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Linking fatty acids in the diet and tissues to quality of larval southern flounder (*Paralichthys lethostigma*)

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Linking fatty acids in the diet and tissues to quality of larval southern flounder (*Paralichthys lethostigma*)

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Dedication

To my wife Linda and parents Roger and Rosie for their constant love and support.

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Abstract

Linking fatty acids in the diet and tissues to quality of larval southern flounder (*Paralichthys lethostigma*)

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Essential fatty acids are necessary for growth, survival, and development of larval fishes, but there is limited information on the essential fatty acid requirements of larval southern flounder (*Paralichthys lethostigma*). The objectives of this study were to elucidate connections between dietary supply of docosahexaenoic acid (DHA) and arachidonic acid (ARA) and deposited fatty acids in the head or body, and then link diet and stored fatty acids in the head or body with larval quality traits. From 4-15 days posthatch (dph), southern flounder larvae were fed rotifers enriched with four different combinations of DHA-rich Algamac 3050 and ARA-rich Algamac ARA. Fatty acid concentrations in the head and body were measured at 15 dph, and relationships between fatty acids in head or body and in the diet were determined. Larval quality traits, including specific growth rate (SGR), survival, and eight behavioral performance variables were measured. Results showed that concentrations of DHA and ARA in the head and in the body were correlated with concentrations of DHA

and ARA in the diet. Growth rate did not vary among the four diets, but survival was positively correlated with the amount of lipid in the diet. Responsiveness to a visual stimulus was positively correlated with the concentration of DHA in the diet, the ratio DHA:EPA in the head, and total energy content of the diet. Turning rate during routine swimming was correlated with body DHA. This study demonstrates the influence of DHA content, total lipid content, and energy levels in the diet of southern flounder and provides a foundation for future studies examining causal factors of recruitment variability or larviculture production success.

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Chapter 1: Larval fish nutrition and southern flounder

In marine environments, high fecundity of fishes is coupled with high mortality during the early life stages (Houde, 2002). Furthermore, despite the absence of many of the adverse conditions that larvae are exposed to in nature, production of an adequate supply of high quality larvae is an impediment to aquaculture (Holt, 2011). In both cases, nutrition is one determinant of mortality, however, nutritional information for larval fishes is limited (Houde, 2002; Holt, 2011). Accordingly, understanding how nutrients, such as fatty acids, influence growth, survival, development, and performance for different species is important for developing a greater understanding of factors that influence mortality in nature and optimizing larval diets in aquaculture.

Fatty acids are saturated or unsaturated carbon chains ending with a carboxylic acid group (Gropper and Smith, 2012). The most important fatty acids are the essential fatty acids (EFA), those that cannot be synthesized internally in sufficient quantities to meet physiological demands and, therefore, must be acquired through the diet (Sargent et al., 1997). Some fatty acids are essential because of the inability of certain organisms to desaturate specific points of the carbon chain. For example, no vertebrates are able to desaturate the third or sixth carbon of 18-carbon fatty acids (Tocher, 2003). As a result, for humans and fish alike, the ω-6 fatty acid 18:2ω6 (meaning an 18-carbon chain with 2 double bonds, the first being on the 6^{th} carbon) and the ω-3 fatty acid 18:3ω3 are considered EFAs (Tocher, 2003; Gropper and Smith, 2012). After consuming 18:2ω6 or 18:3ω3, many vertebrates can build on these structures to create longer chain ω-6 or ω-3 fatty acids. Marine

fishes in general, however, lack the ability to elongate these fatty acids, presumably an evolutionary consequence of readily available ω-6 or ω-3 fatty acids in marine food webs (Brett and Müller-Navarra, 1997; Tocher, 2003).

Highly unsaturated fatty acids (HUFAs) are a subset of EFAs which have three or more double bonds on their carbon chain (Tocher, 2003). HUFAs are important constituents of cell membranes and biologically active compounds that play significant roles in numerous physiological processes (Izquierdo and Koven, 2011). Docosahexaenoic acid (DHA) is one HUFA that is especially well known because of its established importance to the health of fishes and mammals and general deficiency in the western diet of humans (Connor, 2000; Gropper and Smith, 2012). DHA is incorporated into membranes throughout an organism's body, but is most often associated with brain and retina development. DHA deficiency has been shown to cause visual impairment in rhesus monkeys as well as Atlantic herring (*Clupea harengus* L.) (Neuringer et al., 1984; Bell et al., 1995) and to adversely affect the response to visual stimuli in gilthead sea bream (*Sparus aurata*) (Benítez-Santana et al., 2007). In addition, the HUFAs arachidonic acid (ARA) and eicosapentaenoic acid (EPA), along with DHA, are precursors of eicosanoids, which are locally produced in cells to regulate processes such as neural function, inflammatory response, and immune response (Tocher, 2003). Through the eicosanoid pathway, ARA is known to promote inflammation, and higher ARA concentrations are believed to act as a stress-alleviating mediator in small rearing tanks (Harel et al., 2002) or adverse environmental conditions (Connor, 2000; Bell and Sargent, 2003). Multiple studies have shown correlations between dietary ARA levels and growth and survival (Harel et al., 2000; Koven et al., 2001;

Bell and Sargent, 2003). It is also important to recognize that the efficacy of DHA and ARA can be modulated by EPA as well as each other and therefore these fatty acids are frequently analyzed and discussed as ratios of one another (Sargent et al., 1999; Harel et al., 2000).

Southern flounder, *Paralichthys lethostigma*, populations support an economically important commercial and recreational fishery (Wenner and Archambault, 2005). A steady decline in recreational and commercial fishing harvests along the Texas coast from the mid-1980's through 2007 has increased interest in the health of wild populations and in larviculture to support stock enhancement (Froeschke et al., 2011). But, very little nutritional information beyond what is known for other larval fish is available for southern flounder larvae. The influence of EFAs in the diet on larval mortality in nature cannot be ascertained without this knowledge. In addition, lack of nutritional information may be limiting the success of the current flounder stock-enhancement program. To address this shortfall of knowledge, the influence of EFAs on southern flounder growth, survival, and development needs to be investigated.

Broadly, the goal of the research described in this thesis is to examine the relationships between EFAs in larval tissues and those provided in diet as well as relationships between larval quality traits (growth, survival, and behavioral performance) and EFAs in diet or tissues. By examining relationships between quality traits and fatty acids in the head and body, this study determines the linkages through which fatty acids influence larval performance. This new knowledge of the early life history of southern flounder may improve future assessments of mortality in the wild and it may help in the development of optimized diets for larviculture.

Chapter 2: Linking fatty acids in the diet and tissues to quality of larval southern flounder (*Paralichthys lethostigma*)

INTRODUCTION

Mortality is high during the larval period of most marine fishes, both in nature and in aquaculture, and nutrition is one contributing factor. Essential fatty acids (EFA), those that cannot be synthesized by an organism in sufficient quantities to meet metabolic needs, are a nutritional component that is important for structural development, health, and sensory function in larval marine fishes (Izquierdo, 1996). For most marine fishes, highly unsaturated fatty acids (HUFAs; fatty acids with carbon chain lengths $\geq$ 20 and at least 3 double bonds), such as arachidonic acid (20:4ω6, ARA), eicosapentaenoic acid (20:5ω3, EPA), and docosahexaenoic acid (22:6ω3, DHA), are considered essential (Sargent et al., 1999; Tocher, 2003). Concentrations of DHA, ARA, and EPA, individually, as well as their ratios, are related to various measures of larval quality. Ratios are important because one of these EFAs can regulate the efficacy of another (Koven et al., 2001; Izquierdo and Koven, 2011).

While nutritional information is relevant both ecologically and practically for aquaculture, knowledge of the influence of important nutrients, such as essential fatty acids, on the early life stages is lacking for many species. One such fish is the southern flounder (*Paralichthys lethostigma*), an important commercial and recreational food fish found along the mid-Atlantic Coast and the northern Gulf of Mexico. Improved understanding of its nutritional requirements is important because the species is a candidate for commercial

aquaculture and stock-enhancement programs that seek to halt long term declines in wild populations (Miller et al., 2010; Froeschke et al., 2011).

Marine heterotrophs typically store fatty acids with carbon-chain lengths of at least 14 in their tissues at concentrations that are proportional to those of their diet (Cook, 1991; Iverson et al., 2004). When fatty acids are used as an energy source, saturated and monounsaturated fatty acids are used preferentially over EFAs (Wiegand, 1996; Copeman et al., 2002). Of the stored fatty acids, some are conserved preferentially and incorporated into specific structures where they play critical roles in physiological function (Copeman et al., 2002). For example, retinal tissue has an abundance of DHA in polar lipids (Navarro et al., 1997; Sargent et al., 1999).

While accumulation of fatty acids in particular tissues implies a role in organismal function, several studies have found relationships between dietary intake of EFAs and specific larval quality traits. Growth and survival were positively correlated with EFAs in the diet of turbot (*Scophthalmus maximus*), gilthead sea bream (*Sparus auratus*), and Atlantic cod (*Gadus morhua*) (Castell et al., 1994; Bessonart et al., 1999; Galloway et al., 1999). Behavioral studies have shown that DHA-deficient diets reduced visual feeding by herring (*Clupea harengus*) in low light conditions (Bell et al., 1995), delayed the appearance of a response to visual stimuli in gilthead sea bream (Benítez-Santana et al., 2007), and increased escape swimming speed in response to sound stimuli in gilthead sea bream (Benítez-Santana et al., 2014), suggesting the influence of DHA on sensory systems. Growth and responsiveness to visual stimuli are especially valuable quality indicators because they are ecologically relevant (Fuiman et al., 2006).

The goal of this study was to determine how head and body tissue composition and quality of southern flounder larvae varied under different dietary levels of DHA and ARA. Tissue concentrations of EFAs were expected to be correlated with diet concentrations. It was hypothesized that individual EFAs accumulate in tissues (head or body) in direct proportion to their concentration in the diet, and that concentrations of DHA would be higher in the head than in the body because of their association with neural tissue and the greater amount of neural tissue in the head. For a better indication of the functional role of fatty acids, larval quality traits (growth, survival, and behavioral performance) were compared with concentrations of fatty acids in the diet and head and body tissues. Concentrations of EFAs in the diet and in the head and body were hypothesized to be positively correlated with larval quality traits.

METHOD

Animal care

Viable southern flounder eggs were acquired from three individuals of a captive broodstock population (n=55) maintained in a 36 kL recirculating aquaculture system with controlled temperature (18-20 °C), salinity (33-35 ppt), and photoperiod (10 light: 14 dark) at the Fisheries and Mariculture Laboratory of the University of Texas Marine Science Institute in Port Aransas, Texas. The broodstock were collected from the Lydia Ann Ship Channel near Port Aransas, TX, and maintained in captivity for > 2 years. Beginning one month before the start of the project, the broodstock were fed three times per week approximately 400 g of Spanish sardines (*Sardinella aurita*) and 400 g of shrimp (*Litopenaeus setiferus* or

Farfantepenaeus aztecus). Both food items were previously frozen and wild caught from the Gulf of Mexico. Three separate spawns from different males and females served as replicates. Females were strip spawned 48 h following injection (0.5 ml kg^{-1} wet weight of fish) with Ovaprim (Western Chemical, Inc., Ferndale, WA), a synthetic hormone and dopamine inhibitor that stimulates spawning.

Between 24 and 30 h after fertilization, approximately 2,800 eggs (volumetrically determined, approximately 2 ml) were placed in each of four, 60-L conical tanks. Larvae were reared under identical conditions of constant salinity (33.5 ± 0.5 ppt), temperature (17.9 ± 0.9 °C), photoperiod (12h light: 12h dark), and aeration (no filtration or water exchange) for 15 days. The four tanks raised from each spawn differed only in the diet they received. At the onset of first feeding (4 days post-hatching [dph]) through 15 dph, larvae in each tank were fed one of four live prey diets (rotifers, *Brachionus plicatilis*) fed at 5 ml^{-1}. Rotifers were enriched for 12 h using different combinations of two commercially available lipid emulsions (Algamac 3050 and Algamac ARA; Aqua-fauna Bio-Marine, Hawthorn, CA, USA). The enrichment combinations were selected to produce diet fatty acid profiles in which concentrations of DHA and ARA varied widely (Table 2.1), while minimizing differences in energy content and proximate composition. Diets are identified as A, B, C, and D in order of increasing DHA:ARA content and were prepared per million rotifers as follows: Diet A was 0.18 g Algamac 3050 + 0.12 g Algamac ARA; Diet B was 0.24 g Algamac 3050 + 0.06 g Algamac ARA; Diet C was 0.03 g Algamac 3050; and Diet D was 0.3 g Algamac 3050 (manufacturers recommended amount).

Larval quality traits

Larval quality was assessed by measuring growth, survival, and behavioral performance (routine swimming and escape response to a visual stimulus) on 15-dph larvae. To determine growth, 30 larvae were randomly selected at 5 dph and at 15 dph from each treatment in each replicate and photographed under a dissecting microscope for subsequent measurement of standard length (SL, in mm) using image analysis (ImageJ, National Institute of Health, Bethesda, MD, USA). Daily specific growth rate (SGR, % d^{-1}) was calculated for the 10-day period as: $SGR = (\ln(SL_{15})-\ln(SL_5))/10 \cdot 100$, where ln is the natural logarithm, SL_{15} is the mean SL on 15 dph, SL_5 is the mean SL on 5 dph, and 10 is the number of days of growth measured (Ricker, 1979).

To estimate survival, larvae in the rearing tanks for each diet were completely mixed using high aeration, and larvae were counted in five, 0.5-L subsamples from each tank at 5 dph. The mean of these five values was used to estimate the number of fish in the entire 60-L tank. At 15 dph, all surviving larvae were counted in each tank and survival (%) was determined.

Behavioral performance was measured in two assays, routine activity and visual escape response, following established methods (Fuiman and Ojanguren, 2011). To conduct these trials, 24 randomly selected larvae from each replicate were gently transferred from their rearing tank to individual acrylic testing chambers (4.1 x 4.1 x 5.6 cm) that had white walls on three sides and the bottom, but a transparent fourth wall. Larvae were allowed to acclimate in the chamber for 30 min before the chamber was moved to the testing arena, where the transparent wall was in front of a liquid crystal display (LCD) showing a white

screen. After 3 min of acclimation, the routine swimming assay was conducted by recording routine activity of the fish for 34 s at 30 frames per second (fps). Following routine swimming, an animation of a black ellipse that increased in size was played on the LCD to simulate the silhouette of a rapidly approaching predator, and larval escape behavior was recorded on high speed video at 240 fps. Subsequent frame-by-frame analysis was conducted using image analysis software (ImageJ).

