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Chapter 1

THE EFFECTS OF DIETARY ARACHIDONIC ACID (20:4N-6) ON GROWTH, SURVIVAL AND HYPERSALINE STRESS RESISTANCE IN LARVAL SOUTHERN FLOUNDER *PARALICHTHYS LETHOSTIGMA* (JORDAN AND GILBERT 1884)

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ABSTRACT

The effects of dietary arachidonic acid (ARA) on survival, growth, and hypersaline stress resistance of southern flounder larvae were determined from first feeding through metamorphosis. Larvae were fed rotifers and *Artemia* nauplii enriched with emulsions containing 10% docosahexaenoic acid (DHA) and five different levels of ARA (0, 1, 2, 4, and 6%). Larvae in a sixth treatment were fed unenriched rotifers cultured with *Nannochloropsis oculata* (0% DHA, 31% eicosapentaenoic acid, EPA and 3.94% ARA).

There were no significant differences ($P > 0.05$) among treatments in larval survival (41.9% ± 6.3), growth (notochord length = 6.8 mm ± 0.2, dry weight = 0.5 mg ± 0.06) or median survival time (41 min.) following hypersaline (60 g L^{-1}) challenge on d26 -d27ph. ARA and DHA levels in d27ph larvae reflected those of live prey, except for larvae fed unenriched prey (0% DHA), which contained 2.5% DHA, but with no diminution in performance. Results indicated that DHA and ARA prey enrichment was not required for southern flounder larvae fed rotifers cultured with *N. oculata*, suggesting sufficient basal levels of ARA in live prey and derivation of DHA from maternally-derived yolk reserves, or via biosynthesis from the precursor fatty acids α-linolenic acid (ALA), EPA, or docosapentaenoic acid (DPA).

INTRODUCTION

The nutritional aspects of essential fatty acids (EFA) in fish have been extensively studied with considerable attention focused on the n-3 polyunsaturated fatty acids (PUFA) requirements of marine fish, particularly eicosapentaenoic acid 20:5n-3 (EPA) and docosahexaenoic acid 22:6n-3 (DHA) (Watanabe, Izquierdo, Takeuchi, Satoh and Kitajima 1989; Takeuchi, Toyota, Satoh and Watanabe 1990; Watanabe 1993; Takeuchi, Masuda, Ishizaki, Watanabe, Kanematsu, Imaizumi and Tsukamoto 1996; Ibeas, Cejas, Fores, Badia, Gomes and Hernandez 1997). The essentiality of these fatty acids is based on their roles as necessary components of cell membranes and as precursors for prostaglandin hormones.

Most marine fish studied to date whose diets are rich in DHA and EPA are usually unable to chain elongate and further desaturate α-linolenic acid 18:3n-3 (ALA) to the more highly unsaturated EPA and DHA (Tacon, 1990; Besson-nart, Izquierdo, Salhi, Hernandez-Cruz, Gonzalez and Fernandez-Palacios 1999). This is caused by a deficiency in one of two enzymes in the conversion

pathway from ALA to EPA, i.e., the C18 to C20 elongase multienzyme complex or the Δ5 fatty acid desaturase (Bell and Sargent 2003). Since both EPA and DHA are required for good growth, they are considered as essential dietary requirements. However, in all of the larval marine fish species examined to date, DHA has been demonstrated to be superior to EPA in providing vitality to the larvae (Watanabe 1993). Essential fatty acid enrichment of live feed diets (rotifers and *Artemia* nauplii) is a routine aspect of larval marine fish aquaculture. Commercial preparations used to enrich live foods typically contain abundant amounts of DHA and lower levels of EPA (Koven, Barr, Lutzky, Ben-Atia, Weiss, Harel, Behrens and Tandler 2001).

Given that DHA is naturally found at high levels in neural tissue, it is thought to play a critical role in neural membrane structure and function, including visual acuity for optimum hunting success (Copeman, Parrish, Brown and Harel 2002). Elevated dietary EPA relative to DHA has been shown to have a negative impact on larval neural function leading to lower growth and survival (Copeman et al.,2002). In juvenile turbot *Scophthalmus maximus* (Linnaeus 1758), the most significant aspect of lipid metabolism in the developing brain was the accumulation of DHA, while no other fatty acid increased in percentage in total lipid (Mourente, Tocher and Sargent 1991). Futhermore, increased mortalities were shown in juvenile turbot when the DHA: EPA ratio in the diet was decreased from 13.8 to 2.2, without changing the percentage of total n-3 PUFA (Bell, Henderson, Pirie and Sargent 1985). Turbot larvae exhibited lower pigmentation success with lower DHA: EPA ratio in their diet (Rainuzzo, Reiten and Olsen 1997). In starved gilthead seabream *Sparus aurata* (Linnaeus 1758) larvae, DHA was highly conserved, indicating its particular importance during the larval growth stage (Koven, Kissil and Tandler 1989).

Another series of essential fatty acids, the n-6 series, are generally found in smaller proportion than the n-3 series in marine fish with a ratio of n-3: n-6 PUFA of 10 to 15:1 (Ackman, 1979) and have therefore received little attention in marine fish nutrition. Despite the relatively small amount of arachidonic acid (20:4n-6, ARA) represented in the tissue, it is also essential for marine carnivorous fish (Koven et al., 2001), because they are unable to chain elongate and further desaturate linoleic acid (18:2n-6, LA) to the highly unsaturated n-6 fatty acids such as ARA. Hence, enrichment of live foods with lipids rich in n-6 as well as n-3 fatty acids prior to feeding to marine fish larvae is necessary for optimal growth, survival and resistance to handling stress (Watanabe, Izquierdo, Takeuchi, Satoh, Kitajima 1983; Takeuchi et al., 1990; Koven et al., 2001; Carrier, Watanabe, Harel, Rezek, Seaton and Shafer

2011). The southern flounder is an important commercial fishery species in the US Atlantic coast and a promising candidate for aquaculture. While nutritional requirements of other larval flatfish species such as turbot and summer flounder *Paralichthys dentatus* (Linnaeus 1776) have been reported, relatively little published data is available on the nutritional needs of southern flounder larvae. The objectives of this study were to determine the effects of dietary arachidonic acid on survival, growth and stress resistance of southern flounder larvae raised from first feeding to the metamorphic stages.

METHODS

Experimental Animals

This study was conducted at the University of North Carolina Wilmington Center for Marine Science Aquaculture Facility, Wrightsville Beach, NC. Adult southern flounder were maintained in fiberglass recirculating (tank exchange = 1100% / day, makeup exchange = 10% / day) broodtanks (diameter = 2.5 m, depth = 1 m, volume = 4.9 m^3) under 34 g L^{-1} salinity and a controlled photothermal cycle (Watanabe, Woolridge and Daniels, 2006). Adult females at the post-vitellogenic stage of ovarian development were induced to spawn using luteinizing hormone releasing hormone – analogue (LHRH-a) (Berlinsky, King, Smith, Hamilton and Sullivan, 1996; Smith, McVey, Wallace 1999; Watanabe, Carroll and Daniels, 2001) at a dose of 50 µg kg^{-1} body weight. Floating (viable) eggs were separated from sinking (non-viable) eggs in a 2-L separatory funnel in 34 g L^{-1} seawater. Floating fertilized eggs were transferred to a 15-L separatory funnel before distribution to the rearing tanks.

Experimental System

The experimental system consisted of four temperature-controlled water baths (152 x 61 x 23 cm) each covered by a light hood (152 x 65 x 19 cm) and surrounded by a black polyethylene curtain. Each water bath contained eight cylindrical black plastic tanks (15-L working volume) in which the larvae were reared. Fresh water was continuously re-circulated through the baths and through a heater/chiller to maintain a constant temperature.

Illumination was provided by four 40-W fluorescent bulbs supplying full spectrum lighting. Light intensity was controlled by regulating the height of the hood and with shade cloth. Lights were equipped with automatic timers to control photoperiod.

