35-dph larvae, mean DHA content was significantly correlated with three fatty acids in eggs: 18:3(n-6) ($r = -0.63$, $P = 0.028$), 20:1(n-9) ($r = 0.60$, $P = 0.039$), and 22:5(n-3) (n-3 DPA) ($r = -0.62$, $P = 0.032$, Figure 3.3b). Of these, only (n-3) DPA occurred in eggs in amounts greater than 1.0%.

DISCUSSION

Results from this study indicate that DHA content of larval tissues is important to escape responses and that DHA content of larval tissues is related to egg composition. The strongest correlations between responsiveness and larval fatty acid composition at both ages occurred with fatty acid ratios containing DHA. Levels of DHA are significantly greater in the head of southern flounder larvae than in the body, probably because DHA is concentrated in neural tissue, and there is a large amount of neural tissue in the head (Sargent 1993, Harel et al. 2000, Oberg and Fuiman 2015). DHA is especially concentrated in the retina (Sargent 1993). Thus, the positive relationship between responsiveness to a visual stimulus the amount of DHA relative to other EFAs, in southern flounder may be a result of improved visual function from higher amounts of DHA.

Fatty acid composition of 15-dph larval southern flounder, specifically DHA, is strongly influenced by the fatty acid composition of the larval diet, but results of this study suggest that it also may be influenced by embryonic nutrition. DHA content of larvae at both 15 and 35 dph was negatively associated with (n-3) DPA content of eggs (Figure 3.3). These correlations may be evidence of metabolic or nutritional programming, in which nutrition during early developmental stages has long term effects on metabolic pathways. Metabolic programming has been suggested in red drum, where

the amount of DHA in the egg influences the amount of DHA in the larval body at 21-dph (Perez and Fuiman 2015). Here, lower concentrations of (n-3) DPA in the yolk of southern flounder may facilitate uptake and accumulation of DHA in larval tissues. This, in turn, influences responsiveness of southern flounder to a predatory stimulus.

The role of (n-3) DPA in nutrition of marine fish larvae is not well known. Biochemically, (n-3) DPA is an intermediary in the anabolic and catabolic pathways between EPA and DHA. The enzyme Δ4 fatty acyl desaturase (from the FADS4 gene) adds a double bond to (n-3) DPA to create DHA along the anabolic pathway. Marine fishes and many vertebrates generally lack FADS4 and cannot efficiently create DHA in physiologically meaningful quantities (Li et al. 2010). Thus, (n-3) DPA is usually viewed as a product of DHA catabolism in marine fishes. Recent research on Senegalese sole (*Solea senegalensis*), however, demonstrated the presence of the FADS4 gene in the sole genome, as well as its expression and functionality by transforming (n-3) DPA into DHA (Morais et al. 2012, 2015). Therefore, FADS4 may be present and active in southern flounder, and the capacity for larvae to use the anabolic pathway to synthesize DHA may be programmed by (n-3) DPA content of the egg. Alternatively, it may be that the (n-3) DPA content of the egg programs the larva's capacity to breakdown DHA to DPA. For example, elevated levels of (n-3) DPA in the egg could heighten oxidation of DHA, resulting in lower levels of DHA in larval tissues.

It is interesting that the relationship between egg (n-3) DPA and larval DHA was stronger in larvae at 35 dph than at 15 dph. Differences in FADS4 transcription and Δ4 fatty acyl desaturase activity were only observed in late stage Senegalese sole larvae (Morais 2012). If metabolic programming alters FADS4 expression and enzyme production in southern flounder larvae, differences between ages may be due to developmental changes, in which the pathways altered by the early nutrition may not be

as active at 15 dph as they are at 35 dph. Another possible explanation for the difference in results between the ages is the larval diet. Enriched rotifers fed to 15-dph larvae contain higher amounts of DHA than enriched *Artemia* nauplii fed to 35-dph larvae. It is possible that larvae fed rotifers obtained more DHA from their diet and this was sufficient to mask the effects of metabolic programming. The amount of DHA in the *Artemia* diet may not have been sufficient for larval metabolic needs, where the effects of metabolic programming due to (n-3) DPA in the egg were observable.