These assays produced eight measures of behavioral performance for each fish. Visual responsiveness was the proportion of larvae of the 24 larvae in each replicate for each diet that responded to the visual stimulus, response latency was the time (in milliseconds) between the start of the visual stimulus and start of the escape response. Using X-Y coordinates for the position of the fish's snout and the number of video frames in the response, response distance (mm), mean response speed (mm s^{-1}), maximum response speed (mm s^{-1}), and response duration (s) were determined for each escape response. Mean routine swimming speed (mm s^{-1}) and turning rate (degrees s^{-1}) were calculated in a similar way from the routine swimming assay. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Analyses

Levels of fatty acids (mg g^{-1} dry weight [DW]), energy content (kJ g^{-1} DW), and crude lipid (% DW) were measured for six batches of enriched rotifers for each diet (Table 2.1). Each sample of enriched rotifer was filtered with a 50-μm sieve, thoroughly rinsed with distilled water, stored at -80 °C, and then freeze dried to a constant weight overnight prior to

analysis. Because enrichments contained other fatty acids in addition to DHA and ARA, 26 fatty acids were analyzed according to the method described by Faulk and Holt (2005) using a Shimadzu GC-2014 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Supelcowax 10 capillary column (30 m long, 0.32 mm internal diameter, 0.5 μm thickness; Supelco, Bellefonte, PA, USA). Fatty acid composition of each diet was characterized by the mean (mg g^{-1} DW) of the six measurements for each of 26 fatty acids and three calculated ratios of fatty acids (ARA:EPA, DHA:ARA, and DHA:EPA).

Energy content was measured for three batches of enriched rotifers for each diet using a Parr 6200 oxygen bomb calorimeter with a model 1109A semi-micro oxygen combustion bomb previously calibrated with 0.2 g benzoic acid pellets (Parr Instrument Company, Moline, IL, USA). Dry rotifer samples were 0.045 – 0.067 g and were pressed into a pellet before they were loaded into the bomb for analysis. To determine crude lipid, total lipids were extracted from three batches of enriched rotifers for each diet by homogenizing the dried rotifers in chloroform/methanol (2:1, v/v) and the crude lipid extract was measured gravimetrically, following evaporation of the solvent under nitrogen (Faulk and Holt, 2003).

After conducting the behavioral assays (15 dph), 50 larvae per diet from each replicate were euthanized with an overdose of tricaine methanesulfonate (0.3 mg ml^{-1}) and the heads and bodies were carefully separated and pooled for fatty acid analysis following the method of Estevez et al. (1997). By measuring fatty acid concentrations in the head and body separately, comparisons between the two sections of the body could be made to determine whether fatty acids are preferentially incorporated into sensory structures (head)

versus locomotor and circulatory structures (body). Given the minute size of the larvae, finer micro-dissection was not possible without contaminating samples from nearby tissues. Samples were rinsed with distilled water, stored at -80 °C, and then freeze dried prior to lipid extraction and fatty acid analysis. Total lipid content was extracted and fatty acids were analyzed as described for the diets, except that values were measured as % of total fatty acids due to the inability to measure initial dry weights of the small samples.

To determine the relationship between the amount of each fatty acid in the diet and the amount of the same fatty acid in the head and body, Pearson product-moment correlation coefficients (r) were computed for each fatty acid and ratio. Assessment of relationships between the diet and behavioral traits and between head and body tissues and behavioral traits began with principal component analysis (PCA). Separate PCAs, with varimax rotation, were computed for the fatty acid composition of the diets, head tissue, and body tissue to reduce the dimensions of the fatty acid profiles (fatty acids and ratios) and the number of statistical tests required to assess relationships between fatty acids and larval quality traits. The PCA on the diets was also used to determine the degree to which other fatty acids correlated with DHA and ARA, which would complicate interpretation of the results. Principal components were retained until the cumulative percentage of variance explained was ≥ 90% (Mardia et al., 1979). Then, correlations were computed between the mean principal component scores for diets and quality traits of individual larvae.

Correlations were computed for head fatty acid composition and body fatty acid composition principal component scores and all quality traits except for survival, since only larvae that survived to 15 dph were measured. If a principal component was significantly

correlated with a quality trait, correlations between variables (fatty acids and ratios) that had loadings > 0.7 or < -0.7 (Tabachnick and Fidell, 1989; McGarigal et al., 2000) and the same trait were determined. Correlations were also computed between quality traits and crude lipid and total energy. In all cases, $P < 0.05$ was considered statistically significant. Statistical analyses were completed using R software version 3.1.0 (R Development Core Team, 2014).

RESULTS

At 15 dph, when behavioral performance and tissue analysis were performed larvae were 3.4 ± 0.11 mm SL (mean ± standard error [SE]) and had an SGR of 13.4 ± 0.37 % d^{-1} (mean ± SE). Neither SL nor SGR varied significantly among diet. Therefore, they were not included in further analyses.

Four fatty acid variables (DHA, ARA, ARA:EPA, DHA:ARA, and 18:3ω6 showed significant correlations between levels in the head (Table 2.2) and levels in the diet ($r = 0.67$ - 0.79, Table 2.3, Figure 2.1). For the body tissues (Table 2.2), DHA, ARA, ARA:EPA, DHA:ARA, and 22:5ω6 showed significant correlations with their concentrations in the diet (Table 2.2, $r = 0.62$ - 0.92, Figure 2.1). DHA:ARA was significantly higher in the head than the body, but there were no significant differences in DHA, ARA, or ARA:EPA between the head and body.

PCA characterized 94% of the variation in fatty acid composition of the diets with three principal components, which explained 50%, 27%, and 17% of the total variance, respectively (Figure 2.2). Each of the first three components had a single high loading: DHA for PC1 (loading = -0.99), ARA for PC2 (0.99), and 16:0 for PC3 (0.99). All other loadings

on these axes were between -0.1 and 0.1. The only significant relationship between the principal component scores and any of the larval quality traits was for visual responsiveness, which was highly correlated with PC1 ($r = -0.71$, $P = 0.01$). DHA, which loaded heavily on PC1, was highly correlated with visual responsiveness ($r = 0.71$, $P = 0.01$, Figure 2.3a). Visual responsiveness was also correlated with energy content of the diet ($r = 0.66$, $P = 0.02$, Figure 2.3b). The only other significant relationship between diet composition and larval quality was the correlation between crude lipid content of the diet and larval survival ($r = 0.72$, $P = 0.01$, Figure 2.3c).

The first three components of another PCA characterized 94% of the variance in fatty acid composition of head tissue (55%, 28%, and 11%, respectively, Figure 2.4). Each of the first three components had a single high loading: DHA for PC1 (loading = 0.98), DHA:EPA for PC2 (0.94), and 16:0 for PC3 (-0.98). PC2 for head tissue was significantly correlated with visual responsiveness ($r = 0.73$, $P = 0.01$). Therefore, visual responsiveness was also highly correlated with head DHA:EPA ($r = 0.73$, $P = 0.01$, Figure 2.5).

Four principal components explained 92% of the variance in fatty acid composition of the body (57%, 15%, 12%, and 8%, respectively, Figure 2.6). Each of the first four components had a single high loading: DHA for PC1 (loading = -0.96), DHA:EPA for PC2 (-0.92), ARA for PC3 (-0.75), and 16:0 for PC4 (-0.97). Turning rate during routine swimming was correlated with PC1 ($r = -0.61$, $P = 0.03$) and with DHA ($r = 0.61$, $P = 0.03$, Figure 2.7a). Visual responsiveness was correlated with PC4 ($r = 0.58$, $P = 0.047$) and 16:0 ($r = -0.58$, $P = 0.046$, Figure 2.7b).

DISCUSSION

The results of this study show that larval southern flounder accumulate DHA and ARA (and a few other fatty acids and ratios of fatty acids) in the head and body in proportion to concentrations of DHA and ARA in their diet, at least over the range of concentrations used in the experiment. Furthermore, there were significant relationships between certain larval quality traits (visual responsiveness and turning rate during routine swimming) and concentrations of some fatty acids in the diet, head, and body. In addition, survival was related to lipid content of the diet. The dominant feature of the results is the presence of DHA in almost every connection between the diet, tissues, and larval quality (Figure 2.8). In contrast, ARA is present in the links between the diet and tissues, but it is not linked to any of the measures of larval quality.

As hypothesized, tissue levels of DHA and ARA were positively correlated with diet levels. Several other fatty acids (e.g. 18:0, 18:2ω6, 20:3ω6) varied appreciably among the diets (see Table 2.1), but were not correlated with tissue levels (except for 18:3ω6 in the head and 22:5ω6 in the body). This lack of correlation between diet and tissues could have various causes. Those fatty acids could have passed through the digestive tract unabsorbed, catabolized, excreted when in excess, or synthesized from precursors when deficient. Correlations for DHA and ARA and lack of correlations for other fatty acids reinforces that DHA and ARA is acquired from the diet and preferentially deposited in tissues relative to the amount supplied.

The presence of higher levels of DHA:ARA in the head of flounder larvae, compared with levels in the body is consistent with previous research that has shown that

DHA is concentrated in neural tissues in other species. Concentrations of DHA in the brain and eyes are higher than concentrations of ARA in the same tissues of Atlantic herring (*Clupea harengus* L.) and white bass (*Morone chrysops*) fed diets supplemented with DHA (Bell et al., 1995; Harel et al., 2000). These higher levels of DHA:ARA are specifically in the structural lipids of the brain synaptic membranes and rod photoreceptor outer segments (Harel et al., 2000).

We also found that DHA plays a role in visual function. Responsiveness to the visual stimulus was positively correlated with DHA in the diet and with DHA:EPA in the head (Figure 2.8). Previous research suggests that DHA in retinal tissues increases fluidity of the rod cell membranes and improves visual acuity (Brown, 1994). DHA also activates retinoid X receptors, a nuclear hormone receptor important to retina development and neural function (Urquiza et al., 2000). Further, DHA utilization has been shown to be modulated by EPA (Izquierdo, 1996). Relationships revealed by the experiments on southern flounder larvae are consistent with visual responsiveness being regulated by DHA:EPA in the head (probably the retina) which is, in turn, is controlled by dietary intake of DHA. Since concentrations of EPA (20:5ω3) did not vary substantially among the diets used in this study, it is not possible to determine the sensitivity of this pathway to DHA:EPA. This may be difficult to test because of the abundance of EPA in diets typically fed to marine fish larvae.

In addition to its effect on sensory systems, DHA probably affects motor skills through the central nervous system. The positive correlation between DHA in the body and turning rate could be due DHA concentrations in the spinal cord and an effect of DHA on

neural control of movement within the brain and spinal cord, in agreement with previous studies (Masuda et al., 1999; Benítez-Santana et al., 2014). DHA concentrations in the whole body tissue of gilthead sea bream larvae increased burst swimming speed by increasing activity of the neurons that initiate burst swimming (Benítez-Santana et al., 2014). In addition, a DHA-deficient diet impaired schooling in larval yellowtail (*Seriola quinqueradiata*; Masuda et al., 1998). Visual skills required for schooling were tested and determined to be functional and therefore impairment of schooling was not due to effects of DHA on vision (Masuda et al., 1999). Instead, impairment was attributed to an effect of DHA on neural control of movement because, aside from the eyes, DHA was most concentrated in the brain and spinal cord (Masuda et al., 1999).

Visual responsiveness was also influenced by other factors, including energy content of the diet. Dietary energy fuels activity, but also growth (Daniels and Robinson, 1986). The correlation between visual responsiveness and energy content of the diet could indicate the effect of having more energy available to make an escape response or it could reflect differences in development induced by more available energy throughout the 11 days of feeding. Alternatively, it is possible the relationship is an artifact of the relationship between DHA and visual responsiveness because the diet that was most different in energy had the lowest DHA.

Results also showed that higher concentrations of the saturated fatty acid 16:0 in body tissues were negatively correlated with visual responsiveness. This fatty acid was abundant in all four diets, averaging 28 to 35% of total fatty acids, but there is no known mechanism to explain its apparent effect on visual responsiveness. Levels of 16:0 in the body

were negatively correlated with DHA in the body ($r = -0.78$, $P = 0.047$). Although the correlation between DHA in the body and visual responsiveness was not significant based on an experiment replicated three times ($r = 0.28$, $P = 0.38$), it is possible that with more replication DHA in the body could be shown to have a significant effect on visual responsiveness, just as DHA in the head was found to be implicated in visual responsiveness. Consequentially, the negative correlation between 16:0 in the body and visual responsiveness may be spurious.

Survival is an important concern for larval rearing, and this study found that the amount of crude lipid in the diet was correlated with survival. This is similar to other studies that showed that survival increases with crude lipid up to an asymptote (Chou et al., 2001; Zheng et al., 2010). Interestingly, we found no relationship between survival and levels of individual fatty acids in the diet despite previous research suggesting that regulation of stress by essential fatty acids could affect survival (Watanabe 1993, Izquierdo 2005). This lack of a relationship may indicate that even the diet with the lowest EFA content in this study met the minimum requirements of the larvae for survival. Regardless, the correlation between survival and crude lipid, but lack of correlations between survival and individual fatty acids, suggests that survival was influenced by a combination of lipids in the diet.

There are clear ecological implications for the effects of diet composition on quality indicators for larval southern flounder. Visual responsiveness is a critically important survival skills in nature since a fish must initiate an escape before other measures of escape response performance, such as speed, come into play (Fuiman and Cowan, 2003). In addition, visual responsiveness has been shown to be correlated with escape potential in a study with actual

predators (Fuiman et al., 2006). Mean visual responsiveness of larvae fed the diet with the highest amount of DHA and energy was 33%, which was nearly twice that of larvae fed the lowest amount of DHA and energy (18% responding). Since predators rarely fail to capture a fish larva that does not respond to the attack (Fuiman et al., 2006), this decrease in responsiveness due to lower DHA could result in a 45% decline in survival. This suggests that variations in dietary DHA and/or energy could play an important role in survival in nature. But, for another ecologically relevant trait, growth rate, there were no differences among treatments, although it is possible that slow growing larvae perished so that the effects of DHA on growth could not be measured accurately.

An important result of this study is that DHA and ARA concentrations in larval tissues can be manipulated through the larval diet. This allows for the design of experiments to further clarify the scope of influence of DHA and ARA on larval fish functions. The link between dietary DHA and visual function may not be surprising, given the high concentrations of DHA known to be present in retinal tissue. But, the influence of body DHA on routine turning rate was not expected and it underscores the importance of looking for effects of DHA beyond those associated with vision. Interestingly, while this study was designed to look at two specific fatty acids (DHA and ARA), survival was correlated with total lipids in the diet, which is a reminder that total lipids must be considered in addition to concentrations of individual fatty acids when understanding larval nutritional requirements.

Undoubtedly, EFAs play an important role in the early life history of fishes and the approach of examining the fatty acid linkages between the diet, tissues, and performance revealed connections for at least one fatty acid, DHA, to processes that ultimately have

major implications for larvae in nature and in aquaculture. Results of this study may improve our understanding of how EFAs, especially DHA, influence variability in recruitment in wild populations and production success in aquaculture.

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We thank Jeff Kaiser and Rene Lopez for helping maintain and collect the broodstock. We also thank Texas Parks and Wildlife Department, Marine Development Center for use of their calorimeter. EO would like to thank master's thesis committee members Ben Walther and Andrew Esbaugh. This study is supported by the Perry R. Bass Chair in Fisheries and Mariculture at the University of Texas Marine Science Institute.