Experimental Design

To study the effects of dietary ARA on southern flounder larval growth, survival and hypersaline stress resistance, first-feeding stage larvae were fed rotifers (*Brachionus rotundiformis)* and *Artemia franciscana* that were enriched in emulsions containing 10% DHA and five levels of ARA (0, 1, 2, 4 and 6%). In a sixth treatment, larvae were fed unenriched rotifers cultured with *Nannochloropsis oculata*, which contained no DHA and 3.94% ARA (Reed Mariculture, Campbell CA.) and newly hatched unenriched *Artemia* nauplii. Five replicate tanks were assigned to each treatment.

To begin the experiment, developing embryos were stocked using volumetric methods into tanks at 35 g L^{-1} salinity, a temperature of 17 °C and at a density of 47 embryos L^{-1}. Filtered (10-μm) UV-treated seawater was used for larval rearing experiments.

LIVE PREY ENRICHMENT

The DHA: ARA emulsions were formulated by combining DHAsco (DHA-rich single cell oil) and different ratios of ARAsco (ARA rich single cell oil) with olive oil and water with 5% lecithin, 1% ascorbic acid and 1% Tween-80 (with oil weight) to form a 1:1 oil/water mixture (Advanced Bionutrition, Columbia, MD, US). Emulsions were sealed and refrigerated for daily use. DHAsco and ARAsco are microbial-derived triglyceride oils that are rich in DHA and ARA, respectively. DHAsco was extracted from the heterotrophically grown algae *Crypthecodinium cohnii* and the ARAsco was extracted from the fungus *Mortirella alpina* (Martek Biosciences, Columbia, MD).

Prior to enrichment, rotifers were cultured in 150-L tanks under continuous illumination, 34 g L^{-1} and at temperatures of 22-25 °C. Rotifers were fed a diet of preserved microalgae *N. oculata* (Reed Mariculture, San Jose, California) at a rate of 0.75 g per million rotifers per day.

Artemia (Argent Laboratories, Redmond, WA, US) were hatched from decapsulated cysts 24 h before enrichment. During enrichment, rotifers and *Artemia* were held in 3-L beakers in aerated, UV-treated seawater at 34 g L^{-1}. Photoperiod and temperature were maintained at 24 L: 0 D and 25°C. Dissolved oxygen was maintained above 4 mg L^{-1}.

During enrichment, rotifer density was maintained at 500,000 L^{-1}. Rotifers were enriched for 8 h before feeding to the larvae. The emulsions were added at 0.1 g $500{,}000^{-1}$ rotifers at 0 h and 4 h. During the period of enrichment with DHA: ARA emulsions, the unenriched rotifers were fed preserved *N. oculata* at rate of 0.75 g per million rotifers.

During enrichment, *Artemia* density was maintained at 200,000 nauplii L^{-1}. *Artemia* were enriched for 16 h before feeding to the larvae. Emulsions were added at 0.3 g $250{,}000^{-1}$ nauplii at 0 h and 8 h. Samples of the enriched and unenriched rotifers and *Artemia* were collected on three separate days (N = 3) immediately after the enrichment period and stored at -20 ^{o}C until fatty acid analysis.

Feeding

On day 2 post hatch (d2ph), rotifers were added to the larval rearing tanks at a density of 5 mL^{-1}. Once feeding began, rotifer density was increased to 20 mL^{-1}. Rotifer density was maintained by quantifying the rotifers in each culture vessel using volumetric methods and adding the appropriate number of enriched rotifers to make up the difference on a daily basis. Background algae *N. oculata* was added daily to maintain a density of 300,000 cells mL^{-1}. On d22ph, newly hatched *Artemia* instar 1 nauplii were added at a density of 0.25 mL^{-1} in addition to enriched rotifers. By d24ph, rotifer feeding stopped and larvae were fed *Artemia* metanauplii enriched with the prescribed treatments at a density of 1 mL^{-1}.

Environmental Conditions

Temperature ($\bar{X} \pm$ SEM; 18.7 ± 0.03 °C), salinity (35 ± 0.05 g L^{-1}), dissolved oxygen (7.3 ± 0.01 mg L^{-1}), light intensity (410 ± 4.0 lux), and pH ($\bar{X}$ = 7.96, range = 7.8 - 8.1) were measured daily in each tank. Photoperiod was 18L: 6D (Moustakas, Watanabe and Copeland 2004).

Aeration was supplied at approximately 80-100 mL min^{-1} through diffusers (4 cm x 1.3 cm x 1.3 cm) placed on the bottom at the center of each tank (Mangino and Watanabe 2006). Once feeding began, 30-55% of water in each tank was exchanged daily, and the water surface was skimmed 3 times daily with a paper towel to remove oil films.

Growth and Survival

To monitor growth and survival, a minimum of 10 larvae were randomly sampled from each tank on d2, d5, d9, d12, d16, d19 and d26ph using volumetric methods. Larvae were anesthetized (0.3 g L^{-1} 2-phenoxyethanol in freshwater) then placed into a gridded 100 x 15-mm Petri dish. Living larvae were distinguished from dead ones by the presence or absence of a heartbeat as well as by opacity and appearance. Notochord length was measured with an ocular micrometer. Wet weights were recorded by weighing approximately 10 larvae on a microscope slide and then subtracting the weight of the slide using a Sartorius (Goettingen, Germany) electrobalance to the nearest 10 μg. To determine dry weights, larvae were dried at 60 °C until they reached a constant weight (72 h) and then re-weighed.

The larval density (number of larvae L^{-1}) was determined by dividing the number of larvae sampled by the volume of water collected. On d28ph, all remaining larvae were sampled for carcass fatty acid composition.

Fatty Acid Analysis

Sample preparation of live prey and larvae for fatty acid analysis was similar to Watanabe et al.,(1989). Samples were poured onto a 23-μm mesh screen which was blotted with a sponge from underneath to remove excess water. Samples (approx. 100-150 mg) were then transferred to a 1.2 mL cryogenic vial (Fisher Scientific US), filled with nitrogen, and then frozen (-20 °C) before lipid extraction and fatty acid analysis.

Total lipid and fatty acid analysis was conducted using a revised Folch, Lee and Stanley (1957) method using a chloroform/methanol mixture (1:1 v/v) to extract lipids from tissue. Total lipid wet weight was converted to total lipid dry weight (mg g^{-1}) based on the % dry weight values of larvae and prey of the same treatment groups. Lipid fatty acids were converted to their methyl esters (FAMEs) for GC analysis by the FAME reaction.

The first phase of the FAME reaction is a base catalyzed hydrolysis of the glycerol esters, which required the addition of 0.5 M NaOH/MeOH to the sample, and then heated (70-100 °C) for 30 minutes. The second phase is an esterification of the free fatty acids catalyzed by the addition of 1.5 mL of boron trifluoride-methanol (BF_3) and heated for an additional 30 minutes. FAMEs from all samples were identified using a Hewlett Packard 6890 gas chromatography flame-ionization-dectector (GC FID) (Hewlett Packard, US). Helium was used as the carrier gas. The column temperature profile was: 195 °C, hold for 8 min, increased to 270 at 15 °C min^{-1} and hold at 270 °C for 2 min (Rezek et al.,2010). FAME peaks were integrated using the HP Chemstation software package and individual FAMEs were identified by comparison of retention times to standards: GLC-84 (Nu Chek Prep US), eicosapentaenoic acid methyl ester, arachidonic acid methyl ester (Sigma-Aldrich US), stearidonic acid methyl ester (Cayman Chemical US).

Hypersaline Stress Resistance

On d13 and d27ph larvae were subjected to a hypersaline (60 g L^{-1}) challenge (Dhert, Lavens and Sorgeloos 1991). A minimum of 20 larvae were sampled from each rearing tank and transferred to a 100 x 15-mm Petri dish containing 50 mL of 60 g L^{-1} seawater at 20 °C. Survival was monitored at 5 min. intervals until complete mortality was observed. For each replicate tank, survival (%) was plotted over time and a second order polynomial regression was fitted to the data. Stress sensitivity index (ST50) was calculated as the time at which survival fell to 50%.