Fatty acid	Broodstock diet	
	Spanish sardine	Brown shrimp
12:0	0.1 ± 0.1	0.1 ± 0.1
14:0	4.9 ± 0.9	2.0 ± 0.7
15:0	1.4 ± 0.2	1.3 ± 0.2
16:0	24.7 ± 1.4	13.2 ± 0.6
16:1(n-7)	4.3 ± 1.9	4.1 ± 0.9
16:2(n-4)	1.2 ± 0.1	0.5 ± 0.1
17:0	1.6 ± 0.1	2.1 ± 0.2
16:3(n-4)	0.3 ± 0.3	1.7 ± 0.3
18:0	7.0 ± 0.7	9.5 ± 1.3
18:1(n-9)	8.1 ± 2.1	6.3 ± 1.0
18:1(n-7)	2.8 ± 0.4	3.7 ± 1.0
18:2(n-6)	1.5 ± 0.1	1.6 ± 0.5
18:3(n-6)	0.7 ± 0.1	0.4 ± 0.1
18:3(n-4)	0.1 ± 0.1	0.1 ± 0.1
18:3(n-3)	0.6 ± 0.1	0.5 ± 0.3
18:4(n-3)	0.6 ± 0.2	0.1 ± 0.1
20:1(n-9)	0.3 ± 0.1	0.3 ± 0.1
20:2(n-6)	0.2 ± 0.1	1.0 ± 0.2
20:3(n-6)	0.4 ± 0.1	0.4 ± 0.1
20:4(n-6)	2.8 ± 0.4	7.8 ± 1.5
20:3(n-3)	0.1 ± 0.1	0.2 ± 0.1
20:4(n-3)	0.4 ± 0.1	0.3 ± 0.1
20:5(n-3)	5.9 ± 0.9	13.6 ± 1.7
22:5(n-6)	1.2 ± 0.2	1.0 ± 0.2
22:5(n-3)	0.8 ± 0.4	2.1 ± 0.2
22:6(n-3)	19.9 ± 3.3	10.4 ± 1.2

Table 3.1. Fatty acid composition (% of total fatty acids) of broodstock diet components.

Fatty acid	Larval diet	
	Rotifers	*Artemia*
12:0	0.1 ± 0.1	0.1 ± 0.1
14:0	4.7 ± 1.5	1.6 ± 0.1
15:0	0.5 ± 0.1	0.2 ± 0.1
16:0	18.3 ± 1.7	10.8 ± 0.5
16:1(n-7)	5.6 ± 3.0	1.9 ± 0.1
16:2(n-4)	0.2 ± 0.1	0.1 ± 0.1
17:0	0.4 ± 0.1	0.5 ± 0.1
16:3(n-4)	0.3 ± 0.3	0.5 ± 0.1
18:0	2.2 ± 0.3	3.4 ± 0.1
18:1(n-9)	2.6 ± 0.5	13.4 ± 0.1
18:1(n-7)	1.9 ± 0.5	5.2 ± 0.2
18:2(n-6)	5.5 ± 1.4	3.3 ± 2.2
18:3(n-6)	0.3 ± 0.1	0.3 ± 0.2
18:3(n-4)	0.1 ± 0.1	0.1 ± 0.1
18:3(n-3)	2.3 ± 0.8	21.0 ± 0.7
18:4(n-3)	0.3 ± 0.1	2.9 ± 0.2
20:1(n-9)	0.9 ± 0.4	0.4 ± 0.1
20:2(n-6)	0.4 ± 0.2	0.1 ± 0.1
20:3(n-6)	0.7 ± 0.1	0.2 ± 0.1
20:4(n-6)	2.3 ± 0.3	2.2 ± 0.1
20:3(n-3)	0.4 ± 0.2	0.5 ± 0.1
20:4(n-3)	1.1 ± 0.3	0.7 ± 0.1
20:5(n-3)	7.8 ±3.2	4.9 ± 0.2
22:5(n-6)	7.2 ± 1.3	4.7 ± 0.4
22:5(n-3)	2.3 ± 0.7	0.3 ± 0.1
22:6(n-3)	21.8 ± 4.2	13.3 ± 1.1

Table 3.2. Fatty acid composition (% of total fatty acids) of larval diets. Rotifers and *Artemia* were enriched using a fatty acid emulsion.