Table 2.1. Nutrient composition of diets A, B, C and D. Values are means ± SE expressed as mg g^{-1} DW for fatty acids, Kj g^{-1} DW for energy, and % DW for crude lipid.

	Diet			
Component	A	B	C	D
14:00	7.78 ± 0.62	6.95 ± 1.08	5.12 ± 0.43	8.34 ± 0.49
15:00	0.59 ± 0.05	0.51 ± 0.07	0.48 ± 0.04	0.59 ± 0.03
15:01	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.11 ± 0.07
16:00	34.86 ± 3.11	28.12 ± 4.20	30.17 ± 1.54	32.05 ± 1.09
16:1ω7	4.52 ± 0.34	4.37 ± 0.48	2.52 ± 0.77	4.73 ± 0.24
16:2ω4	0.29 ± 0.02	0.35 ± 0.07	0.34 ± 0.05	0.28 ± 0.03
16:3ω4	0.55 ± 0.17	0.54 ± 0.20	0.43 ± 0.13	0.39 ± 0.14
17:00	0.73 ± 0.08	0.51 ± 0.09	0.44 ± 0.04	0.51 ± 0.05
18:00	8.06 ± 1.03	4.51 ± 0.78	4.08 ± 0.27	3.82 ± 0.04
18:1ω7	2.68 ± 0.24	2.15 ± 0.20	1.77 ± 0.11	2.09 ± 0.12
18:1ω9	12.80 ± 1.67	7.95 ± 1.28	7.44 ± 0.69	6.1 ± 0.61
18:2ω6	14.51 ± 1.62	10.14 ± 1.46	9.25 ± 0.49	8.45 ± 0.76
18:3ω3	3.63 ± 0.48	3.21 ± 0.39	3.78 ± 0.36	3.39 ± 0.42
18:3ω4	0.19 ± 0.05	0.09 ± 0.03	0.02 ± 0.01	0.03 ± 0.00
18:3ω6	2.29 ± 0.40	1.22 ± 0.22	0.51 ± 0.05	0.60 ± 0.09
18:4ω3	0.45 ± 0.04	0.41 ± 0.05	0.28 ± 0.02	0.50 ± 0.06
20:1ω9	2.22 ± 0.15	1.84 ± 0.21	1.72 ± 0.08	1.60 ± 0.15
20:2ω6	1.15 ± 0.14	0.79 ± 0.11	0.70 ± 0.06	0.60 ± 0.07
20:3ω3	0.61 ± 0.14	0.50 ± 0.06	0.54 ± 0.06	0.53 ± 0.10
20:3ω6	3.52 ± 0.50	2.10 ± 0.32	1.57 ± 0.21	1.33 ± 0.12
20:4ω3	1.91 ± 0.17	1.78 ± 0.35	1.81 ± 0.10	2.14 ± 0.18
20:4ω6 (ARA)	27.36 ± 4.10	12.15 ± 2.55	5.17 ± 0.33	5.99 ± 0.39
20:5ω3 (EPA)	7.49 ± 0.57	7.37 ± 0.86	7.36 ± 0.55	8.85 ± 0.29
22:5ω3	2.32 ± 0.50	3.17 ± 0.28	3.29 ± 0.19	3.51 ± 0.27
22:5ω6	14.93 ± 1.59	13.72 ± 1.94	10.09 ± 0.81	19.49 ± 3.27
22:6ω3 (DHA)	45.08 ± 4.36	39.71 ± 6.62	29.55 ± 1.33	57.56 ± 5.90
ARA:EPA	3.65 ± 0.43	1.58 ± 0.16	0.76 ± 0.03	0.77 ± 0.03
DHA:ARA	1.54 ± 0.08	3.40 ± 0.15	5.01 ± 0.30	8.65 ± 0.34
DHA:EPA	6.02±0.00	5.39±0.00	4.01±0.00	5.91±0.00
Energy	25.6±0.54	26.5±0.18	22.1±0.24	26.1±0.19
Crude lipid	32.7±2.59	40.5±3.66	20.8±2.87	32.8±2.79

Table 2.2. Fatty acid composition of heads and bodies for 15 dph larvae fed diets A, B, C and D. Values are means ± SE expressed as % of total fatty acids.

		Diet			
Fatty acid	Section	A	B	C	D
14:00	Head	0.93 ± 0.03	2.38 ± 0.78	1.00 ± 0.56	1.05 ± 0.17
	Body	1.04 ± 0.11	1.08 ± 0.09	1.29 ± 0.36	1.25 ± 0.27
15:00	Head	0.45 ± 0.08	0.53 ± 0.11	0.70 ± 0.33	0.40 ± 0.03
	Body	0.47 ± 0.10	0.50 ± 0.04	0.53 ± 0.06	0.41 ± 0.03
15:01	Head	0.04 ± 0.01	0.42 ± 0.37	0.20 ± 0.16	0.19 ± 0.09
	Body	0.06 ± 0.02	0.21 ± 0.11	0.15 ± 0.08	0.29 ± 0.13
16:00	Head	16.55 ± 0.30	19.96 ± 1.17	11.50 ± 5.22	17.08 ± 0.56
	Body	15.83 ± 0.69	18.14 ± 1.27	18.61 ± 1.30	16.07 ± 0.27
16:1ω7	Head	1.06 ± 0.13	1.64 ± 0.64	0.62 ± 0.28	1.25 ± 0.28
	Body	1.05 ± 0.17	0.96 ± 0.07	0.70 ± 0.28	1.08 ± 0.10
16:2ω4	Head	0.60 ± 0.11	0.39 ± 0.07	0.34 ± 0.17	0.62 ± 0.13
	Body	0.60 ± 0.10	0.48 ± 0.04	0.77 ± 0.17	0.59 ± 0.11
16:3ω4	Head	0.60 ± 0.07	0.34 ± 0.10	0.72 ± 0.08	0.60 ± 0.16
	Body	0.59 ± 0.32	0.63 ± 0.23	0.81 ± 0.19	0.87 ± 0.17
17:00	Head	0.67 ± 0.13	0.81 ± 0.34	0.85 ± 0.30	0.72 ± 0.12
	Body	0.58 ± 0.20	0.85 ± 0.11	0.43 ± 0.08	0.69 ± 0.17
18:00	Head	10.54 ± 0.24	8.41 ± 2.73	7.73 ± 3.04	10.71 ± 0.22
	Body	9.57 ± 0.23	11.03 ± 0.61	11.79 ± 0.93	9.78 ± 0.37
18:1ω7	Head	1.97 ± 0.14	1.56 ± 0.29	4.57 ± 3.22	1.74 ± 0.30
	Body	1.40 ± 0.71	1.78 ± 0.22	1.60 ± 0.13	1.74 ± 0.16
18:1ω9	Head	6.03 ± 0.15	5.19 ± 0.57	3.58 ± 1.51	5.58 ± 0.75
	Body	3.86 ± 0.98	4.54 ± 0.16	4.84 ± 0.09	4.32 ± 0.24
18:2ω6	Head	3.14 ± 0.43	3.95 ± 1.53	1.92 ± 1.06	2.58 ± 0.14
	Body	3.61 ± 0.47	3.02 ± 0.78	3.17 ± 0.70	2.92 ± 0.19
18:3ω3	Head	0.52 ± 0.07	0.99 ± 0.54	0.50 ± 0.21	0.56 ± 0.06
	Body	0.39 ± 0.21	0.63 ± 0.11	0.65 ± 0.09	0.79 ± 0.10
18:3ω4	Head	0.12 ± 0.01	0.06 ± 0.02	0.11 ± 0.03	0.08 ± 0.02
	Body	0.14 ± 0.06	0.10 ± 0.02	0.08 ± 0.01	0.08 ± 0.01
18:3ω6	Head	0.39 ± 0.01	0.34 ± 0.01	0.32 ± 0.05	0.29 ± 0.01
	Body	0.35 ± 0.15	0.38 ± 0.02	0.34 ± 0.03	0.28 ± 0.01
18:4ω3	Head	0.06 ± 0.01	0.09 ± 0.05	0.11 ± 0.02	0.05 ± 0.01
	Body	0.08 ± 0.02	0.09 ± 0.03	0.47 ± 0.38	0.07 ± 0.01

Table 2.2, cont.

20:1ω9	Head	0.89 ± 0.09	1.15 ± 0.11	0.43 ± 0.26	0.78 ± 0.03
	Body	0.86 ± 0.27	0.93 ± 0.13	0.88 ± 0.14	0.87 ± 0.05
20:2ω6	Head	0.80 ± 0.07	0.55 ± 0.08	0.70 ± 0.07	0.65 ± 0.05
	Body	0.65 ± 0.30	0.76 ± 0.18	0.90 ± 0.10	0.76 ± 0.09
20:3ω3	Head	0.37 ± 0.06	0.29 ± 0.07	0.22 ± 0.15	0.33 ± 0.08
	Body	0.48 ± 0.04	0.29 ± 0.11	0.25 ± 0.17	0.39 ± 0.02
20:3ω6	Head	0.93 ± 0.10	0.80 ± 0.17	0.54 ± 0.14	0.67 ± 0.04
	Body	0.79 ± 0.34	0.89 ± 0.16	0.83 ± 0.08	0.76 ± 0.06
20:4ω3	Head	0.63 ± 0.07	0.75 ± 0.31	0.50 ± 0.14	0.62 ± 0.04
	Body	0.69 ± 0.05	0.59 ± 0.14	0.58 ± 0.13	0.69 ± 0.03
20:4ω6 (ARA)	Head	7.33 ± 0.11	4.59 ± 0.81	2.89 ± 1.38	4.79 ± 0.56
	Body	7.93 ± 0.20	5.47 ± 1.05	4.21 ± 0.85	4.80 ± 0.63
20:5ω3 (EPA)	Head	2.00 ± 0.09	3.03 ± 1.35	4.65 ± 2.72	2.31 ± 0.17
	Body	2.10 ± 0.11	1.86 ± 0.46	2.15 ± 0.33	2.15 ± 0.12
22:5ω3	Head	2.62 ± 0.17	2.42 ± 0.48	2.17 ± 0.70	2.66 ± 0.15
	Body	2.83 ± 0.16	3.01 ± 0.61	2.70 ± 0.52	2.68 ± 0.23
22:5ω6	Head	4.79 ± 0.32	4.55 ± 1.04	2.60 ± 1.31	5.18 ± 0.71
	Body	5.62 ± 0.37	4.34 ± 0.68	3.73 ± 1.15	6.32 ± 0.92
22:6ω3 (DHA)	Head	25.91 ± 0.82	20.04 ± 1.43	13.12 ± 1.31	25.57 ± 1.28
	Body	22.11 ± 0.99	16.86 ± 2.08	15.84 ± 2.86	21.9 ± 1.77
ARA:EPA	Head	3.68 ± 0.20	2.23 ± 0.80	0.70 ± 0.00	2.07 ± 0.14
	Body	3.81 ± 0.28	3.00 ± 0.14	1.94 ± 0.16	2.22 ± 0.19
DHA:ARA	Head	3.54 ± 0.11	4.51 ± 0.43	4.71 ± 0.18	5.43 ± 0.41
	Body	2.79 ± 0.07	3.16 ± 0.23	3.80 ± 0.10	4.63 ± 0.30
DHA:EPA	Head	13.01 ± 0.90	9.80 ± 3.70	5.93 ± 2.67	11.14 ± 0.55
	Body	10.62 ± 0.84	9.53 ± 1.01	7.34 ± 0.39	10.20 ± 0.52

Table 2.3. Significant correlations ($P < 0.05$) between concentrations of fatty acids in the diet and in head and body tissue. r is the Pearson product-moment correlation coefficient.

Fatty acid	Tissue	r	P
18:3ω6	Head	0.67	0.017
20:4ω6 (ARA)	Head	0.73	0.007
22:6ω3 (DHA)	Head	0.73	0.008
ARA:EPA	Head	0.73	0.007
DHA:ARA	Head	0.68	0.015
20:4ω6 (ARA)	Body	0.79	0.002
22:5ω6	Body	0.62	0.031
22:6ω3 (DHA)	Body	0.62	0.030
ARA:EPA	Body	0.89	<0.001
DHA:ARA	Body	0.92	<0.001

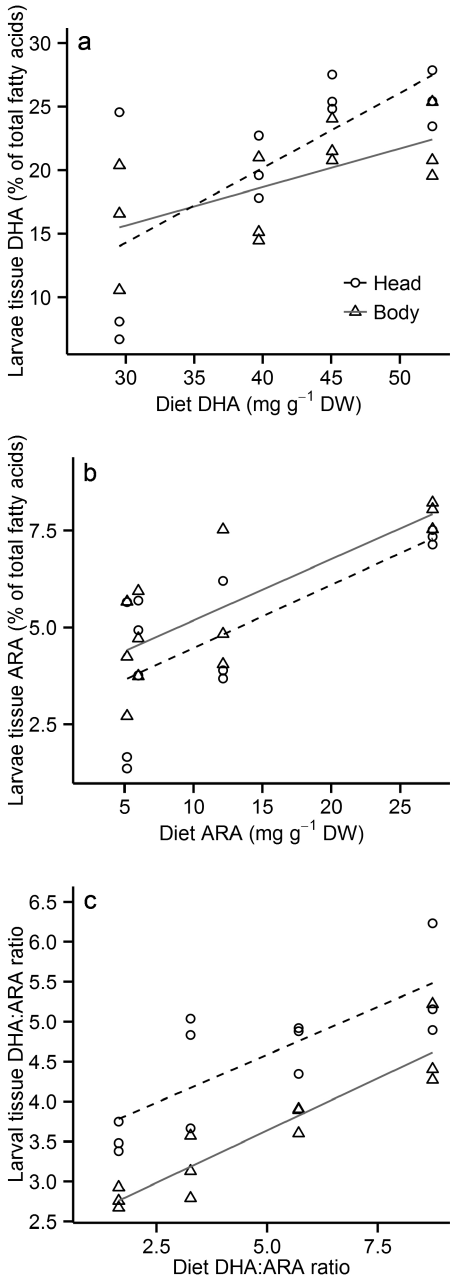

Figure 2.1. Relationships between head (circles and dashed line) or body (triangles and solid line) tissue concentrations of a) DHA, b) ARA, and the c) ratio of DHA:ARA and concentrations in the diet.