Analytical Methods

The effects of ARA on growth, survival, stress resistance and fatty acid composition were expressed as treatment means ± standard error of the mean (SEM). For percentage data, arcsine transformation was performed before analysis. To meet the assumptions of ANOVA, data were analyzed using Levene's test for homogeneity of variances. For data that met the assumptions of ANOVA, a Tukey-Kramer honesty significant difference test was used for multiple comparisons among means. If the data violated the assumptions of ANOVA a Kruskal-Wallis nonparametric pairwise comparison with Bonferoni adjustment factor was used for multiple comparisons among means. Analyses

were performed using the JMP IN version 7 statistical software (SAS Institute Inc.).

RESULTS

Fatty Acid Composition of Enriched Rotifers

Lipid and fatty acid composition of enriched rotifers are shown in Table 1. No significant differences ($P > 0.05$) were observed in total lipid (4.4 ± 0.68% or 255 ± 40.3 mg g^{-1} dry wt.). Saturated fatty acids (SFA) were significantly higher in unenriched rotifers (40.6 ± 1.3% TFA) compared to rotifers enriched with 10% DHA and 0, 1, 2, 4%, or 6% ARA (29.3 ± 1.1% TFA). Levels of monounsaturated fatty acids (MFA) in rotifers decreased significantly from 43.8 ± 1.5% TFA to 34.4 ± 0.4% TFA as levels of dietary ARA increased from 0 to 6% and were lowest in unenriched rotifers fed only *N. oculata* (25.3 ± 0.4% TFA).

Total n-3 PUFA's were significantly higher in unenriched rotifers (27.4 ± 1.0% TFA) compared to rotifers enriched with 10% DHA and 0, 1, 2, or 4% ARA (23.5 ± 0.65% TFA). Of the n-3 PUFA's, levels of ALA (5.8 ± 0.9% TFA), EPA (15.3 ± 0.5% TFA) and 22:5n-3 docosapentaenoic acid (DPA) (6.3 ± 0.4% TFA) were significantly higher in unenriched rotifers compared to all other treatments (2.38 ± 0.21%, 7.9 ± 1.0, 2.9 ± 0.2% TFA, respectively). Unenriched rotifers fed only *N. oculata* contained no detectable levels of DHA, while no significant differences in DHA levels were observed among rotifers enriched with 10% DHA and 0, 1, 2, 4, or 6% ARA (10.5 ± 1.1% TFA).

There was a trend toward higher total n-6 PUFA's as level of ARA enrichment increased, from 4.2 ± 0.3% at 0% ARA to 12.0 ± 0.6 % ARA at 6% ARA. Total n-6 PUFA's were significantly higher in rotifers enriched with 6% (12.0 ± 0.6% TFA) and 4% ARA (10.1 ± 0.7% TFA) compared to 2% ARA (7.4 ± 0.1% TFA), 1% ARA (5.4 ± 0.1% TFA) and 0% ARA (4.2 ± 0.3% TFA). Unenriched rotifers had an intermediate n-6 PUFA level of 8.6 ± 0.7%. Likewise, concentration of ARA increased from 1.5 ± 0.2% TFA at 0% ARA to 8.2 ± 0.5% TFA at 6% ARA, while unenriched rotifers contained an intermediate ARA level (3.5 ± 0.3% TFA).

Table 1. Total lipid and fatty acid composition (% of total fatty acid) (mean ± SEM, *N* = 3) of rotifers *Brachionus rotundiformis* enriched with five different oil emulsions consisting of 10% DHA and varying levels of ARA (0%, 1%, 2%, 4%, 6%) and an unenriched group

Rotifer Treatments (DHA:ARA)						
Fatty acids	10:0	10:1	10:2	10:4	10:6	Unenriched
14:0	3.70 ± 0.30	3.9 ± .2	4.5 ± 0.2	4.2 ± 0.1	4.7 ± 0.1	4.0 ± 0.7
16:0	15.3 ± 0.8^{a}	14.6 ± 0.9^{a}	17.6 ± 1.4^{a}	17.2 ± 0.9^{a}	17.3 ± 0.9^{a}	25.1 ± 1.5^{b}
16:1n-7	7.2 ± 0.9^{a}	7.3 ± 0.9^{a}	8.4 ± 1.2^{a}	8.3 ± 1.1^{a}	8.4 ± 1.2^{a}	15.3 ± 1.6^{b}
18:0	8.4 ± 0.3^{b}	8.1 ± 0.2^{ab}	6.3 ± 0.4^{ab}	7.1 ± 0.5^{ab}	5.7 ± 0.2^{a}	8.2 ± 1.3^{ab}
18:1n-9	35.9 ± 2.5^{c}	36.1 ± 1.8^{c}	30.3 ± 2.4^{bc}	26.5 ± 1.5^{b}	25.3 ± 1.4^{b}	10.0 ± 1.2^{a}
18:2n-6	2.7 ± 0.1^{a}	2.8 ± 0.1^{ab}	3.7 ± 0.1^{abc}	4.2 ± 0.5^{bc}	3.8 ± 0.1^{abc}	5.1 ± 0.5^{c}
18:3n-3	2.7 ± 0.3^{a}	2.6 ± 0.2^{a}	2.4 ± 0.2^{a}	2.2 ± 0.2^{a}	2.0 ± 0.1^{a}	5.8 ± 0.9^{b}
20:0	1.4 ± 0.7^{a}	1.1 ± 0.3^{a}	1.8 ± 0.2^{ab}	1.8 ± 0.2^{ab}	1.7 ± 0.1^{ab}	3.3 ± 0.5^{b}
20:1	0.7 ± 0.3	Undetected	Undetected	0.6 ± 0.3	0.7 ± 0.3	Undetected
20:4n-6	1.5 ± 0.2^{a}	2.6 ± 0.1^{b}	3.7 ± 0.1^{b}	5.9 ± 0.5^{c}	8.2 ± 0.5^{d}	3.5 ± 0.3^{b}
20:5n-3	7.4 ± 0.9^{a}	7.2 ± 0.8^{a}	8.2 ± 1.0^{a}	8.4 ± 1.1^{a}	8.1 ± 1.0^{a}	15.3 ± 0.5^{b}
22:5n-3	2.8 ± 0.3^{a}	2.8 ± 0.1^{a}	3.1 ± 0.3^{a}	2.8 ± 0.3^{a}	3.1 ± 0.1^{a}	6.3 ± 0.4^{b}
22:6n-3	10.4 ± 0.9	10.8 ± 0.8	10.0 ± 1.3	10.4 ± 1.6	11.1 ± 1.1	Undetected
∑SFA	28.8 ± 0.6^{a}	27.7 ± 0.6^{a}	30.2 ± 1.6^{a}	30.4 ± 1.4^{a}	29.3 ± 1.2^{a}	40.6 ± 1.3^{b}
∑MFA	43.8 ± 1.5^{d}	43.4 ± 0.9^{cd}	38.8 ± 1.3^{bc}	35.3 ± 1.0^{b}	34.4 ± 0.4^{b}	25.3 ± 0.4^{a}
∑n-3 PUFA	23.2 ± 0.9^{a}	23.4 ± 0.3^{a}	23.6 ± 0.4^{a}	23.8 ± 1.0^{a}	24.2 ± 0.4^{ab}	27.4 ± 1.0^{b}
∑n-6 PUFA	4.2 ± 0.3^{a}	5.4 ± 0.1^{ab}	7.4 ± 0.1^{bc}	10.1 ± 0.7^{de}	12.0 ± 0.6^{e}	8.6 ± 0.7^{cd}
n3/n6 PUFA	5.5 ± 0.3^{d}	4.3 ± 0.1^{c}	3.2 ± 0.1^{b}	2.4 ± 0.2^{ab}	2.0 ± 0.1^{a}	3.2 ± 0.4^{bc}
DHA/EPA	1.5 ± 0.3	1.6 ± 0.3	1.3 ± 0.4	1.4 ± 0.4	1.4 ± 0.3	na
DHA/ARA	7.1 ± 1.2^{b}	4.2 ± 0.4^{ab}	2.7 ± 0.3^{a}	1.8 ± 0.1^{a}	1.4 ± 0.1^{a}	na
EPA/ARA	4.8 ± 0.3^{c}	2.8 ± 0.4^{b}	2.2 ± 0.3^{ab}	1.5 ± 0.3^{ab}	1.0 ± 0.2^{a}	4.4 ± 0.4^{c}
Total lipid %	5.3 ± 0.3	5.6 ± 1.2	4.0 ± 0.6	4.9 ± 0.4	3.9 ± 1.1	2.5 ± 0.5
Total lipid mg g^{-1}	311 ± 18.3	327 ± 67.8	233 ± 34.4	283 ± 23.9	228 ± 65.1	147 ± 32.1

Column titles refer to the percentage of DHA: ARA in each enrichment emulsion.