	Principal component			
Fatty acid	1	2	3	4
14:0	-0.630	0.433	-0.553	-0.085
15:0	0.284	-0.581	-0.615	-0.006
16:0	**0.816**	-0.311	0.072	-0.234
16:1(n-7)	0.566	0.538	-0.012	0.214
16:2(n-4)	-0.306	-0.687	-0.382	0.331
17:0	-0.497	-0.299	-0.588	0.048
16:3(n-4)	0.071	0.239	**-0.796**	0.245
18:0	0.642	-0.499	0.184	-0.485
18:1(n-9)	**0.805**	-0.139	-0.185	-0.194
18:1(n-7)	**0.876**	0.237	0.226	0.006
18:2(n-6)	**0.709**	0.588	-0.028	0.030
18:3(n-6)	0.150	-0.488	0.463	0.421
18:3(n-3)	-0.611	0.681	-0.197	-0.137
18:4(n-3)	-0.078	0.543	-0.170	-0.679
20:1(n-9)	0.666	0.242	0.201	-0.369
20:2(n-6)	-0.213	0.442	0.617	-0.336
20:3(n-6)	**0.704**	0.122	0.304	0.456
20:4(n-6)	0.539	0.361	0.130	0.596
20:3(n-3)	-0.682	0.496	0.160	-0.358
20:4(n-3)	-0.647	0.602	0.152	0.210
20:5(n-3)	0.264	**0.871**	-0.252	0.253
22:5(n-6)	**-0.751**	0.119	0.591	0.156
22:5(n-3)	0.678	0.600	-0.020	0.225
22:6(n-3)	-0.697	0.263	0.401	0.319
DHA:EPA	-0.479	-0.696	0.416	0.019
DHA:ARA	**-0.874**	0.006	0.181	-0.126
Σ (n-3): Σ (n-6)	**-0.854**	0.220	-0.275	0.092
Variance explained (%)	38.1	21.5	13.1	8.9

Table 3.3. Principal component loadings for fatty acid composition (% of total fatty acids) of 15-dph larvae (n = 15 spawns). Boldface type identifies fatty acids that load heavily on each principal component (|loading| > 0.700) and used for in correlations with responsiveness (PC1) and routine swimming speed (PC2).

	Principal component		
Fatty acid	1	2	3
14:0	0.350	0.619	0.442
15:0	-0.230	0.699	0.203
16:0	-0.581	-0.606	0.513
16:1(n-7)	**0.833**	-0.259	0.129
16:2(n-4)	-0.332	**-0.844**	0.193
17:0	**-0.710**	-0.191	0.289
16:3(n-4)	**-0.745**	-0.173	0.212
18:0	-0.536	**-0.753**	0.332
18:1(n-9)	0.693	**-0.701**	-0.092
18:1(n-7)	0.543	**-0.815**	0.008
18:2(n-6)	0.512	0.233	**0.781**
18:3(n-6)	**0.936**	0.166	-0.206
18:3(n-4)	**0.879**	-0.075	0.222
18:3(n-3)	**0.948**	0.010	-0.303
18:4(n-3)	**0.812**	0.349	-0.427
20:1(n-9)	**0.795**	-0.514	0.195
20:2(n-6)	0.111	-0.167	**0.700**
20:3(n-6)	0.157	**0.704**	0.251
20:4(n-6)	-0.590	0.643	0.346
20:3(n-3)	**0.943**	-0.120	-0.274
20:4(n-3)	0.686	0.662	-0.032
20:5(n-3)	0.640	0.650	0.284
22:5(n-6)	**-0.854**	0.366	-0.166
22:5(n-3)	0.376	0.579	0.673
22:6(n-3)	**-0.818**	0.497	-0.163
DHA:EPA	**-0.960**	0.061	-0.222
DHA:ARA	-0.545	0.245	-0.550
Σ(n-3): Σ(n-6)	**0.855**	0.050	-0.470
Variance explained (%)	48.2	23.8	13.4