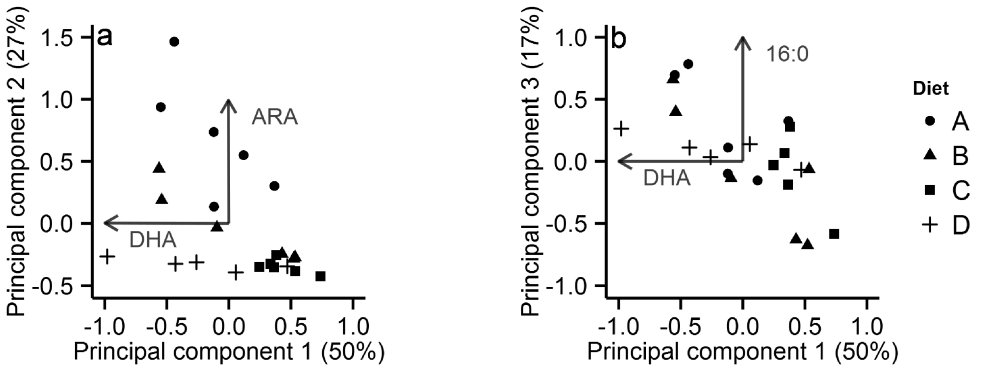

Figure 2.2. Principal component analysis for diet fatty acid composition. Data points show principal component scores for pooled larvae from each diet for each of three replicate spawns. Vectors show loadings of variables (fatty acids) that had high (> 0.7 or <-0.7) loadings on each axis. Percentages on the axis titles indicate the amount of variance explained by the component.

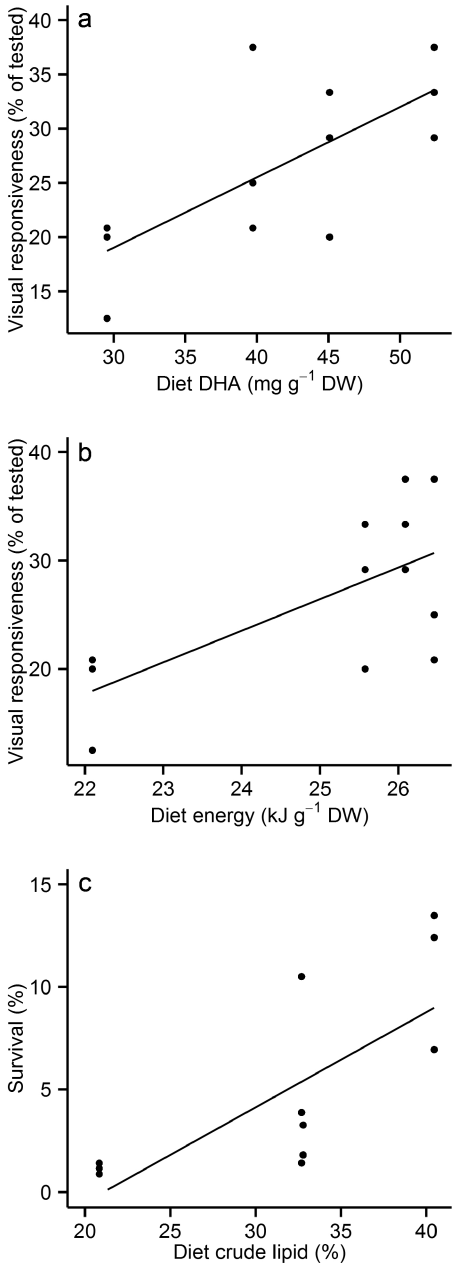

Figure 2.3. Relationship between a) visual responsiveness and dietary DHA concentration, b) visual responsiveness and dietary energy content, and c) survival and dietary crude lipid content at 15 dph for southern flounder larvae on four diets.

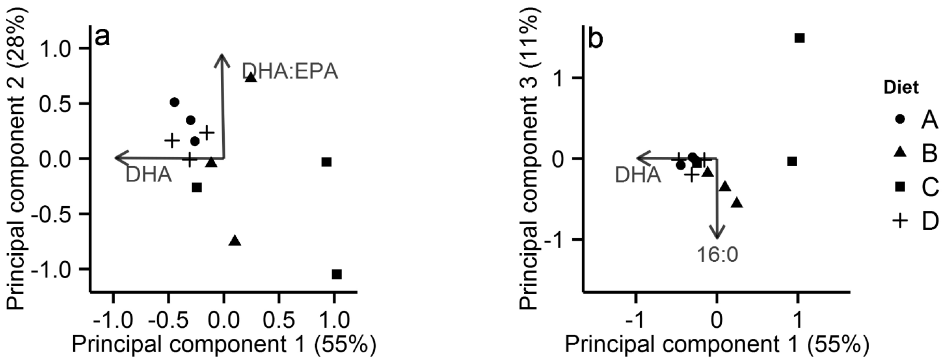

Figure 2.4. Principal component analysis for fatty acid composition of head tissue. Data points show principal component scores for pooled larvae from each diet for each of three replicate spawns. Vectors show loadings of variables (fatty acids) that had high (> 0.7 or <-0.7) loadings on each axis. Percentages on the axis titles indicate the amount of variance explained by the component.

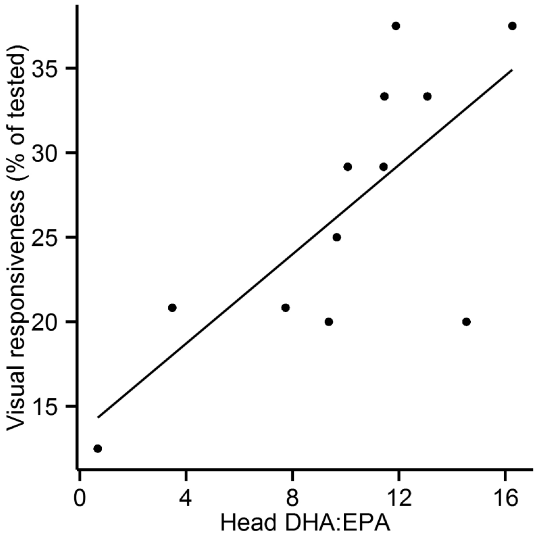

Figure 2.5. Relationship between visual responsiveness and DHA:EPA in the head of southern flounder larvae. Each point represents pooled larvae from each diet for each of three replicate spawns.

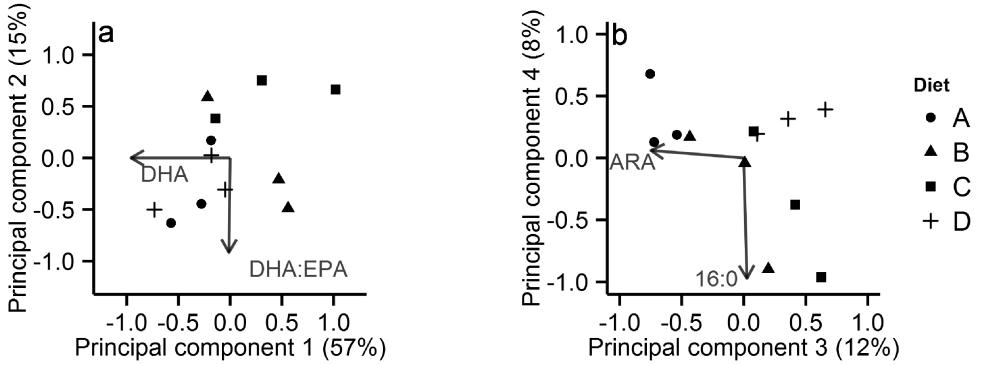

Figure 2.6. Principal component analysis for fatty acid composition of body tissue. Data points show principal component scores for pooled larvae from each diet for each of three replicate spawns. Vectors show loadings of variables (fatty acids) that had high (> 0.7 or <-0.7) loadings on each axis. Percentages on the axis titles indicate the amount of variance explained by the component.

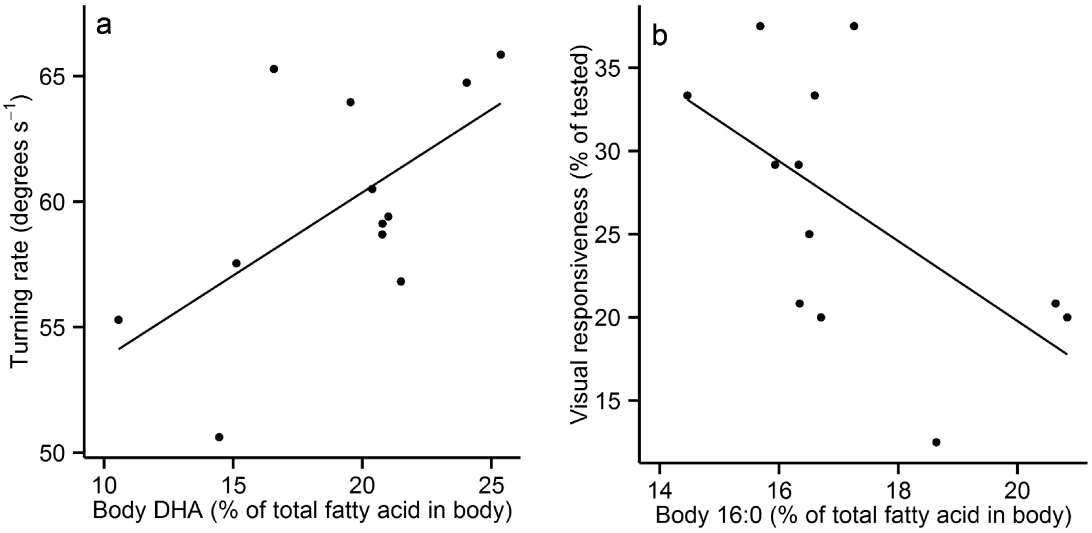

Figure 2.7. Relationship between a) turning rate and DHA concentration in body tissue and b) visual responsiveness and 16:0 concentration in body tissue. Each point represents pooled larvae from each diet for each of three replicate spawns.

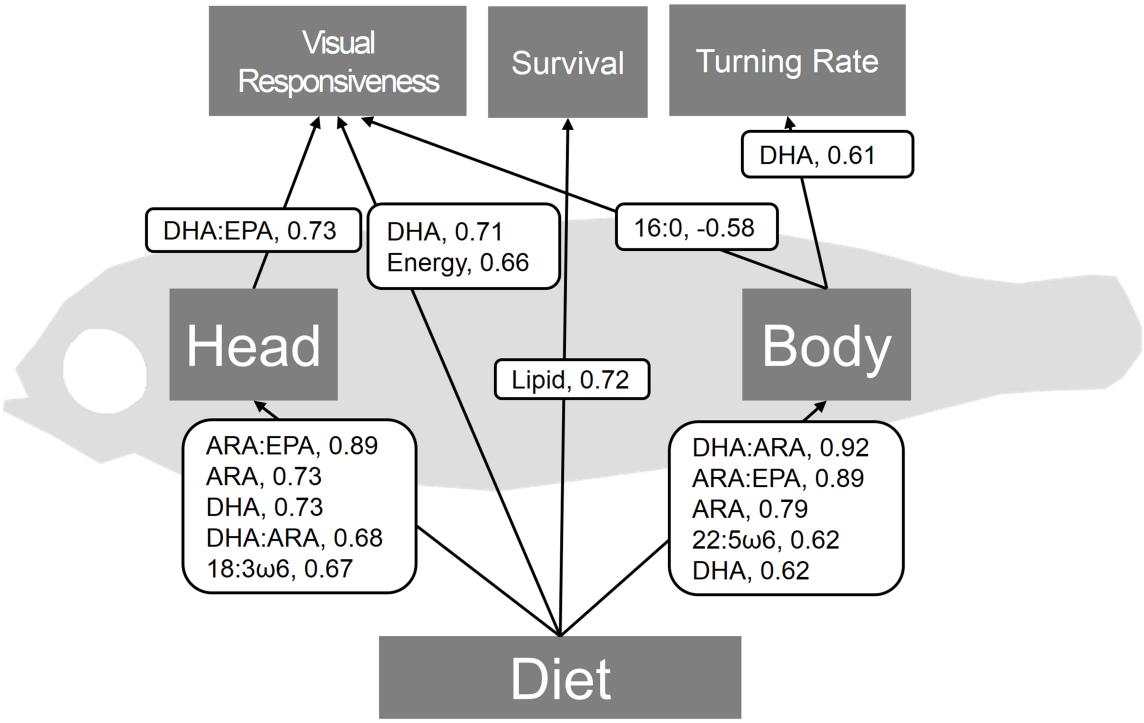

Figure 2.8. Summary of linkages between diet, tissues, and larval quality traits in southern flounder. White boxes show the fatty acids that were correlated and correlation coefficient (r).

Appendix A: Additional analysis of egg fatty acid composition and differences among nutrients in the diet

Fatty acid composition of a subsample of eggs for each replicate was analyzed using the same method described for the diet and tissues. Eggs were characterized by 26 individual fatty acid values and three calculated ratios (Table A1).

Diets were characterized by 26 individual fatty acids values, three calculated ratios, crude lipid, and dietary energy (Table A2). Multivariate analysis of variance (MANOVA) was used to test for differences among diets in the amounts of individual fatty acid, and individual ANOVAs were used to test for differences in ratios, energy, and crude lipid. There were significant differences among the diets for 14 of 26 fatty acids measurements (Table A2).

Table A1. Egg fatty acid composition of the three replicates. Values are expressed as mg g^{-1} DW.

	Replicate		
Component	1	2	3
14:00	4.10	4.40	4.90
15:00	1.60	1.30	1.20
15:01	0.50	0.00	0.00
16:00	36.50	37.90	31.30
16:1ω7	10.00	10.80	11.20
16:2ω4	1.90	1.40	1.60
16:3ω4	1.50	1.30	1.20
17:00	1.70	1.50	1.60
18:00	6.10	6.20	4.90
18:1ω7	19.40	19.30	19.60
18:1ω9	6.50	4.90	5.30
18:2ω6	2.20	1.90	1.90
18:3ω3	0.40	0.20	0.30
18:3ω4	0.30	0.00	0.00
18:3ω6	0.60	0.40	0.60
18:4ω3	0.70	0.60	0.90
20:1ω9	0.60	0.40	0.50
20:2ω6	0.30	0.50	0.40
20:3ω3	0.20	0.20	0.20
20:3ω6	4.90	4.70	3.90
20:4ω3	0.30	0.20	0.20
20:4ω6 (ARA)	0.60	0.60	0.70
20:5ω3 (EPA)	7.80	7.30	8.80
22:5ω3	1.40	1.60	1.50
22:5ω6	7.60	8.30	8.00
22:6ω3 (DHA)	44.60	40.40	34.30
ARA:EPA	0.63	0.64	0.44
DHA:ARA	9.10	8.60	8.79
DHA:EPA	5.72	5.53	3.90

Table A2. Fatty acids and ratios of fatty acids in the diet. * indicates fatty acids with at least one significant difference among the means of diets A, B, C, and D.