Fatty acids in trace amounts (< 0.5%) are not reported.

Treatment means are compared using ANOVA ($P < 0.05$), and a Tukey-Kramer HSD was used for multiple comparisons among means.

Means not sharing a common letter are significantly ($P < 0.05$) different.

Total lipid is reported in mg g^{-1} dry weight.

ANOVA, analysis of variance; ARA, arachidonic acid; DHA docosahexaenoic acid; PUFA, polyunsaturated fatty acids.

The ratio of n3/n6 PUFA was significantly higher in the rotifers enriched 10% DHA and 0% ARA (5.5 ± 0.3) than in all other treatments (3.02 ± 0.18).

No significant differences in the ratio of DHA/EPA (1.4 ± 0.3) were observed. The ratio of DHA/ARA was significantly higher in rotifers enriched with 10% DHA and 0% ARA (7.1 ± 1.2) than in rotifers enriched with 2, 4 and 6% ARA (2.0 ± 0.2).

The ratio of EPA/ARA was significantly higher in rotifers enriched with 10% DHA and 0% ARA (4.8 ± 0.3) and in unenriched (4.4 ± 0.4) rotifers than in those enriched with 1, 2, and 4% ARA (2.17 ± 0.3), while rotifers enriched with 6% ARA (1.0 ± 0.2) showed the lowest level (Table 1).

Fatty Acid Composition of Enriched *Artemia*

Lipid and fatty acid composition of enriched *Artemia* are shown in Table 2. No significant differences were observed in total lipid (3.6 ± 0.37% or 264 ± 27.1 mg g^{-1} dry wt.). Total SFA was significantly higher in the 4% ARA (19.8 ± 0.1% TFA) treatment compared to the 0, 1, 2, and 6% ARA (17.8 ± 0.4 TFA) and the unenriched (18.0 ± 0.1) treatments. A trend toward decreasing MFA with increasing dietary ARA was observed from 49.6 ± 2.0 TFA at 0% ARA to 41.1 ± 0.9 TFA at 6% ARA. Unenriched *Artemia* had an intermediate MFA of 31.8 ± 0.6%.

Total n-3 PUFA levels in enriched *Artemia* were significantly higher in the 4% ARA (36.3 ± 0.4% TFA) and in the unenriched (39.8 ± 0.1% TFA) treatments compared to the 0, 1, 2 and 6% ARA treatments (28.4 ± 0.9% TFA) (Table 2).

Of the n-3 PUFA's, ALA was significantly higher in unenriched *Artemia* (34.3 ± 0.4% TFA) compared to the enriched *Artemia* (20.5 ± 0.9 TFA). A small amount (1.2 ± 0.6% TFA) of 18:4n-3 stearidonic acid (STD) was detected in all treatments, except in *Artemia* fed 0% and 4% ARA. Levels of EPA were significantly higher in the unenriched *Artemia* (5.5 ± 0.3% TFA) than in *Artemia* enriched with 0, 1 and 4% ARA (3.4 ± 0.3% TFA), but not with 2 and 6% ARA (4.1 ± 0.2 TFA).

DHA levels in *Artemia* (range = 3.8 – 8.7% TFA) were considerably lower than provided in their emulsions diets (10%), with significant variation among treatments. DHA levels were significantly higher in *Artemia* enriched with 0, 1 and 4% ARA (7.1 ± 0.6 % TFA) than in those enriched with 2% (3.8 ± 0.7% TFA) and 6% ARA and (4.3 ± 0.4% TFA). DHA was not detected in unenriched *Artemia*.

Table 2. Total lipid and fatty acid composition (% of total fatty acid) (mean ± SEM, *N* = 3) of *Artemia* enriched with five different oil emulsions consisting of 10% DHA and varying levels of ARA (0%, 1%, 2%, 4%, 6%) and an unenriched group

Artemia Treatments (DHA:ARA)						
Fatty acids	10:0	10:1	10:2	10:4	10:6	Unenriched
14:0	2.1 ± 0.3^{ab}	2.1 ± 0.3^{ab}	1.6 ± 0.1^{ab}	2.4 ± 0.5^{b}	1.6 ± 0.1^{ab}	1.1 ± 0.03^{a}
16:0	10.8 ± 0.3	10.8 ± 0.3	11.1 ± 0.4	10.6 ± 0.2	11.0 ± 0.1	11.7 ± 0.1
16:1n-7	3.8 ± 0.3^{ab}	4.0 ± 0.2^{ab}	4.6 ± 0.4^{ab}	3.7 ± 0.1^{a}	4.9 ± 0.2^{b}	6.4 ± 0.1^{c}
18:0	3.8 ± 1.1	5.4 ± 0.1	5.2 ± 0.1	6.1 ± 0.4	5.1 ± 0.1	5.2 ± 0.1
18:1n-9	45.8 ± 1.7^{c}	42.0 ± 0.7^{bc}	38.0 ± 2.2^{b}	29.9 ± 0.6^{a}	36.1 ± 1.1^{b}	25.4 ± 0.7^{a}
18:2n-6	4.7 ± 0.1^{a}	4.8 ± 0.2^{a}	5.3 ± 0.02^{a}	4.9 ± 0.1^{a}	5.5 ± 0.1^{ab}	6.5 ± 0.5^{b}
18:3n-3	17.4 ± 1.2^{a}	17.6 ± 0.2^{ab}	21.8 ± 1.5^{bc}	24.7 ± 0.3^{c}	20.9 ± 1.1^{abc}	34.3 ± 0.4^{d}
18:4n-3	Undetected	0.88± 0.4	1.2 ± 0.6	Undetected	1.1 ± 0.6	1.5 ± 0.9
20:4n-6	1.1 ± 0.1^{a}	1.9 ± 0.2^{b}	2.5 ± 0.3^{b}	5.2 ± 0.2^{c}	5.2 ± 0.6^{c}	2.1 ± 0.2^{b}
20:5n-3	3.5 ± 0.3^{a}	3.8 ± 0.4^{a}	4.2 ± 0.3^{ab}	3.0 ± 0.3^{a}	4.1 ± 0.1^{ab}	5.5 ± 0.3^{b}
22:6n-3	6.3 ± 0.3^{cd}	6.4 ± 0.3^{cd}	3.8 ± 0.7^{b}	8.7 ± 1.1^{d}	4.3 ± 0.4^{bc}	Undetected
∑SFA	16.9 ± 0.4^{a}	18.3 ± 0.5^{ab}	18.2 ± 0.5^{a}	19.8 ± 0.1^{b}	17.8 ± 0.3^{a}	18.0 ± 0.1^{a}
∑MFA	49.6 ± 2.0^{c}	46.1 ± 0.5^{bc}	42.5 ± 1.8^{b}	33.6 ± 0.5^{a}	41.1 ± 0.9^{b}	31.8 ± 0.6^{a}
∑n-3 PUFA	27.3 ± 1.7^{a}	27.8 ± 0.3^{a}	30.0 ± 0.7^{a}	36.3 ± 0.4^{b}	29.2 ± 0.6^{a}	39.8 ± 0.1^{b}
∑n-6 PUFA	5.6 ± 0.2^{a}	6.7 ± 0.3^{ab}	7.7 ± 0.3^{b}	10.0 ± 0.1^{cd}	10.7 ± 0.7^{d}	8.6 ± 0.7^{bc}
n3/n6 PUFA	4.8 ± 0.1^{c}	4.2 ± 0.1^{bc}	3.9 ± 0.2^{bc}	3.6 ± 0.1^{ab}	2.8 ± 0.2^{a}	4.7 ± 0.4^{c}
DHA/EPA	1.8 ± 0.1^{ab}	1.7 ± 0.3^{ab}	0.9 ± 0.1^{a}	3.1 ± 0.7^{b}	1.0 ± 0.1^{a}	na
DHA/ARA	6.2 ± 0.1^{c}	3.4 ± 0.4^{b}	1.5 ± 0.1^{a}	1.7 ± 0.2^{a}	0.8 ± 0.1^{a}	na
EPA/ARA	3.4 ± 0.1^{d}	1.2 ± 0.1^{b}	1.7 ± 0.1^{b}	0.6 ± 0.1^{a}	0.8 ± 0.1^{ab}	2.6 ± 0.1^{c}
Total lipid %	3.5 ± 0.2	2.8 ± 0.1	3.2 ± 0.4	4.1 ± 0.4	3.4 ± 0.6	4.3 ± 0.5
Total lipid mg g^{-1}	262 ± 13.3	211 ± 10.2	239 ± 31.8	302 ± 27.1	250 ± 43.0	321 ± 37.4