Table 3.4. Principal component loadings for fatty acid composition (% of total fatty acids) of 35-dph larvae (n = 12 spawns). Boldface type identifies fatty acids that load heavily on each principal component (|loading| > 0.700).

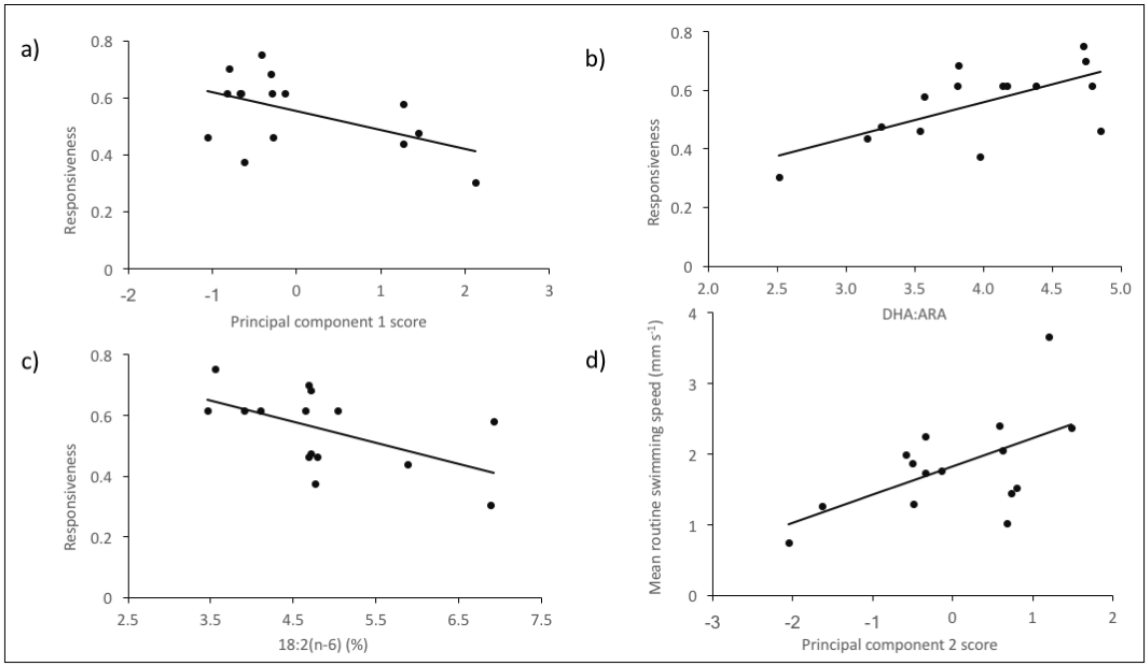

Figure 3.1. Relationships between responsiveness of 15-dph southern flounder larvae to a visual predator stimulus and fatty acids in their body. (a) Principal component 1 scores; (b) DHA:ARA; (c) 18:2(n-6). (d) Relationship between routine swimming speed and whole body fatty acid composition (PC2 scores) of 15-dph southern flounder larvae. Solid line denotes significant correlation ($P < 0.05$).