Component	Significance
14:00	*
15:00	
15:01	
16:00	
16:1ω7	*
16:2ω4	
16:3ω4	
17:00	*
18:00	*
18:1ω7	*
18:1ω9	*
18:2ω6	*
18:3ω3	
18:3ω4	*
18:3ω6	*
18:4ω3	*
20:1ω9	
20:2ω6	*
20:3ω3	
20:3ω6	*
20:4ω3	*
20:4ω6 (ARA)	*
20:5ω3 (EPA)	
22:5ω3	
22:5ω6	*
22:6ω3 (DHA)	*
ARA:EPA	*
DHA:ARA	*
DHA:EPA	*
Energy	*
Crude lipid	*

Appendix B: Carbon dioxide is an effective anesthetic for multiple marine fish species

E.W. Oberg, K.O. Perez, L.A. Fuiman

ABSTRACT

Fisheries research involving surgery is aided by and, sometimes, requires anesthesia, but health and safety regulations limit the anesthetic methods that can be used on species considered food fish. Carbon dioxide is one anesthetic that the United States Food and Drug Administration (FDA) tolerates when certain guidelines are met, and it complies with Institutional Animal Care and Use Committee protocols. But, there is very little published work that characterizes its utility on marine fishes and no studies have compared its effectiveness across species or sizes. We used acetic acid and sodium carbonate to create a carbon dioxide rich seawater bath to induce anesthesia and measured induction time and recovery time for five species and several sizes of marine fishes. We found that carbon dioxide quickly and effectively anesthetizes these marine fishes to stage-4 anesthesia, a level acceptable for minor surgery. Induction time was positively related to body size (total length or wet mass), but recovery time was independent of size. Using red drum, we also found differences between rested and fatigued fish. These results provide needed documentation of the effectiveness of carbon dioxide on marine fishes and are useful for planning field studies that involve minor surgery on marine food fish.

INTRODUCTION

Important and widely used methods of fisheries research, such as making size measurements, tagging, obtaining tissue biopsies, and minor surgery, have been facilitated by the use of anesthetics. Proper anesthesia calms the fish, reduces its movement, and minimizes stress and pain. These attributes increase researchers' ability to handle fish without injuring themselves or the fish. Furthermore, anesthesia is required by Institutional Animal Care and Use Committees (IACUC) following guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and regulations provided by the Animal Welfare Act (Garber et al., 2011).

Many different methods of anesthetization have been tested on fishes, but technical or legal reasons limit options. When tagging or surgery is involved, stage-4 anesthesia should be induced, which is characterized by complete loss of equilibrium, loss of swimming motion, and weakened opercular movements (Summerfelt and Smith, 1990). Some methods of inducing anesthesia are ineffective or inconsistent, impractical, or do not meet regulatory requirements. A review of 16 different anesthetization methods for a freshwater fish, rainbow trout (*Oncorhynchus mykiss*), found that effectiveness can vary widely among methods(Gilderhus and Marking, 1987). Two products, tricaine methanesulfonate (MS-222) and clove oil (eugenol, AQUI-S®20E), are commonly used because they induce high stages of anesthetization rapidly (< 3 min) and have quick recovery times (< 10 min) (Gilderhus and Marking 1987, reviewed by Neiffer and Stamper 2009; Javahery et al. 2012). Electroanesthesia can be used in fresh water, but it requires a significant initial investment

for equipment, which could be prohibitive for many fisheries professionals (Trushenski et al., 2012a).

A limited number of anesthetic drugs have been approved for use on food fish, and special precautions have been prescribed for the approved drugs (Bowker and Trushenski, 2012; Trushenski et al., 2013). The United States Food and Drug Administration (FDA) authorized tricaine methanesulfonate for use on Ictaluridae, Salmonidae, Esocidae, and Percidae with a 21-day withdrawal period (Kelsch and Shields, 1996; Bowker and Trushenski, 2012). Eugenol and benzocaine based anesthetic drugs, AQUI-S®20E and BENZOAK, are "Investigational New Animal Drugs" for freshwater finfish (Trushenski et al., 2013). Since these policies exclude all non-salmonid marine fishes, field researchers who study marine food fishes and wish to release the fish alive immediately after handling must use an anesthetic technique that does not violate FDA regulations (Trushenski et al., 2013).

Carbon dioxide is the only alternative anesthetic that the FDA has explicitly stated it is unlikely to oppose, provided that conditions regarding appropriate and safe use are met (USFDA, 2011). As carbon dioxide is a 'generally recognized as safe' (GRAS) food ingredient, carbon dioxide anesthesia poses no risk to humans if an animal is released immediately after recovery from anesthesia then recaptured and consumed. Finally, carbon dioxide complies with IACUC requirements.

Carbon dioxide concentrations in water can be increased by bubbling carbon dioxide gas directly into the holding tank, although this requires carrying inconvenient and potentially dangerous gas cylinders. Carbon dioxide can also be created by reacting sodium bicarbonate ($NaHCO_3$) or sodium carbonate (Na_2CO_3) with an acid (e.g., sulfuric or acetic)

to form carbonic acid (H_2CO_3), which then releases carbon dioxide into the water (Gelwicks et al., 1998). Total carbon dioxide in sea water is normally around 95 mg L^{-1}, whereas the solubility of carbon dioxide is estimated to be 1365 mg L^{-1} in 35 ppt aqueous NaCl at 24 ^{o}C (Duan and Sun, 2003; Keeling et al., 2005). Elevated concentrations of carbon dioxide in water (for example, 750 mg L^{-1} total carbon dioxide; Trushenski et al. (2012b), induce hypercapnia (elevated levels of carbon dioxide in the blood) and decrease blood pH (Post, 1979), which reduces oxygen transport to the brain and results in anesthetization. When a fish is returned to water with a normal carbon dioxide concentration, carbon dioxide diffuses out of the fish, blood pH increases, and equilibrium and swimming return (Gelwicks et al., 1998).

Use of carbon dioxide as a fish anesthetic has had a long and controversial history in fisheries biology (Fish, 1943; Summerfelt and Smith, 1990). Although carbon dioxide anesthesia satisfied the criteria that define an effective anesthetic for small fishes, Gilderhus and Marking (1987) found that this method was slow to act on adult fishes and suggested it be used only in situations where a low level of anesthesia is acceptable. Others have found that carbon dioxide can completely anesthetize a fish, causing disequilibrium, loss of muscle control, and possibly an analgesic effect (Post, 1979; Summerfelt and Smith, 1990; Prince et al., 1995). Interestingly, a comparison of five different anesthetization techniques found that carbon dioxide caused the smallest decrease in blood oxygen concentrations during induction of deep stages of anesthesia (Iwama et al., 1989). Additionally, plasma cortisol levels (indicators of stress) were no higher in steelhead trout (*Oncorhynchus mykiss*) after anesthetization using carbon dioxide than when using MS-222 or clove oil (Pirhonen and

Schreck, 2003), and plasma cortisol levels decreased to pre-handling concentrations sooner when using carbon dioxide compared with clove oil (Wagner et al., 2002). Carbon dioxide anesthesia has been found to be effective across a broad range of temperatures (Gelwicks et al., 1998). Through various delivery systems, carbon dioxide has been successfully used on multiple freshwater species, including yellow perch (*Perca flavescens*), burbot (*Lota lota*), carp (*Cyprinus carpio*), walleye (*Sander vitreus*), salmon, and trout (salmonids), where it was used while conducting minor surgical procedures, such as implanting telemetry transmitters (Yoshikawa et al., 1991; Prince et al., 1995; Erikson et al., 2006; Vandergoot et al., 2011). To our knowledge, the only documented uses of carbon dioxide as an anesthesia for marine finfishes was to reduce stress during harvesting and prior to processing of aquacultured Atlantic salmon (*Salmo salar*; Erikson et al. 2006) and a comparison of anesthetization techniques on juvenile cobia (*Rachycentron canadum*; Trushenski et al. 2012b).

Despite concerns regarding the effects of carbon dioxide anesthesia, it is currently the only option for many fisheries professionals, particularly those working under the more restrictive regulations of academia. Therefore, the goal of this study was to assess the utility of carbon dioxide for inducing stage-4 anesthesia in a broad range of marine fishes by examining its effectiveness. An ideal anesthetic works quickly to minimize stress and injury and allows the fish to recover quickly and be returned to the wild immediately. Here, we assess effectiveness using induction time and recovery time. We evaluate variability in these measures related to species, size, and energetic state (fatigued versus rested). Our successful experimentation with carbon dioxide anesthesia opens the door for field research that

requires anesthesia for minor surgery on marine food fishes, such as tissue biopsy and implantation of telemetry transmitters.

METHODS

We performed anesthetization experiments on five marine fish species acquired from laboratory-maintained stocks or the wild. Because this study is directly relevant to field studies, where the targeted individuals are within a size range that commercial or recreational fishers are allowed to catch, we focused our efforts on evaluating whether carbon dioxide was an effective anesthetic for four game fishes: red drum (*Sciaenops ocellatus*), southern flounder (*Paralichthys lethostigma*), common snook (*Centropomus undecimalis*), and Florida pompano (*Trachinotus carolinus*). We also tested larval red drum and young-of-the-year inland silversides (*Menidia beryllina*) to extend our results to smaller body sizes.

The goal of this study was to determine whether methods used on freshwater fishes were effective for marine species. Therefore, our experiment was designed to test the effectiveness of carbon dioxide as an anesthetic at one concentration using methods of Prince et al. (1995) and Trushenski et al. (2012b).We combined 1.33 g L^{-1} sodium carbonate and 0.75 ml L^{-1} glacial acetic acid to 30-55 L of sea water. Using a Corning 965 carbon dioxide analyzer (Ciba Corning Diagnostics Corporation, Medfield, Massachusetts), we determined that this method immediately elevated total carbon dioxide levels in the sea water to 669 ± 32 mg L^{-1} (mean ±SE) and decreased pH from 8.0 to 6.7, which remained constant throughout the use of a bath. If a species did not reach stage-4 anesthesia within 10 min, we doubled the concentrations of sodium carbonate and acetic acid. This higher concentration

raised total carbon dioxide to 1248 ± 178 mg L^{-1}, which was likely saturation. We aerated baths of water prior to addition of sodium carbonate and acetic acid, but not after the chemicals were added and fish were being tested, following Trushenski et al. (2012b).

To test each fish, we carefully netted individual larval red drum, juvenile flounder, adult snook, adult flounder, or adult pompano from laboratory tanks and immediately placed the fish in the prepared anesthesia bath. We collected inland silversides and red drum in the wild by net or hand line and allowed each fish to recover in an oxygenated tank for at least 1 h before testing. To evaluate carbon dioxide anesthesia under conditions common during field sampling, we landed adults using rod and reel and immediately placed the fish in the anesthesia bath. These fish were referred to as "fatigued." While fish were in the bath, pH was between 6.7 and 7.2. For fish tested in the laboratory, we maintained water temperature at 24 ^{o}C. Fatigued red drum were tested at ambient water temperatures (27-29 ^{o}C). Due to the potential for loss of carbon dioxide a new bath was prepared if the bath was used for > 30 min.

We conducted trials on most species in a covered clear glass container (91.5 x 30.5 x 40.5 cm) and monitored the fish continuously. Due to their large size, however, we tested adult red drum and common snook in a covered, opaque plastic container (142 L "ice chest") and opened the container approximately every 15 s to monitor the level of anesthesia. Several stages of anesthesia have been formally defined for fishes (Summerfelt and Smith 1990, Prince et al. 1995). At Stage 3, the fish exhibits partial loss of equilibrium and increased opercular rate. At Stage 4, muscle control and equilibrium are lost and opercular movements are weak. We recorded induction time (in min) until Stage-4

anesthesia. We then measured total length (in cm; TL) and wet mass (in g; WM) before returning the fish to oxygenated sea water. We measured recovery time (min) as the time between immersion in oxygenated sea water and return of equilibrium. A single observer made all the timed observations to limit bias, since assessing induction and recovery times can potentially be subjective, as noted by Trushenski et al. (2012b). We verified 24-h survival for red drum, flounder, and snook. In addition, most flounder and all snook were maintained at our facility for at least a month following experimentation. Other species were released into the wild following recovery or euthanized as part of other projects.

We define anesthetic effectiveness as achieving stage-4 with induction time < 10 min, recovery time < 5 min (Summerfelt and Smith 1990), and < 5% mortality. We used linear regressions of induction time or recovery time on TL and WM to determine the effects of body size for all rested individuals or all adult red drum (rested and fatigued). All variables were log-transformed before analysis to linearize relationships and decrease heteroscedasticity. We tested for differences between species or between rested and fatigued adult red drum after correcting for differences in size (residuals from regression of induction or recovery time on body size) using ANOVA and Scheffe's post-hoc test (for species) and Student's t-test (rested vs. fatigued).

RESULTS

Carbon dioxide successfully induced stage-4 anesthesia in all five marine fish species (Tables B1 and B2). For red drum, flounder, pompano, and silverside, the original concentration was sufficient to reach stage-4 anesthesia in < 10 min. Snook, however, did

not reach stage-4 at this concentration, but they did reach stage-4 when the carbon dioxide concentration was doubled. Juvenile flounder, unlike adult flounder, did not reach stage-4 anesthesia within 10 min in either the original or the doubled carbon dioxide concentration. Only one of 96 anesthetized fish, a larval red drum, did not survive the anesthetization process, whereas, all individuals that we were able to monitor after recovery of equilibrium resumed pre-test behavior (schooling and feeding) in less than 24 h (n = 22 red drum, n = 29 snook). We tested fish sequentially in the same anesthetization bath and there was no effect of the order in which a fish was tested on induction or recovery time after correcting for fish size ($r = -0.13$, $P = 0.23$ for induction time, $r = 0.02$, $P = 0.88$ for recovery time).

There was a strong linear relationship between the logarithm of induction time and both logarithm of TL and logarithm of WM across all species, such that log induction time = $0.65 \times \log(TL) - 0.51$ or log induction time = $0.22 \times \log(WM) - 0.08$ ($R^2 = 0.69$, $P < 0.001$, Figure B1a for TL; $R^2 = 0.69$, $P < 0.001$, Figure B1c for mass). Expressed another way, induction time is proportional to $TL^{0.64}$ and to $WM^{0.22}$. Mean induction time for the smallest fish, larval red drum, was 17 s on average, while adult red drum averaged 3.11 min. Neither log TL nor log WM were significantly related to recovery time across all species ($R^2 = 0.04$, $P = 0.07$, Figure B1b for TL; $R^2 = 0.04$, $P = 0.07$ for mass, Figure B1d for mass).

Species differed in both induction time and recovery time after accounting for differences in body size. Total length residuals (observed minus predicted) for induction time differed significantly among species (ANOVA $P \leq 0.001$). Pairwise comparisons of TL residuals showed that flounder had significantly longer induction time than all other species ($P < 0.05$). Silverside, snook, red drum, and pompano were not significantly different from

each other ($P > 0.05$). Because recovery time was not significantly related to body size, we compared species based on unadjusted values and found one difference. Recovery time for silverside, the species with the longest recovery time, was significantly different from pompano, which had the shortest recovery time (Table B2). The range of induction times (maximum - minimum) was greatest for adult red drum and the range for other species was < 5 min (Table B2). The range of recovery times for rested fish was greatest for common snook (Table B2).