Column titles refer to the percentage of DHA:ARA in each enrichment emulsion. Fatty acids in trace amounts (< 0.5%) are not reported.

Treatment means are compared using ANOVA ($P < 0.05$), and a Tukey-Kramer HSD was used for multiple comparisons among means.

Means not sharing a common letter are significantly ($P < 0.05$) different.

Total lipid is reported in mg g^{-1} dry weight.

ANOVA, analysis of variance; ARA, arachidonic acid; DHA docosahexaenoic acid; PUFA, polyunsaturated fatty acids.

The n-6 PUFA's in *Artemia* increased with ARA levels in the emulsions and were significantly higher at the 4% (10.0 $\pm$ 0.1% TFA) and 6% (10.7 $\pm$ 0.7% TFA) ARA levels than in the 1% (6.6 $\pm$ 0.3% TFA) and 2% ARA (7.7 $\pm$ 0.3% TFA) levels and were lowest at 0% ARA (5.6 $\pm$ 0.2% TFA).

Unenriched *Artemia* had an intermediate level of n-6 PUFA's (8.6 ± 0.7 TFA). Likewise, concentration of ARA in enriched *Artemia* increased significantly with increasing level of ARA enrichment from 1.1 ± 0.1% TFA at 0% ARA to 5.2 ± 0.6% TFA at 6% ARA. Unenriched *Artemia* contained an intermediate ARA concentration (2.1 ± 0.2% TFA).

The ratio of n3/n6 PUFA significantly declined with increasing level of ARA enrichment from 4.8 ± 0.1 at 0% ARA to 2.8 ± 0.2 at 6% ARA. The n3/n6 PUFA ratio was significantly higher in *Artemia* enriched with 10% DHA and from 0% (4.8 ± 0.1) to 2% ARA (3.9 ± 0.2) than in those enriched with 6% ARA (2.8 ± 0.2). The n3/n6 ratio in unenriched *Artemia* (4.7 ± 0.4) was not significantly different from those enriched with 0% ARA. The DHA/EPA ratio was significantly higher in *Artemia* enriched with 10% DHA and 4% ARA (3.1 ± 0.7) than in *Artemia* enriched with 2% (0.9 ± 0.1) or 6% ARA (1.0 + 0.1), but was not different from *Artemia* enriched with 0% or 1% ARA (1.8 ± 0.2).

The ratio of DHA/ARA declined significantly as level of ARA enrichment increased, from 6.2 ± 0.1 in *Artemia* enriched with 10% DHA and 0% ARA to 0.8 ± 0.1 in those enriched with 6% ARA. The EPA/ARA ratio was significantly higher in *Artemia* enriched with 10% DHA and 0% ARA (3.4 ± 0.1) than in *Artemia* enriched with 1%, 2% and 6% ARA (1.2 ± 0.1), while lowest values were observed 4% ARA (0.6 ± 0.1). Unenriched *Artemia* had a relatively high EPA/ARA ratio of 2.6 ± 0.1, significantly lower than the 0% ARA treatment.

Fatty Acid Composition of D27ph Larvae

Whole body lipid and fatty acid composition of southern flounder larvae d27ph are shown in Table 3. No significant differences were observed in total lipid (3.5 ± 0.5% or 272 ± 42.4 mg g^{-1} dry wt.). Concentration of SFA was significantly higher in larvae fed the unenriched prey (30.9 ± 0.2% TFA) than in the other treatments (26.8 ± 0.5% TFA). Total MFA was not significantly different among larvae fed prey enriched with 0%, 1% or 2% ARA (29.2 ± 1.3% TFA). MFA was significantly higher in larvae fed prey enriched with 1% ARA (32.6 ± 0.6% TFA) than in the 4% and 6% ARA, or unenriched prey treatments (23.9 ± 1.1% TFA). Total n-3 PUFA levels in d27ph larval tissue were significantly lower in larvae fed prey enriched with 1% ARA (29.1 ± 1.0% TFA) than in all other treatments (33.7 ± 0.6% TFA).

Table 3. Total lipid and fatty acid composition (% of total fatty acid) (mean ± SEM, N = 5) of d27ph larvae fed enriched rotifers *Brachionus rotundiformis* and *Artemia,* using 5 different oil emulsions consisting of 10% DHA and varying levels of ARA (0%, 1%, 2%, 4%, 6%) and an unenriched group