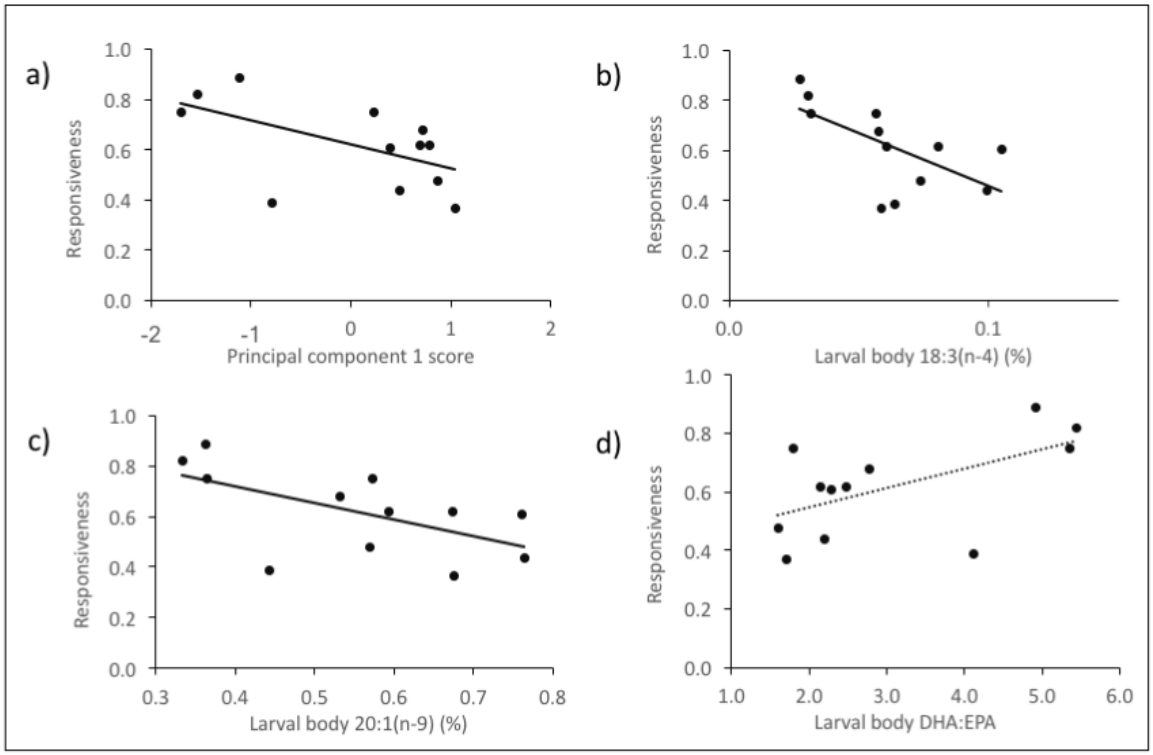

Figure 3.2. Relationships between responsiveness of 35-dph southern flounder larvae to a visual predator stimulus and fatty acids in their body. (a) Principal component 1 scores; (b) 18:3(n-4); (c) 20:1(n-9); (d) DHA:EPA. Solid line denotes significant correlation ($P < 0.05$); broken line indicates significant relationship ($P < 0.10$).