Within the relatively small range of body sizes for adult red drum, neither log TL nor log WM were significantly related to induction or recovery time. Therefore, comparisons of rested and fatigued fish did not require adjustment for differences in body size. Rested red drum had significantly longer induction times than fatigued fish ($P < 0.05$, Figure B2a), but there was no difference in their recovery time ($P = 0.86$, Figure B2b). The range for induction time for fatigued fish was much less than for rested fish. The trend for recovery was the opposite; fatigued fish had a much larger range in recovery than rested fish.

DISCUSSION

We found that carbon dioxide can be used to induce stage-4 anesthesia for five marine fish species in < 10 min, with recovery of equilibrium in < 2 min, and < 5% mortality after 24 h. Although these general results support the utility of carbon dioxide as an anesthetic for marine fishes, we did find that its effectiveness varied with life stage in southern flounder and that one species (snook) required a higher carbon dioxide concentration. For all individuals successfully anesthetized, induction time was positively

correlated with body size, but there was no relationship between recovery time and body size. Woody et al. (2002) also observed a positive relationship between body size and induction time but not recovery time for sockeye salmon using clove oil as the anesthetic. Shorter induction times for fatigued red drum indicate that fatigued fish may be more sensitive to carbon dioxide anesthesia than rested fish. However, variability in induction time and recovery time, which was especially apparent for fatigued red drum, highlights the importance of physiological state (preexisting oxygen debt from excessive activity) to this method (Summerfelt and Smith 1990).

Our findings are comparable to the only other timed carbon dioxide anesthetization study completed on a marine fish in sea water that has been published. Trushenski et al. (2012b) found juvenile cobia (38 ± 0.5 cm and 297 ± 9 g) in a ~750 mg L^{-1} carbon dioxide bath were anesthetized within 2.7 ± 0.1 min. The relationships we found for TL or WM predict induction times of 3.29 or 2.91 min, respectively, which is only 0.59 and 0.21 min longer than what was observed in a carbon dioxide concentration 81 mg L^{-1} higher. We agree with Trushenski et al. (2012b) that higher concentrations of carbon dioxide are needed in saltwater nevertheless, our induction times are also similar to previous freshwater research. Prince et al. (1995) reported the carbon dioxide concentration produced by adding 1.33 g L^{-1} sodium bicarbonate and 0.5 mL L^{-1} acetic acid to river water was 328 mg L^{-1} total carbon dioxide. With this method, 50-60 cm TL sockeye salmon reached stage-4 anesthesia after 6.35 min (Prince et al. 1995). Peake (1998) found 2.66 g L^{-1} sodium bicarbonate sufficient to anesthetize walleye within 7.0 min in fresh water. Finally, Vandergoot et al. (2011) used 2.66

mg L^{-1} sodium bicarbonate and 1.0 mL L^{-1} acetic acid to bring walleye (n= 10; 56.7 $\pm$ 1.6 cm TL) to stage-4 anesthesia after 4.76 minutes.

Interestingly, mean induction time for rested red drum was similar to that for sockeye salmon and walleye (Prince et al. 1995, Vandergoot et al. 2011); however, snook of comparable size required twice the concentration of the acetic acid and sodium carbonate solution. These species-specific differences in induction time are probably due to differing tolerances to low oxygen conditions. Common snook, which are known to inhabit hypoxic mangrove swamp habitat when they are of a size similar to the smaller snook in our study, have a relatively low routine metabolic rate (O_2 consumption = 0.40 mg $O_2 g^{-1} h^{-1}$, Table B2, Peterson and Gilmore 1991, Peterson et al. 1991, but see Stevens et al. 2007). They move to the surface when exposed to low oxygen conditions. This differs from both Florida pompano and inland silverside, which are not tolerant of low oxygen concentrations due to their high routine metabolic rates (0.79 and 0.76 mg $O_2 g^{-1} h^{-1}$, respectively, Cunha et al. 2009, Peck et al. 2003). Juvenile southern flounder have the lowest metabolic rate of the species tested (0.08 mg $O_2 g^{-1} h^{-1}$, Taylor and Miller 2001), which may explain our unsuccessful anesthetization attempt. The general positive relationship between metabolic rate and stage-4 induction time suggests that knowledge of a species' metabolic rate is a useful guide for using carbon dioxide as an anesthetic (Taylor and Miller 2001, Gregory Tolley and Torres 2002).

Our study demonstrates that carbon dioxide anesthesia can be used on a range of marine fish species and sizes. Concentrations of 0.33 g L^{-1} sodium carbonate and 0.75 ml L^{-1} glacial acetic acid were sufficient to attain stage-4 anesthesia within 10 min. Beneficial

qualities of this method are that it is harmless to the researcher when used in an open area, inexpensive, portable in small plastic containers, and can be easily disposed of with no anticipated environmental impact (Summerfelt and Smith 1990). These positive attributes, as well as the rapid induction time, enabled us to perform ovarian biopsies on adult red drum in the field without harm to the investigators or the fish. Future studies may find the relationships we determined between size and induction time as helpful initial guidelines, but for different species we recommend carefully evaluating how quickly the organism displays signs of stage-4 anesthesia prior to conducting formal research on the organism. Investigators should also remain cognizant of the variability in induction time displayed within species and at a common size.

We emphasize this work was not an attempt to examine adverse physiological effects of carbon dioxide beyond short term survival and concerns remain. For example, stress as indicated by cortisol levels may be elevated more by carbon dioxide than other anesthetic methods (Trushenski et al. 2012a,b). Also, the brief exposure to the reduced pH created by the addition of carbon dioxide may have adverse consequences that require further research. Despite these concerns, until more anesthesia options become available (see Trushenski et al. 2013), carbon dioxide anesthesia is an acceptable method. We conclude that sea water enriched with carbon dioxide, made with sodium carbonate and acetic acid, can be a safe, effective, and convenient anesthetic method for minimally invasive surgery on marine fishes in the field.

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Table B1. Species and mean ± SE sizes (total length, TL and wet mass, WM) of marine fishes and carbon dioxide concentration ± SE used to test anesthetization for marine fishes. Subgroup is listed for species when multiple size classes or fish states were evaluated.

Species	Subgroup	TL (cm)	WM (g)	Number tested
Common snook		23.8 ±0.62	97.9 ± 11.32	27
Inland silverside		6.6 ± 0.3	2.5 ± 0.24	8
Southern flounder	Adult	40.1 ± 1.5	615.2 ± 85.84	10
Southern flounder	Juvenile	1.9 ± 0.1		10
Red drum	rested adult	69.8 ± 6.0	4150 ± 838.1	9
Red drum	fatigued adult	82.6 ± 6.1	8200 ± 1626.02	8
Red drum	Larva	0.81 ± 0.03	$7.8x10^{-3}$ ± $0.8x10^{-3}$	22
Florida pompano		27.7 ± 1.7	247.20 ± 37.96	10

Table B2. Carbon dioxide anesthetic effectiveness for five marine fish species. Subgroup is listed for species when multiple size classes or fish states were evaluated. Induction, handling, and recovery times are means ± SE. VO_2 is routine oxygen consumption rate extracted from published reports for southern flounder (Taylor and Miller 2001), red drum adults (Neill et al., 2004), and Florida pompano (Cunha 2009), or estimated for an individual of mean weight using models described by Peterson and Gilmore (1991) for common snook, Peck et al. (2003) for inland silversides, and Donnelly and Harvey (1996) for larval red drum.

Species	Subgroup	Mean Induction time (min±SE)	Mean Handling time (min±SE)	Mean Recovery time (min±SE)	VO_2 (mg O_2 g^{-1} h^{-1})
Common snook		2.42 ± 0.19	1.60±0.27	2.42 ± 0.19	0.40
Inland silverside		0.85 ± 0.05	1.28 ± 0.24	1.26 ± 0.21	0.76
Southern flounder	adult	9.61 ± 0.23	1.63 ± 0.21	0.72 ± 0.17	
Southern flounder	juvenile	NA	NA	NA	0.08
Red drum	rested adult	4.26 ± 1.11	3.37 ± 0.74	1.06 ± 0.27	0.60
Red drum	fatigued adult	1.20 ± 0.26	2.70 ± 0.59	1.99 ± 1.16	
Red drum	larva	0.28 ± 0.03	1.28 ± 0.08	0.81 ± 0.06	1.14
Florida pompano		2.12 ± 0.52	0.84 ± 0.05	0.52 ± 0.06	0.79

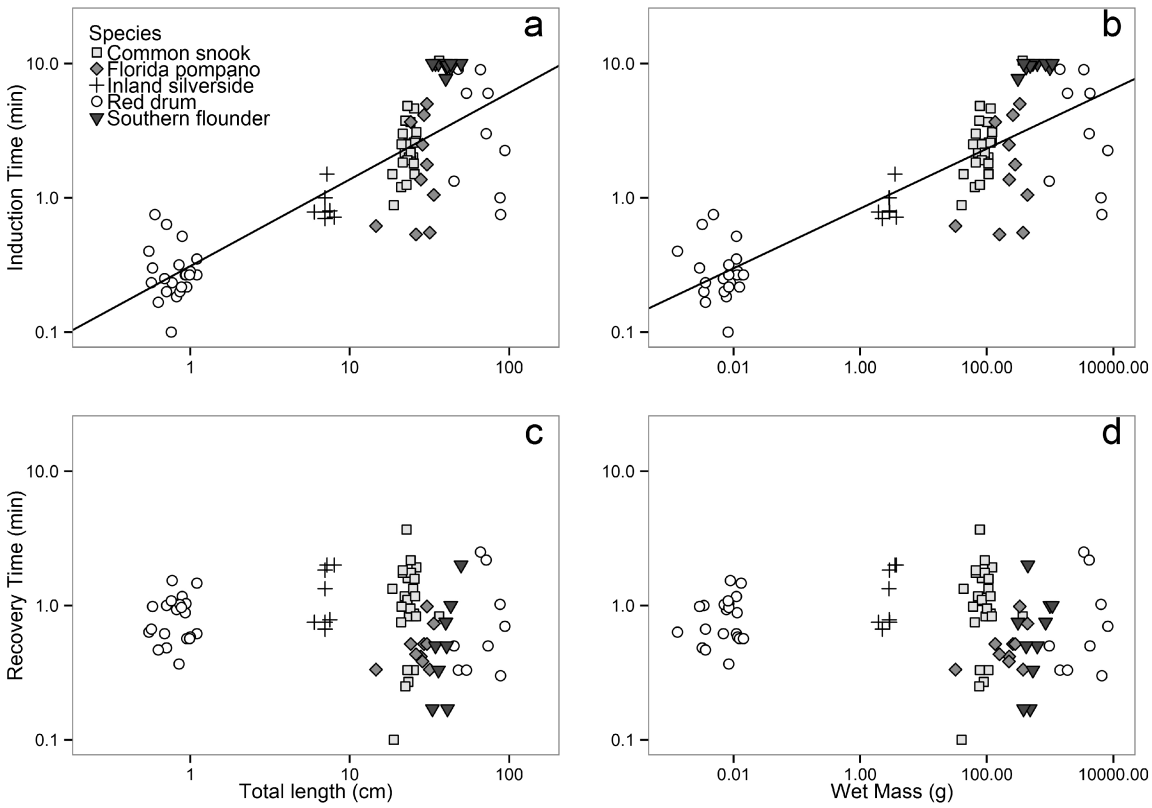

Figure B1. Relationship between fish size, species and (a,b) induction time and (c,d) recovery time. Axes are logarithmic.

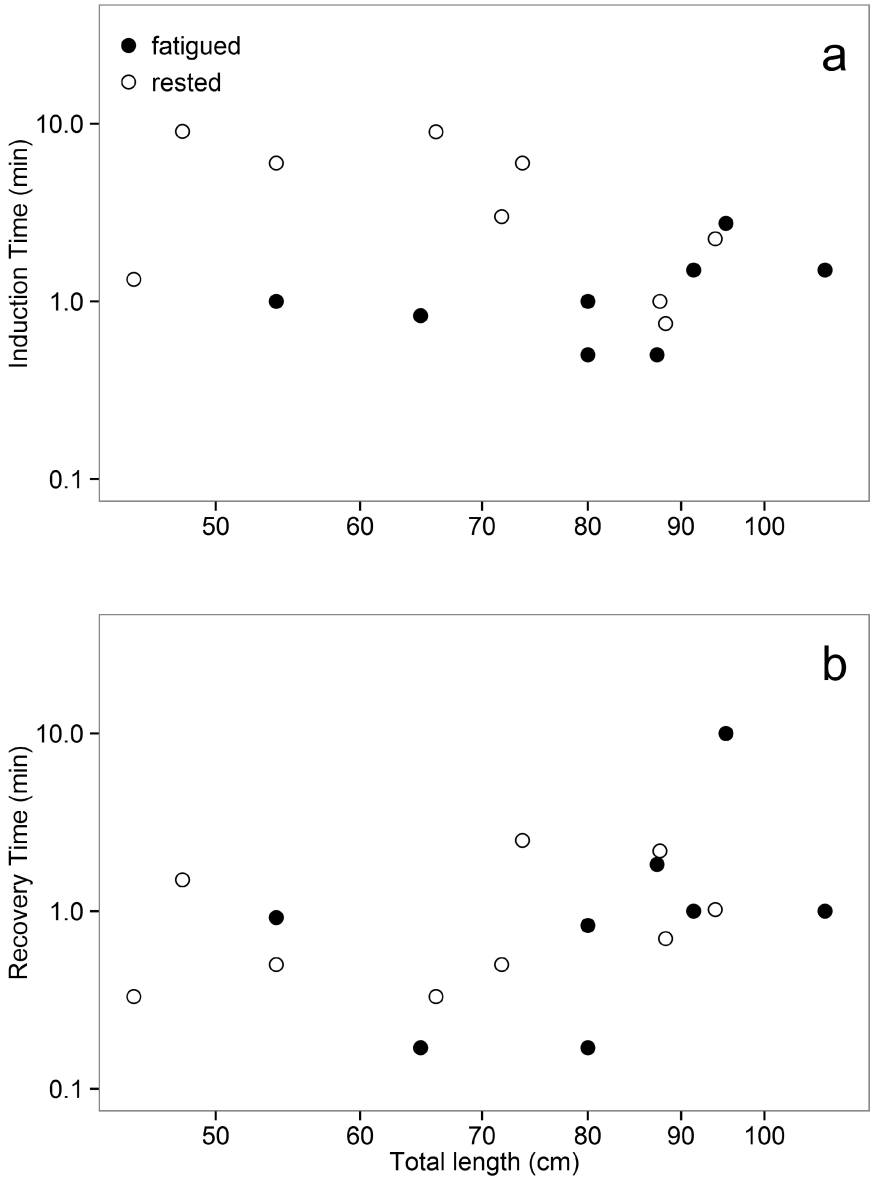

Figure B2. Relationship between fish size, physiological state (rested or fatigued), and (a) induction time and (b) recovery for red drum. Axes are logarithmic.