Enriched diets received by larvae (DHA:ARA)						
Fatty acids	10:0	10:1	10:2	10:4	10:6	Unenriched
14:0	1.0 ± 0.5	1.3 ± 0.1	1.3 ± 0.0	1.4± 0.1	1.0 ± 0.4	1.0 ± 0.5
16:0	16.4 ± 0.5	16.2 ± 0.1	16.5 ± 0.6	17.7 ± 0.3	17.2 ± 0.7	19.9 ± 1.8
16:1n-7	3.9 ± 0.3^{ab}	4.3 ± 0.1^{ab}	4.2 ± 0.2^{ab}	3.9 ± 0.0^{a}	2.6 ± 1.3^{b}	4.5 ± 0.1^{c}
18:0	5.7 ± 0.2	6.7 ± 0.7	5.7 ± 0.1	5.1 ± 0.1	6.0 ± 0.5	6.7 ± 0.7
18:1n-9	23.2 ± 1.1^{b}	25.8 ± 0.4^{ab}	21.9 ± 0.9^{ab}	20.3 ± 0.3^{ab}	22.2 ± 1.7^{ab}	18.1 ± 1.7^{a}
18:2n-6	8.2 ± 0.3^{ab}	7.7 ± 0.2^{a}	7.7 ± 0.3^{ab}	8.7 ± 0.1^{ab}	9.1 ± 0.4^{ab}	10.2 ± 1.1^{b}
18:3n-3	16.0 ± 1.8	13.3 ± 2.1	15.1 ±1.5	13.4 ± 0.2	13.6 ± 2.9	17.0 ± 1.6
20:0	3.2 ± 0.2	2.9 ± 0.2	3.1± 0.2	3.9 ± 0.5	2.1 ± 1.1	3.2 ± 1.8
20:4n-6	3.1 ± 0.3^{a}	3.3 ± 0.1^{b}	4.2 ± 0.4^{b}	5.7 ± 0.1^{c}	6.8 ± 0.8^{c}	3.9 ± 0.3^{b}
20:5n-3	6.0 ± 0.1^{a}	5.0 ± 0.0^{a}	6.1 ± 0.1^{ab}	5.0 ± 0.1^{a}	5.5 ± 0.2^{ab}	8.2 ± 0.4^{b}
22:5n-3	3.1 ± 0.4	2.7 ± 0.2	3.7 ± 0.4	3.2 ± 0.1	4.0 ± 0.6	4.6 ± 2.3
22:6n-3	9.3 ± 2.3^{cd}	8.1 ± 1.0^{cd}	9.4 ± 1.7^{b}	11.6 ± 0.1^{d}	10.7 ± 2.0^{bc}	2.6 ± 1.3^{a}
∑SFA	26.3 ± 0.2^{a}	27.2 ± 0.5^{a}	26.5 ± 0.3^{a}	28.1 ± 0.8^{a}	25.7 ± 0.7^{a}	30.9 ± 0.2^{b}
∑MFA	27.8 ± 1.6^{ab}	32.6 ± 0.6^{b}	27.1 ± 1.7^{ab}	24.3 ± 0.3^{a}	24.8 ± 1.2^{a}	22.6 ± 1.8^{a}
∑n-3 PUFA	34.5 ± 1.1^{b}	29.1 ± 1.0^{a}	34.3 ± 0.7^{b}	33.2 ± 0.5^{b}	33.9 ± 0.6^{b}	32.5 ± 1.0^{ab}
∑n-6 PUFA	11.4 ± 0.6^{a}	11.0 ± 0.1^{ab}	12.0 ± 0.7^{b}	14.4 ± 0.1^{cd}	16.1 ± 1.2^{d}	14.1 ± 1.4^{bc}
n3/n6	3.0 ± 0.1^{c}	2.7 ± 0.1^{abc}	2.9 ± 0.1^{bc}	2.3 ± 0.0^{ab}	2.2 ± 0.2^{a}	2.4 ± 0.3^{abc}
DHA/EPA	1.5 ± 0.4^{ab}	1.6 ± 0.2^{b}	1.5 ± 0.3^{ab}	2.3 ± 0.0^{b}	1.9 ± 0.4^{b}	0.3 ± 0.2^{a}
DHA/ARA	2.9 ± 0.4^{c}	2.5 ± 0.2^{bc}	2.2 ± 0.2^{bc}	2.0 ± 0.0^{bc}	1.5 ± 0.1^{ab}	0.7 ± 0.4^{a}
EPA/ARA	1.9 ± 0.2^{cd}	1.5 ± 0.1^{bc}	1.4 ± 0.1^{b}	0.9 ± 0.0^{a}	0.8 ± 0.1^{a}	2.1 ± 0.1^{d}
Total lipid %	3.5 ± 0.2	3.7 ± 0.1	3.0 ± 0.6	3.5± 0.7	3.5 ± 1.0	3.8 ± 0.5
Total lipid mg g^{-1}	207 ±12.8	296 ± 12.2	239 ± 43.9	286± 58.4	296 ± 83.2	309 ± 44.0

Column titles refer to the percentage of DHA: ARA in each enrichment emulsion. Fatty acids in trace amounts (< 0.5%) are not reported.

Treatment means are compared using ANOVA ($P < 0.05$), and a Tukey-Kramer HSD was used for multiple comparisons among means.

Means not sharing a common letter are significantly ($P< 0.05$) different.

Total lipid is reported in mg g^{-1} dry weight.

ANOVA, analysis of variance; ARA, arachidonic acid; DHA docosahexaenoic acid; PUFA, polyunsaturated fatty acids.

ALA ranged from 13.3 $\pm$ 2.1 to 17.0 $\pm$ 1.7% TFA among treatments, with no significant differences.

EPA levels were significantly higher in larvae fed unenriched prey (8.2 ± 0.4% TFA) than in the 0, 1, and 4% ARA treatments (5.3 ± 0.1% TFA), but not the 2 and 6% ARA (5.8 ± 0.2% TFA) treatments.

DHA concentration in larvae fed prey enriched with 10% DHA ranged from 8.1 ± 1.0% at 1% ARA to 11.6 ± 0.1% TFA at 4% ARA, significantly higher than in larvae fed unenriched prey (2.6 ± 1.3% TFA). DPA ranged from 2.7 ± 0.2 to 4.6 ± 2.3% TFA among treatments, with no significant differences.

The total n-6 PUFA concentration was significantly higher in larvae fed 4% and 6% ARA (15.3 ± 0.7% TFA) than in larvae fed 0%, 1% or 2% ARA (11.5 ± 0.5% TFA), or unenriched (14.1 ± 1.4% TFA) prey.

Concentration of ARA in the larval tissue increased with increasing dietary ARA and was significantly higher in larvae fed 4% and 6% ARA (6.3 ± 0.5% TFA) than in those fed 1% and 2% ARA or unenriched prey (3.8 ± 0.3% TFA) prey and lowest at 0% ARA (3.1 ± 0.3% TFA).

Growth

For the duration of the experiment (d2ph through d26ph), no significant treatment effects were found on larval notochord length, wet weight or dry weight (Table 4). By d26ph, mean notochord length, wet weight and dry weight among treatments were 6.77 ± 0.17 mm, 3.87 ± 0.21 mg and 0.51 ± 0.06 mg, respectively.

Survival and Hypersaline Stress Evaluation

There were no significant differences in survival among treatment groups from d2ph (71.6 ± 2.7%) to d26ph (41.9 ± 2.1%) (Table 5). On d26ph, mean survival in all treatment groups receiving rotifers and *Artemia* enriched with 10% DHA was 41.3 ± 2.4%, while survival in larvae fed unenriched prey was 44.9 ± 5.9%.

During the hypersaline challenge, median survival time (ST50) among larvae in all treatments averaged 12.6 ± 3.0 min on d13ph (Table 6), but increased to 40.2 ± 7.0 min on d27ph, with no significant differences.

Table 4. Notochord length (NL, mm), wet weight (WW, mg) and dry weight (DW, mg) (mean ± SEM, *N* = 5) of larvae fed rotifers *Brachionus rotundiformis* and *Artemia*, enriched with five different oil emulsions consisting of 10% DHA and varying levels of ARA (0%, 1%, 2%, 4%, 6%) and an unenriched group. No significant treatment differences were observed for any parameter on any sampling date