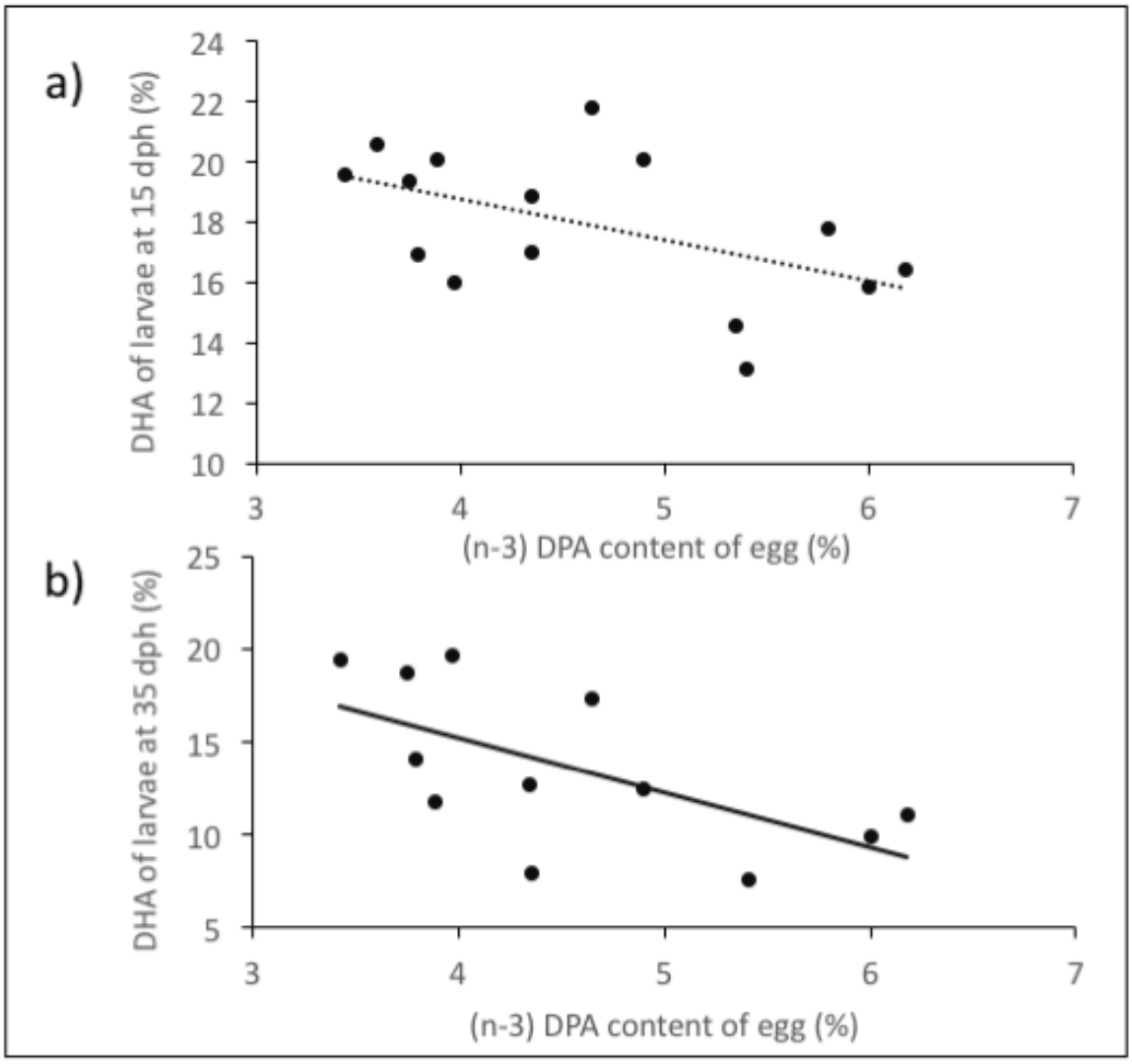

Figure 3.3. Relationships between (n-3) DPA content of eggs and DHA content of larvae at (a) 15 and (b) 35 dph. Solid trend line denotes significant correlation ($P < 0.05$); broken line denotes significant correlation ($P < 0.10$).