Appendix C: Optimal dietary ration for juvenile pigfish, *Orthopristis chrysoptera*, grow-out

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ABSTRACT

Pigfish (*Orthopristis chrysoptera*) have been identified as a good candidate for marine baitfish aquaculture. Initial research on the species has focused on captive spawning and larval rearing, but optimizing juvenile grow-out is also essential for economical production. We conducted an experiment to determine the optimal ration (R_{opt}) for maximizing growth rate while minimizing size variability and overfeeding. We measured total length (TL), wet weight (WW), specific growth rate (SGR), gross feed conversion efficiency (GFCE), and survival of juvenile pigfish (initial size: 2.6 ± 0.4 cm TL) using six ration levels (4, 8, 12, 16, 20, or 24 % WW d^{-1}) for four weeks at 24.7 ± 0.2 °C. Final size (TL and WW) increased with increasing ration at lower rations, reaching a plateau at intermediate levels (8-16% WW d^{-1}). Survival increased with ration from 74% at the lowest ration to a plateau of 96.0% at rations >10.7 % WW d^{-1}. GFCE decreased with increasing ration from 149% to 47%. To identify R_{opt} and its change with fish size, we modeled SGR as a function of WW, ration, and their interaction and found that $R_{opt} = 11.19 \cdot WW^{-0.26}$ ($R^2 = 0.70$, $P < 0.05$). This equation provides a guide for producing pigfish quickly and efficiently and, with further research on culture requirements, can be used to establish an efficient pigfish grow-out protocol.

INTRODUCTION

Pigfish (*Orthopristis chrysoptera*) is a popular marine baitfish throughout its range from the Atlantic Coast of Massachusetts to Florida and throughout the Gulf of Mexico (Darcy, 1983). Currently, pigfish are supplied to the baitfish industry by trapping from the wild and availability varies seasonally (Darcy, 1983). During times of high demand or seasons when pigfish cannot be trapped, supply is poor and the demand for inshore and offshore live bait is not met (Adams et al., 1998). In Florida and Texas, retail prices for live pigfish are typically $6 to $8 dollars per dozen, but can be as much as $15 per dozen (Adams et al., 1998; DiMaggio et al., 2013). Preferred bait size ranges from 6 to 11 cm, which corresponds to young-of-the-year that are available from May-August in Texas (Darcy, 1983). Demand for the fish, its market value, and relatively small length at market, as well as the economic pressure pushing U.S. aquaculture to expand into new markets, have led some to advocate for pigfish aquaculture (Oesterling et al., 2004; Cassiano et al., 2010; DiMaggio et al., 2013).

Pigfish have been spawned in captivity through either photoperiod and temperature control or hormone induction (Ohs et al., 2011; DiMaggio et al., 2013). DiMaggio et al. (2013) hatched pigfish from captive spawned eggs and raised the larvae through metamorphosis to 30 d posthatch (1.5 cm total length [TL]) using a feeding regime that included copepods (*Pseudodiaptomus pelagicus*), rotifers (*Brachionus plicatilis*), and *Artemia*. DiMaggio et al. (2014) examined the effects of stocking density on juvenile pigfish. But, a feeding regime to grow pigfish from early juveniles to market size (6-11 cm TL) has not been established.

Determining optimal ration (R_{opt}) for juvenile grow-out is the next step in developing pigfish aquaculture. Identifying R_{opt} enables producers to grow healthy fish as quickly and/or efficiently as desired and using an R_{opt} avoids overfeeding, which is costly and reduces water quality (Sumagaysay, 1998; Puvanendran et al., 2003). In this study, we use a range of daily rations (% wet weight [WW] d^{-1}) of a dry pelleted feed to grow pigfish from early juvenile (2.6 cm TL) for four weeks to determine R_{opt} by evaluating growth rate, survival, size variability, and gross feed conversion efficiency. Optimal ration is expected to be proportional to mass-specific metabolic rate, so we expected the optimum ration would decrease with size according to a power function with an exponent of -0.25.

MATERIALS AND METHODS

Pigfish were raised from eggs spawned by broodstock (n = 15) that were induced to spawn by photoperiod and temperature manipulation. Larvae were reared under controlled salinity (36.1 ± 0.6 ppt), temperature (20.0 ± 0.7 °C), and photoperiod (10 h light: 14 h dark) conditions at the Fisheries and Mariculture Laboratory of the University of Texas Marine Science Institute. Eggs were collected on mornings following evening spawning events and placed (5-10 eggs ml^{-1}) into a recirculating aquaculture system consisting of six 265-L light blue round tanks partially submerged in a 4,500-L rectangular tank. The recirculating system was configured so that water was pumped from the outer rectangular tank through a bio-filter, protein skimmer, sand filter, and heat pump into the round tanks from which water flowed back into the rectangular tank through a standpipe in the center of each round tank.

Hatched larvae were reared in the round tanks using green water culture (with *Isochrysis galbana*, 60,000 cells ml^{-1}) and feedings of rotifers (*Brachionus plicatilis,* 7 ml^{-1}) from 3-21 days posthatch (dph). To maintain live algae and rotifer concentrations, water flow was suspended throughout the rotifer feeding period. From 15-19 dph, newly hatched *Artemia* nauplii (Great Salt Lake-origin, Brine Shrimp Direct, Ogden, UT, USA) were added to the tanks once daily (0.1 ml^{-1}). From 19-30 dph, enriched *Artemia* (Algamac 3050, Aqua-fauna Bio-Marine, Hawthorn, CA, USA) were fed and water flow was gradually increased from 1 L min^{-1} to 3 L min^{-1}. Feeding levels were increased as larvae grew and consumed more prey, eventually reaching 0.4 ml^{-1} of enriched *Artemia* fed three times daily. This feeding regime insured a constant presence of enriched live food. At 30 dph, fish were weaned onto a 250-μm microdiet (52% crude protein; Otohime, Reed Mariculture, Campbell, CA, USA); granule size was gradually increased to 840-1410 μm as the fish grew. Temperature, salinity, and dissolved oxygen were recorded throughout larval rearing (YSI Inc., Yellow Springs, OH, USA) and maintained at 20.7 ± 0.3 °C, 36.0 ± 0.7 ppt, and > 6.0 mg L^{-1}, respectively. Photoperiod was 10 h light and 14 h dark throughout larval rearing. Ammonia, nitrite, and nitrate levels were monitored using API and Hach test kits (Pentair AES, Apopka, FL, USA) and remained below detectable limits throughout larval rearing.

Ration Study

The study began when individuals were approximately 2.6 cm TL and weighed 0.4 g WW. At that time, 150 randomly selected fish were placed into each of six dark blue round tanks (250 L) partially submerged within a 4,500 L rectangular tank (as described above for

larval rearing). Each tank of juvenile pigfish was fed one of six daily rations: 4, 8, 12, 16, 20, or 24% WW d^{-1}. Automatic feeders (Lifegard Aquatics, Cerritos, CA, USA) dispensed daily rations over the 14 h that lights were on. The initial diet contained 52% crude protein and 11% crude fat in 840-1410-µm granules (Otohime, Reed Mariculture, Campbell, CA, USA). When mean WW exceeded 1.5 g, a larger (1.7-mm) extruded pellet composed of 48% crude protein and 14% crude fat (Otohime) was used. The experiment lasted 4 weeks and was replicated three times. Each replicate consisted of juveniles from a different spawn. Temperature, salinity, and photoperiod were 24.7 ± 0.2 °C, 35.3 ± 0.6 ppt, and 14 h light and 10 h dark, respectively, with water flowing into each round tank at 3 L min^{-1}. Temperature (HOBO Pendant® temperature loggers; Onset Computer Corporation, Bourne, MA, USA), dissolved oxygen (5.7 ± 0.02 mg L^{-1}), and salinity were monitored daily; nitrogen compounds (nitrate, nitrite, and ammonia; < 10, 0.3 ± 0.1, and 0.4 ± 0.0 ppm, respectively) twice weekly; and pH (7.7 ± 0.1) once weekly. Uneaten food was siphoned from the tanks daily or as needed.

Fish were randomly selected and weighed on a balance (MXX-612; Denver Instruments, Bohemia, NY, USA) in a beaker of sea water immediately before starting each replicate and weekly from each tank thereafter. After weighing, fish were placed onto a mesh screen with a ruler and a digital photograph was taken for subsequent measurement of TL using image analysis (ImageJ, National Institute of Health, Bethesda, MD, USA). Fish were then returned to their tanks. Feed amounts were adjusted weekly to maintain the nominal ration levels, using the new mean individual WW. After four weeks, the experiment was terminated and the number of fish remaining in each tank and their final TL and WW were

recorded. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Data analysis

One-way analysis of variance (ANOVA) with six treatment levels was performed, followed by post-hoc Tukey's HSD test for comparisons of final sizes (TL and WW) among rations. The relationship between SGR and WW was modeled as an exponential function for each ration, so daily specific growth rate (SGR, % d^{-1}) was calculated weekly for each ration as: $SGR = (\ln(WW_t)-\ln(WW_{t-1}))/7 \cdot 100$, where ln is the natural logarithm, WW_t is the average WW for a given week, WW_{t-1} is the average WW for the preceding week, and 7 is the number of days between t and t-1 (Ricker, 1979). Gross feed conversion efficiency (GFCE) over the entire experiment was calculated for each ration as the change in WW divided by total dry weight of feed delivered to the tank (Stickney, 2005). Since GFCE increases with ration to a point, then decreases as ration exceeds maximum intake, a second order polynomial (quadratic function) was used to model the relationship between GFCE and ration (Zeitoun et al., 1976; Khan and Abidi, 2010). Survival was high for most rations, but decreased linearly with decreasing ration when levels became limiting. Therefore, a piecewise regression was used to relate survival to ration and to determine the minimum ration for maximal survival. The effect of ration on size variability was examined using the standard deviation of the log-transformed final TL and log-transformed final WW for each treatment group as a scale-independent measure of variability (Lewontin, 1966; Jobling, 1983). In all analyses $P < 0.05$ was considered statistically significant.

To derive an expression that defines R_{opt} as the minimum ration that maximizes SGR for a given WW while accounting for a change in R_{opt} with WW, SGR was modeled as a function of WW, ration, and their product (an interaction term):

$$SGR = \begin{cases} (k_1 \cdot e^{k_2 \cdot WW}) + (k_3 + k_4 \cdot R) + \left((k_1 \cdot e^{k_2 \cdot WW}) \cdot (k_3 + k_4 \cdot R)\right), & for\ R < k_5 \cdot WW^{k_6} \\ (k_1 \cdot e^{k_2 \cdot WW}) + (k_3 + k_4 \cdot k_5 \cdot WW^{k_6}) + \left((k_1 \cdot e^{k_2 \cdot WW}) \cdot (k_3 + k_4 \cdot k_5 \cdot WW^{k_6})\right), & for\ R \geq k_5 \cdot WW^{k_6} \end{cases} \quad \text{(eqn. 1)}$$

where, k_1, k_2, k_3, k_4, k_5, and k_6 are empirical constants solved iteratively by nonlinear regression, R is ration, and e is the base of the natural logarithm. This model is the embodiment of the complex interaction among growth rate, size, and ration alluded to by (Brett and Shelbourn, 1975). The model defines SGR as the sum of the exponential relationship between SGR and WW ($k_1 \cdot e^{k_2 \cdot WW}$), plus the linear relationship between SGR and R ($k_3 + k_4 \cdot R$), plus the interaction (product) of these two relationships for cases where R < R_{opt}. When R exceeds the R_{opt}, excess feed goes uneaten and SGR remains constant and equal to the value of SGR at R_{opt}. This model allows R_{opt} to vary with WW according to a power function:

$$R_{opt} = k_5 \cdot WW^{k_6} \quad \text{(eqn. 2)}$$

as scaling and bioenergetics theory predict, assuming R_{opt} is proportional to mass-specific metabolic rate (Kleiber, 1947; Kitchell et al., 1977; Jobling, 1983). Statistical analyses were completed using R software version 3.0.0 (R Development Core Team, 2014).

RESULTS

Pigfish increased in length by 2.0-2.5 times and in weight by 6-14 times over the 28-day experiment (Table C1). Final TL and WW in the 4% ration were statistically different

from all other rations, but there were no significant differences between other rations (Table C1). Uneaten food was observed daily on the bottom of the tanks in the 16%, 20%, and 24% ration tanks, but not in the 4%, 8%, and 12% rations.

SGR decreased exponentially as pigfish grew and generally increased with increasing ration from between 4 and 16 % WW d^{-1} but stabilized at higher rations (Figure C1). GFCE decreased with increasing ration, where the lower rations (4% and 8%) had significantly higher GFCE than the higher rations ($P < 0.05$, Figure C2a). Survival increased with increasing ration, the trend reached a plateau of 96.0% at a ration of 10.7 ± 1.5 % WW d^{-1} ($P < 0.05$, Figure C2b). There was much greater variability in survival among replicates at the 4% ration than at higher rations (Figure C2b). Variability of final WW and final TL decreased as ration increased, but there were no significant differences among rations (ANOVA and post-hoc Tukey's HSD, Figure C2c, d).

Weekly estimates of SGR for all rations (n = 72) were combined into a single model (equation 1), which accounted for ($R^2 = 0.70$) 70% of the observed variation in SGR ($P < 0.05$, Figure C3). Estimates for the empirical constants were: $k_1 = 0.86$, $k_2 = -0.17$, $k_3 = 2.12$, $k_4 = 0.43$, $k_5 = 11.19$, and $k_6 = -0.26$. Using these values, $R_{opt} = 11.19 \cdot WW^{-0.26}$ (Figure C3). The exponent is consistent with bioenergetics and scaling theory which predicts that R_{opt} is proportional to $WW^{-0.25}$ (Kleiber, 1947; Kitchell et al., 1977; Brett and Groves, 1979).

DISCUSSION

An optimal ration maximizes growth rate and minimizes size variability and food waste. Pigfish at the 8-24% ration levels grew from approximately 2.6 cm TL to market size

(approximately 6.0 cm) in four weeks and final TL and WW were not significantly different among these ration levels (Table C1). But, GFCE decreased as ration increased, and we removed uneaten food daily from the higher ration tanks, suggesting that some tanks were overfed (Figure C2a). Conversely, GFCE was high and no food went uneaten in the 4% treatment, but fish were significantly smaller at the end of four weeks (Figure C2a, Table C1). In addition, survival was lower and more variable and growth was more variable in the low ration treatments (Figure C2b, c, and d). Consistent growth and survival is important in baitfish production as fish are marketed based on size and count, like ornamental fishes (Olivier and Kaiser, 1997; Johnston et al., 2003). Thus, overfeeding is inefficient and could reduce water quality, and underfeeding results in lower and inconsistent growth and survival.

The optimal ration for pigfish grown at 24.7 °C as a function of their size is $R_{opt} = 11.19 \cdot WW^{-0.26}$. This equation indicates that fish at the beginning of the experiment required 14.7% WW day^{-1} to achieve maximum SGR, which decreased to 7.2% WW day^{-1} for 5.5 g fish at the end of the experiment R. Also, when WW is less than 1.15 g, R_{opt} is more than 10.7% WW day^{-1}, which was the minimum ration that maximized survival (Figure C2b). This is important because mortality was highest during the first week when fish were smallest.