	Age (dph)	10:0	10:1	10:2	10:4	10:6	*N. oculata*
NL	2	3.42 ± 0.03	3.39 ± 0.04	3.37 ± 0.01	3.29 ± 0.02	3.36 ± 0.04	3.33 ± 0.01
WW	2	0.24 ± 0.02	0.24 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.26 ± 0.01
DW	2	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
NL	5	3.39 ± 0.02	3.40 ± 0.03	3.39 ± 0.02	3.38 ± 0.02	3.37 ± 0.05	3.38 ± 0.01
WW	5	0.22 ± 0.02	0.25 ± 0.01	0.24 ± 0.01	0.24 ± 0.02	0.26 ± 0.01	0.23 ± 0.01
DW	5	0.03 ± 0.00	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.02	0.04 ± 0.00
NL	9	3.85 ± 0.03	3.80 ± 0.03	3.82 ± 0.04	3.76 ± 0.07	3.74 ± 0.06	3.79 ± 0.06
WW	9	0.40 ± 0.01	0.38 ± 0.01	0.42 ± 0.03	0.37 ± 0.02	0.38 ± 0.01	0.41 ± 0.02
DW	9	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
NL	12	4.19 ± 0.13	4.19 ± 0.07	4.24 ± 0.03	4.25 ± 0.04	4.20 ± 0.07	4.28 ± 0.10
WW	12	0.63 ± 0.05	0.58 ± 0.05	0.55 ± 0.05	0.55 ± 0.05	0.53 ± 0.04	0.62 ± 0.04
DW	12	0.08 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
NL	16	4.96 ± 0.10	5.02 ± 0.05	4.92 ± 0.07	5.04 ± 0.06	5.07 ± 0.12	4.96 ± 0.22
WW	16	1.10 ± 0.17	1.20 ± 0.07	1.14 ± 0.09	1.17 ± 0.09	1.21 ± 0.08	1.22 ± 0.14
DW	16	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.02
NL	19	5.67 ± 0.11	5.71 ± 0.13	5.98 ± 0.10	5.69 ± 0.10	5.82 ± 0.22	5.87 ± 0.14
WW	19	1.99 ± 0.06	1.82 ± 0.12	2.01± 0.06	1.17 ± 0.12	1.99 ± 0.2	2.0 ± 0.18
DW	19	0.25 ± 0.01	0.23 ± 0.01	0.26 ± 0.01	0.22 ± 0.01	0.25 ± 0.02	0.23 ± 0.01
NL	26	6.70 ± 0.15	6.75 ± 0.17	6.84 ± 0.28	6.82 ± 0.09	6.76 ± 0.16	6.73 ± 0.18
WW	26	3.53 ± 0.18	4.07± 0.12	4.27 ± 0.44	3.80 ± 0.15	3.59 ± 0.22	3.94 ± 0.20
DW	26	0.59 ± 0.17	0.51± 0.02	0.54 ± 0.07	0.46 ± 0.02	0.45 ± 0.01	0.49 ± 0.05

DISCUSSION

Fatty Acid Composition of Enriched Rotifers

Rotifers enriched with 10% DHA contained a mean of 10.5% TFA DHA, while the unenriched rotifers contained no detectable DHA, consistent with the lack of DHA in *N. oculata*. A number of workers have reported a direct relationship between PUFA levels in rotifers and in their emulsion diets (Watanabe et al., 1983; Rainuzzo, Olson and Rosenlund 1989; Frolov, Pankov,

Geradze, Pankova and Spektorova 1991; Rezek, Watanabe, Harel, Seaton 2010; Carrier et al., 2011). Rotifers were cultured on *N. oculata*, which naturally contains ARA (3.94% TFA) (Reed Mariculture, Campbell, CA, US), causing the levels of ARA in rotifers from all ARA treatments to be slightly higher than emulsion levels, increasing from 1.5 to 8.2% TFA at treatment levels increasing from 0 to 6% ARA. The unenriched rotifers, on the other hand, contained an ARA level of 3.5% TFA, similar to their diet of *N. oculata* (3.94% TFA). Rotifers enriched with 10% DHA and 0% ARA had a lower ARA level (1.5%) than unenriched rotifers fed only *N. oculata* (3.5%), consistent with a reduction of relative ARA concentrations by enrichment with an emulsion devoid of ARA.

Table 5. Survival (mean ± SEM, *N* = 5) of larvae fed enriched rotifers *Brachionus rotundiformis* and *Artemia*, using 5 different oil emulsions consisting of 10% DHA and varying levels of ARA (0%, 1%, 2%, 4%, 6%) and an unenriched group. No significant treatment differences were observed for any parameter on any sampling date

Dph	10:0	10:1	10:2	10:4	10:6	Unenriched
2	80.1 ± 8.6	66.2 ± 8.4	63.6 ± 3.3	71.4 ± 7.5	78.2 ±4.0	70.0 ± 7.6
5	76.1 ± 7.7	58.9 ± 5.1	63.0 ± 2.9	70.6 ± 7.5	71.5 ± 4.1	69.7 ± 7.7
9	70.3 ± 7.9	57.2 ± 5.4	62.0 ± 2.5	68.9 ± 8.1	64.5 ± 4.2	69.5 ± 7.8
12	63.9 ± 6.9	55.4 ± 5.7	63.3 ± 3.2	60.1 ± 7.5	55.1 ± 2.5	58.3 ± 2.8
16	56.9 ± 8.4	49.6 ± 5.3	51.4 ± 8.1	58.5 ± 8.3	50.1 ± 3.3	52.4 ± 6.2
19	54.0 ± 7.8	48.9 ± 5.1	50.3 ± 8.1	54.2 ± 7.7	46.7 ± 4.3	49.0 ± 7.7
26	36.0 ± 8.6	39.0± 4.5	50.1 ± 9.2	42.3 ± 3.9	39.1 ± 5.9	44.9 ± 5.9

Table 6. Hypersaline stress resistance (ST-50) (mean ± SEM, *N* = 5) of larvae fed enriched rotifers *Brachionus rotundiformis* and *Artemia*, using 5 different oil emulsions consisting of 10% DHA and varying levels of ARA (0%, 1%, 2%, 4%, 6%) and an unenriched group. Larvae were subjected to a hypersaline stress test of 60 g L^{-1}. No significant treatment differences were observed

dph	10:0	10:1	10:2	10:4	10:6	Unenriched
13	12.1 ± 2.77	10.8 ± 0.89	13.6 ± 5.65	8.52 ± 1.69	20.1 ± 4.97	10.7 ± 1.68
27	37.7 ± 7.36	45.5 ± 9.23	31.7 ± 4.58	40.0 ± 4.33	47.3 ± 8.45	38.9 ± 7.30

Mean EPA was 7.9% TFA in rotifers fed the enrichment emulsions and 15.3% TFA in the unenriched rotifers. While EPA was not supplied in the enrichment emulsions, rotifers were cultured before enrichment on *N. oculata,* which contains 31.4% EPA, which contributed to substantial EPA in enriched rotifers.

The lower than expected amount of EPA (15.3% TFA) in the unenriched rotifers relative to their diet of *N. oculata* could be a result of the rotifers converting EPA (20:5n-3) to DPA (22:5n-3), which averaged 6.3% TFA, a significantly higher amount than in rotifers fed the enrichment emulsions (2.9% DPA TFA).

The presence of EPA in the emulsion-enriched rotifers (originating from *N. oculata*) resulted in an average DHA/EPA ratio of 1.4, which is lower than the recommended minimum of 2:1 DHA: EPA for marine finfish larvae (Tocher and Sargent 1984; Sargent et al., 1999).

Fatty Acid Composition of Enriched *Artemia*

In this study, *Artemia* enriched with 10% DHA contained a mean of only 7.3% DHA TFA, while the unenriched *Artemia* contained no detectable DHA. Compared to rotifers, *Artemia* enrichment is difficult due to their rapid development and catabolism of DHA (Sorgeloos, Dhert and Candreva 2001). It is possible that higher levels of DHA in *Artemia* could be achieved if the enrichment period is extended from 16 h (as used in this study) to 19-24 h (McEvoy, Navarro, Bell and Sargent 1995).

Enrichment success in *Artemia* also depends on genetic characteristics. Chinese strains of *Artemia* accumulated higher levels of DHA than the Great Salt Lake strain or the San Francisco Bay strain used in this study (Dhert, Sorgeloos and Devresse 1993; Evjemo, Coutteau, Olsen and Sorgeloos 1997).

The higher (1.7) DHA/EPA ratio in *Artemia* is attributed to lower EPA levels, which averaged 3.7% TFA in *Artemia* enriched with emulsions and 5.5% TFA in unenriched *Artemia* nauplii. ARA levels in enriched *Artemia* were slightly higher than supplied in the prescribed enrichments, except in the 6% ARA treatment which contained 5.2% ARA.

The higher than prescribed ARA levels are likely attributable to endogenous ARA (0.8% TFA) in *Artemia* (Coutteau and Mourente 1997). *Artemia* fed 0% ARA had a lower ARA level (1.5%) than unenriched *Artemia* fed only *N. oculata* (3.5%), again consistent with a reduction of relative ARA concentrations by enrichment with an emulsion devoid of ARA.