Chapter 4: Conclusions

The commonality throughout this research has been a focus on maternal effects and fatty acid composition of the eggs by addressing two broad questions: (1) How quickly are nutrients transferred from the maternal diet into the egg? and (2) What are the consequences of variable egg composition on offspring quality? This research demonstrates that southern flounder females use an income breeding strategy during the reproductive season, but under conditions where diet does not provide adequate nutrition, females can utilize a capital breeding or mixed breeding strategy and use stored reserves to build yolk. Docosahexaenoic acid (DHA) was used to trace nutrient transfer in these experiments because it is an essential fatty acid and cannot be efficiently biosynthesized within the body. Therefore, any DHA in the egg must have come from the maternal diet. Females fed low DHA diets during the experimental period had significantly lower amounts of DHA in white muscle tissues than females maintained on a control diet, suggesting that DHA was mobilized from white muscle. Interestingly, female flounder fed a diet high in DHA had significantly lower amounts of total fatty acids (and presumably total lipids) in white muscle tissue than females maintained on the control diet. This suggests that females fed a high DHA diet drew upon stored reserves of other fatty acids, such as arachidonic acid (ARA) or eicosapentaenoic acid (EPA), during vitellogenesis. These results suggest that the proportion of DHA in southern flounder eggs may change over one spawning season if there is a change in diet at the beginning of the spawning migration and that change is maintained over a period of time. Females switched to a high DHA diet produced eggs with significantly higher proportions of DHA than the week 0 mean after only 3 weeks.

Future work should examine preferred prey of southern flounder in both their estuarine (non-spawning) and offshore (spawning) environments in order to determine if there is a change in diet and how such a shift may affect egg composition. This experiment only looked at changes in DHA, but it would be important to observe the dynamics of other essential fatty acids, such as ARA and EPA, to see whether their proportions show high variability among diets or whether the proportions are highly conserved. Literature discussing the income–capital breeding continuum provides a final question to consider for interpretation of these results as well as for consideration of future work. The task of categorizing species as income breeders or capital breeders is "no longer as important as the question of to what extent endogenous stores are used in breeding, contributing quantitatively to breeding success." (Stephens et al. 2009).

Chapter 3 addressed how variability of fatty acid composition of the eggs can influence larval quality. Larval responsiveness to a simulated approaching predator at 15 and 35 dph was most strongly correlated with the ratio in the larval body of DHA to another essential fatty acid (ARA, and EPA, respectively). Differences in larval southern flounder DHA content has been shown to be a result of differences in the amount of dietary DHA in larval diet (Oberg and Fuiman 2015). All larvae in this experiment were raised under common garden conditions, therefore differences in larval body DHA were not due to dietary differences. The strongest correlations between larval body DHA at both 15 and 35 dph, and an individual fatty acid in the egg were with (n-3) docosapentaenoic acid (DPA). These results are evidence of metabolic programming in southern flounder. DPA is not often discussed in marine fish fatty acid literature, and was an unexpected result in this study. DPA is the intermediate fatty acid along the most direct biosynthesis pathway between EPA and DHA, and in the reverse catabolic pathway. Results from this study suggest a physiological connection between the amount

of (n-3) DPA in the egg and the amount of DHA in the larval body 4 weeks after yolk absorption. Most marine fishes cannot efficiently biosynthesize DHA from smaller chain fatty acids, such as (n-3) DPA and EPA, due to the lack of the *FADS4* gene that codes for the enzyme Δ4 fatty-acyl desaturase. It seems that the relationship between (n-3) DPA in the egg and larval body DHA might be due to changes in catabolic pathways that increase or decrease the breakdown of DHA that are set early in development. Recent work from Morais et al. (2011, 2015) has provided evidence of the *FADS4* gene and functional Δ4 fatty-acyl desaturase synthesis of DHA from DPA in Senegalese sole, another flatfish species. Future work on southern flounder should investigate whether *FADS4* is expressed in the genome and if so, whether Δ4 fatty-acyl desaturase is being produced.

This research shows how maternal diet can have trans-generational effects on offspring quality. Southern flounder act as income breeders, quickly transferring DHA, and more than likely other fatty acids, from the maternal diet into developing oocytes. If conditions become unfavorable, females can transfer fatty acids from somatic reserves in tissues such as the liver and white muscle, into future eggs. Variability in egg composition due to diet, primarily in DPA, can affect lipid metabolic pathways that alter amounts of larval body DHA weeks after the yolk has been absorbed. Differences in larval body DHA between spawns are associated with differences in the proportion of larvae that respond to a simulated visual predatory stimulus, and this suggests differential survival between these batches of larvae in the wild due to differences in mortality from predation.

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