R_{opt} defined for pigfish is within the range one would expect for fish 0.4 - 6.0 g WW, based on rations fed to other juvenile marine fishes. For example, the recommended ration for red drum (*Sciaenops ocellatus*) < 5 g WW is at least 7% WW d^{-1} (Gatlin, 2002). For red sea bream (*Pagrus major*), feeding guidelines suggest 11% at 0.3 g WW, 9% at 0.9 g, and 5.3 - 6.5% WW d^{-1} between 5 and 20 g (Koshio, 2002). Generally, summer flounder (*Paralichthys dentatus*), southern flounder (*Paralichthys lethostigma*), and winter flounder (*Pseudopleuronectes*

americanus) that weigh 1 - 2 g WW, require 15% WW d^{-1}, whereas these species require 5 % WW d^{-1} when they reach 8 g WW (Daniels and Gallagher, 2002). R_{opt} for freshwater Eurasian perch (*Perca fluviatilis*) between 0.22 – 18.9 g WW was expressed as $R_{opt} = 8.22 \cdot WW^{-0.32}$(Fiogbé and Kestemont, 2003), which is lower than our findings and a stronger effect of size. Differences in R_{opt} among species may be caused by species-specific differences or differences in temperature or feed type (Brett and Groves, 1979). Further research is needed to examine the effects of temperature and feed composition on R_{opt}, growth, and survival of juvenile pigfish.

Projected growth based on our equation for SGR highlights a beneficial characteristic of pigfish aquaculture: short time to market. By maintaining an optimal ration, 1.5 cm TL pigfish could reach to the low end of the market size range, 6 cm (equivalent to 4.9 g based on the length-weight relationship in this study: $TL = 3.715 \cdot WW^{0.31}$, $R^2 = 0.99$, $P < 0.001$), in 33 days and they could reach the high end of the market size range, 11 cm (32.6 g), in 69 days at 24.7°C. Considering that DiMaggio et al. (2013) grew larval pigfish from hatching to 1.5 cm TL in 30 days, pigfish could be grown from hatching to market in approximately two months. This would be achieved during grow-out by adjusting the ration weekly (using the equation for R_{opt}) from an initial level of 15% WW d^{-1} to 8% WW d^{-1} at 6.1 cm TL. Time to market would be even shorter if 24.7°C is not the optimal temperature for growth. By comparison, food fish must be grown to larger sizes and require longer grow-out times. For example, a 10 g juvenile cobia (*Rachycentron canadum*) has an SGR of 4.7% when fed 9% WW d^{-1} and reaches market size in a year or more (Sun et al., 2006; Benetti et al., 2008).

Raising captive spawned pigfish to market size for the bait industry could increase availability of this live bait to anglers during peak seasons and extend the period of availability. Cultured pigfish could also reduce pressure on wild pigfish populations to help maintain healthy ecosystems and promote sustainable fisheries. Combined with further research on optimal temperature, a definitive juvenile pigfish grow-out protocol can be developed. This protocol, along with refined captive spawning and larval rearing techniques, will create opportunities for commercial pigfish aquaculture.

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Table C1. Mean final total length (TL) and wet weight (WW) ± S.D. of juvenile pigfish reared on different rations (% WW d^{-1}) over a 4-week period. Initial TL and WW for all rations were 2.6 ± 0.36 cm and 0.4 ± 0.13 g, respectively. For each variable, ration levels with different superscript letters are significantly different (Tukey's HSD, $P < 0.05$).

Ration	Final size		Growth	
(% d^{-1})	TL (cm)	WW (g)	TL (cm)	WW (g)
4	5.1 ± 0.21^{a}	2.5 ± 0.61^{a}	2.5 ± 0.32	2.1 ± 0.48
8	6.1 ± 0.34^{b}	4.9 ± 1.20^{b}	3.5 ± 0.03	4.5 ± 1.07
12	6.4 ± 0.36^{b}	5.3 ± 0.61^{b}	3.8 ± 0.41	5.0 ± 0.53
16	6.3 ± 0.38^{b}	5.7 ± 0.80^{b}	3.7 ± 0.36	5.3 ± 0.70
20	6.3 ± 0.29^{b}	5.4 ± 0.84^{b}	3.7 ± 0.20	5.1 ± 0.72
24	6.4 ± 0.20^{b}	5.7 ± 0.55^{b}	3.8 ± 0.22	5.3 ± 0.44

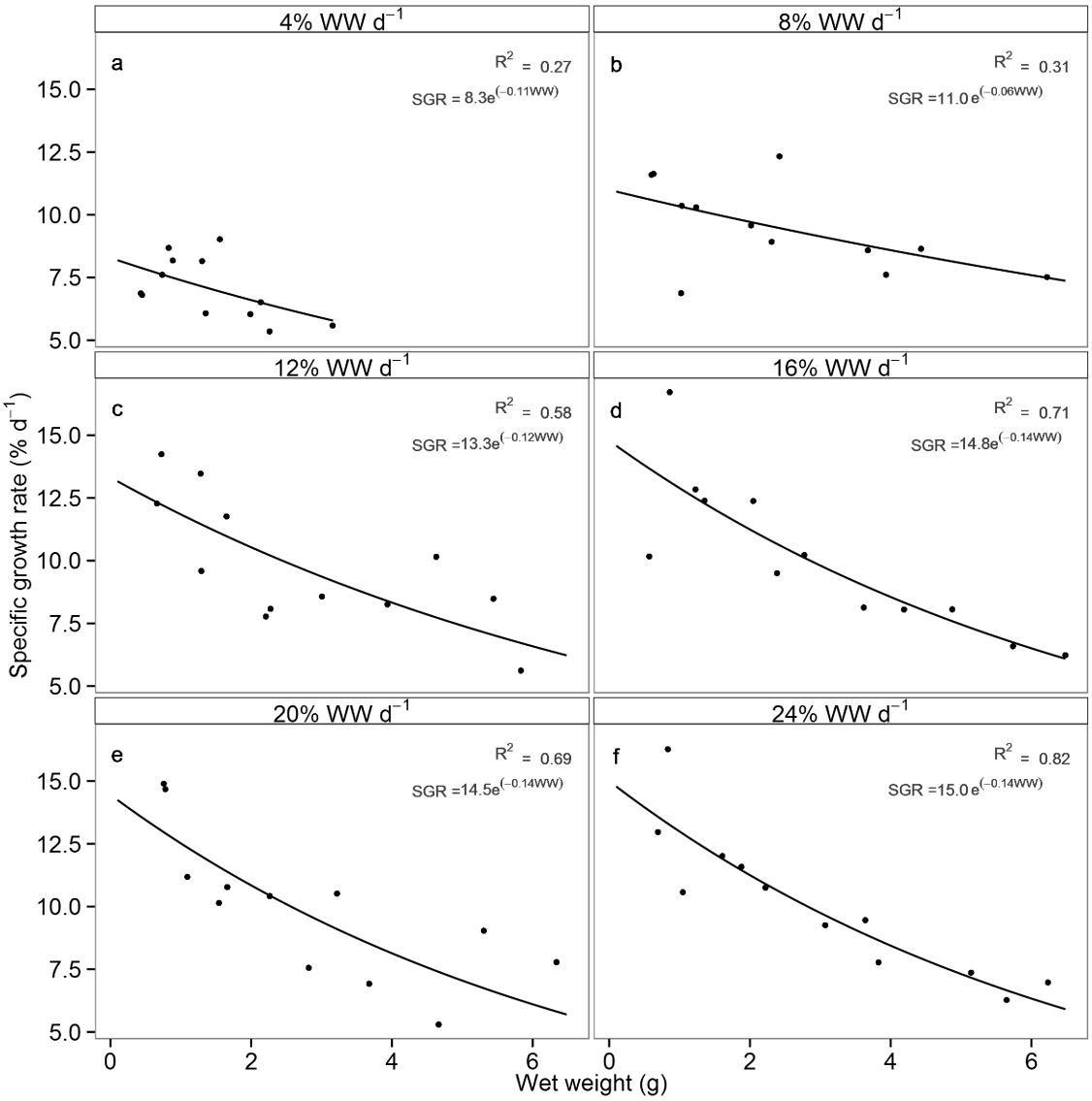

Figure C1. Change in daily specific growth rate (SGR, determined weekly) with wet weight (WW) for juvenile pigfish fed six ration levels (% WW d^{-1}) over a 4-week period. Ration level is shown above each graph. All regressions are significant ($P < 0.05$).

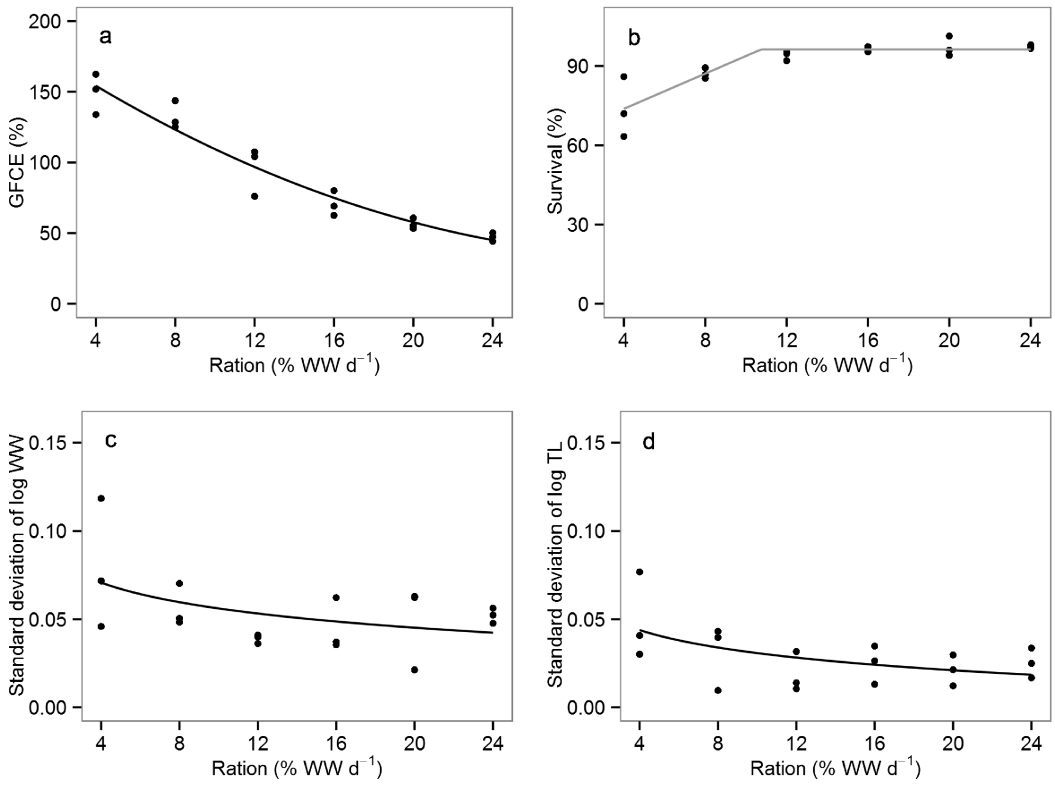

Figure C2. Effect of ration on (a) gross feed conversion efficiency (GFCE), (b) survival, and variability in (c) wet weight (WW) and (d) total length (TL) over 4 weeks. A second order polynomial equation described the relationship between GFCE and ration (GFCE = ration · 0.152 + ration · -9.47 + 189.79, R2 = 0.93, P<0.05). Piecewise regression was used for survival (survival = 3.33 · ration + 60.44 for ration < 10.75; survival = 96.27 for ration > 10.75, R2=0.79, P<0.05). Power functions were used to fit the data for (c) and (d) (standard deviation of the log final WW = -0.91 + ration-0.017, R2 = 0.22, P < 0.05; standard deviation of the log final TL = -0.94 + ration-0.015, R2 = 0.29, P < 0.05).

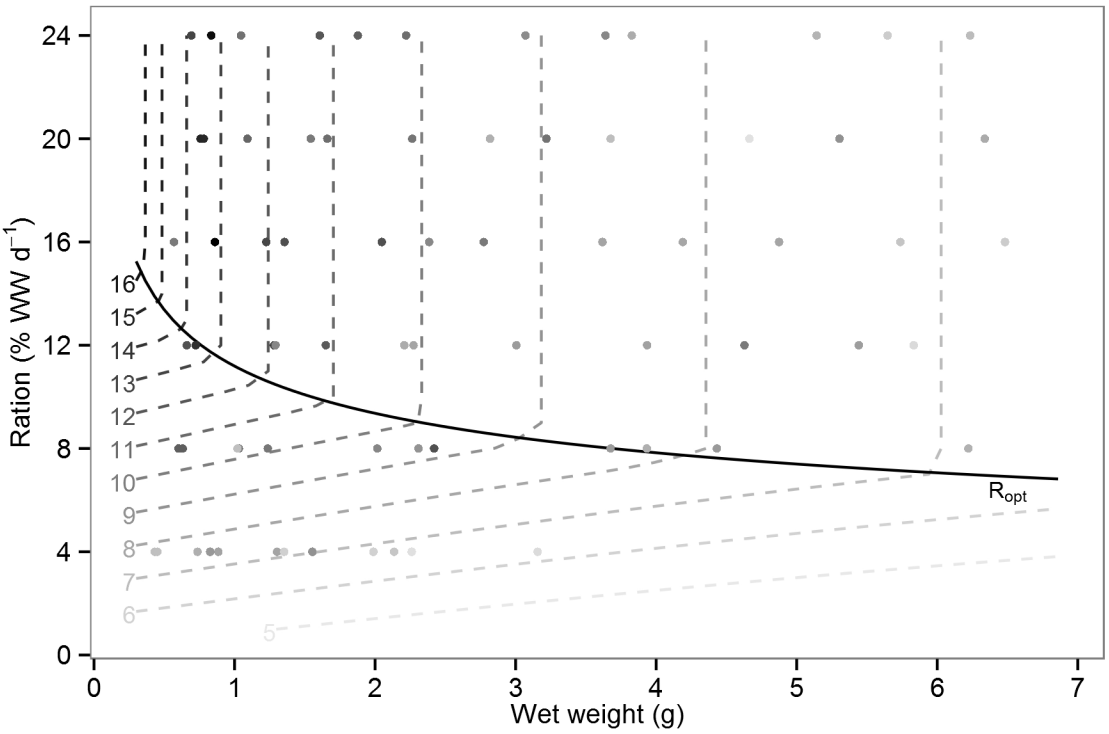

Figure C3. Response surface relating SGR (% d^{-1}) to ration and wet weight (dashed contour lines; $R^2 = 0.70$, $P < 0.05$), which defines the change in optimal ration (R_{opt}) with wet weight (solid line). Points are data from three replicate groups of pigfish raised on six different rations for four weeks (n = 72). Contour lines show SGR at intervals of 1% WW d^{-1}. The curve for R_{opt} is the continuous function ($R_{opt} = 11.19 \cdot WW^{-0.26}$) that connects the breakpoints at each ration level.

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