Fatty Acid Composition of D27ph Larvae

On d27ph, mean DHA levels in larvae fed prey enriched with 10% DHA was 9.8% TFA, corresponding to the levels provided during the rotifer feeding stage. The presence of DHA (2.6% TFA) in southern flounder larvae fed unenriched prey, which contained no detectable DHA, is similar to what was found in larval turbot (Estevez, McEvoy, Bell, Sargent 1999), yellowtail flounder *Limanda ferruginea* (Storer 1839) (Copeman et al., 2002), summer flounder (Willey, Bengtson, Harel, 2003) and Senegal sole *Solea senegalensis* (Kaup 1858) (Villalta, Estevez, Bransden, Bell 2005, Morais, Narciso, Dores, and Pousao-Ferreira 2004) when fed prey containing no DHA. As suggested by previous studies (Morais et al., 2004), it is possible that these larvae fed 0% DHA derived a significant amount of DHA from endogenous yolk reserves, since marine fish eggs normally contain high concentration of PUFA's, especially DHA (Tocher, Fraser and Sargent 1985). Furthermore, DHA levels of 26.1% have been reported in high quality southern flounder eggs (Woolridge 2005). Alternatively, DHA may have been synthesized from precursor fatty acids 18:3n3, 20:5n3 or 22:5n3, which were at significantly higher concentrations in the unenriched rotifers and *Artemia* compared to prey enriched with emulsions. Conversion of 18:3n3 and 20:5n3 to DHA, while limited in most marine carnivorous fish species, has also been reported in the juvenile turbot (Linares and Henderson 1991; Mourente et al., 1991) and gilthead seabream (Mourente, Rodriguez and Tocher 1993).

Carcass ARA levels on d27ph were higher than levels provided in enriched rotifers and *Artemia*. This suggests that dietary ARA was accumulated in larval tissues to levels above those provided in their diet, as reported in larval turbot (Linares and Henderson 1991) and mahi mahi *Coryphaena hippurus* (Linnaues 1758) (Ako, Kraul and Tamaru 1991). As reported in gilthead seabream larvae (Bessonart et al., 1999), tissue levels of ARA in southern flounder larvae increased with increasing dietary levels, suggesting that larval requirements for ARA can be met through dietary manipulations.

GROWTH AND SURVIVAL

In this study, growth in terms of notochord length and wet and dry weight was not different in larvae fed prey enriched with 10% DHA from those fed unenriched prey with 0% DHA. This is in contrast to what was reported in red

sea bream *Pagrus major* (Temmink and Shlegel 1843), where larvae fed rotifers enriched with high levels of DHA (≥ 20%) grew faster than those fed rotifers enriched with ≤ 2% DHA (Watanabe et al., 1989). Improved growth with higher dietary DHA has also been reported in larval yellowtail *Seriola quinqueradiata* (Temmink and Shlegel 1845) (Furuita, Takeuchi, Watanabe, Fujimoto, Sekiya and Imaizumi 1996, Copeman et al., 2002), yellowtail snapper *Ocyurus chrysurus* (Block 1791) (Faulk and Holt 2005a), cobia *Rachycentron canadum* (Linnaeus 1776) (Faulk and Holt 2005b), and black sea bass *Centropristis striata* (Linnaeus 1758) (Rezek et al., 2010). As previously reported for other flatfish species, including Senegal sole (Morais et al., 2004, Villalta et al., 2005) and Japanese flounder *Paralichthys olivaceus* (Temminck and Schlegel 1843) (Izquierdo, Arakawa, Takeuchi, Haroun, Watanabe 1992), a diet devoid of DHA was sufficient for southern flounder larval growth. These flatfish species apparently derive a significant amount of DHA from endogenous yolk reserves, or from elongation of precursor fatty acids 18:3n3, 20:5n3 or 22:5n3 (Mourente et al., 1993, Linares and Henderson 1991; Mourente et al., 1991).

Southern flounder larvae fed diets containing 0 – 6% ARA showed no significant differences in growth up to d26ph. Similar findings were reported for other species such as Japanese flounder (Estevez, Kaneko, Seikai, Tagawa and Tanaka 2001), larval turbot (Estevez, et al., 1999) and larval gilthead seabream (Koven et al., 2001) when fed diets containing 0 - 15% ARA. In larval white bass *Morone chrysops* (Rafinesque 1820), dietary ARA levels of 0 to 26% TFA also had no effect on growth, suggesting bioconversion of 18:2n-6 linoleic acid (LA) to ARA at sufficient levels for larval performance (Harel and Place 2003). From the results of the present study in southern flounder, it appears that the levels of dietary ARA originating from *N. oculata* fed to rotifers and those naturally found in *Artemia* instar 1 nauplii are sufficient for the growth of larval southern flounder.

In this study, *N. oculata* was added daily as background algae (greenwater) to the larval rearing tanks, a standard practice for larval rearing of southern flounder (Daniels and Watanabe 2003), as well as for many marine finfish species (Reitan, Rainuzzo, Øie and Olsen 1997; Faulk and Holt 2005b). *N. oculata* in the rearing tanks likely helped maintain the nutritional value of prey to the fish larvae. While larval southern flounder are considered strict carnivores, incidental ingestion of *N. oculata* cells by larvae is possible and may have contributed to dietary ARA in this study.

In agreement with the growth data, there were no significant differences in survival of southern flounder larvae fed diets containing 0 – 6% ARA up to

d26ph. Larval turbot fed prey containing from 3 to 15% ARA also showed no significant differences in survival to d43ph (Estevez et al., 1999). In larval summer flounder, dietary ARA within the range of 0% to 12% TFA had no effect on growth or survival to d23ph (Willey et al., 2003).

Contrary to these findings, black seabass larvae fed prey containing 10% DHA, with increasing ARA within the range of 0-6%, showed improved growth and survival from first feeding through metamorphic stages (Rezek et al., 2010) when grown under greenwater conditions using *N. oculata* as a background microalgal species. These results emphasize species-specific differences in ARA requirements of marine finfish larvae.

There were no significant differences among treatments in the ability for southern flounder larvae to survive an acute hypersaline challenge of 60 g L^{-1}. Mean ST-50 increased from 12.6 min among treatments on d13ph to 40.2 min on d27ph, suggesting that resistance of larvae to hypersalinity stress increased during larval development (Bodinier, Sucre, Lecurieux-Belfond, Blondeau-Bidet and Charmantier 2010; Watanabe, Kuo and Huang 1985). The use of ARA supplementation during the early larval stages improved survival of larvae through post-metamorphic stages in gilthead seabream and summer flounder (Koven et al., 2001; Willey et al., 2003). Hence, it is possible that ARA effects would be manifested if survival were monitored through later post-metamorphic stages.

Interestingly, low levels of ARA were detected in the 0% ARA enriched rotifers (1.5% TFA) and unenriched *Artemia* nauplii (1.0% TFA) suggesting that even small amounts of ARA in the diet of southern flounder larvae were sufficient for normal stress resistance, growth and survival. Larval summer flounder fed a diet containing 6% ARA (TFA) showed increased hypersaline stress resistance when exposed to 70 gL^{-1} on d18ph and to 80 gL^{-1} on d45ph (Willey et al., 2003). Black sea bass larvae fed diets supplemented with 6-12% ARA demonstrated a significant increase in Na^+/K^+ ATPase mRNA 24 h after a sublethal salinity (42 g L^{-1}) challenge compared to larvae fed 0% ARA (Carrier et al., 2011). It is possible the acute hypersaline challenge (60 g L^{-1}) used in the present study overwhelmed the larvae and did not allow the effects of dietary ARA supplementation on osmoregulation to be clearly resolved. Lower or fluctuating salinity challenge, analysis of Na^+ K^+ ATPase gene expression (Carrier et al., 2011, Ostrowski, Watanabe, Montgomery, Rezek, Shafer and Morris 2011), or cortisol production analysis may enable better resolution of the effects of dietary DHA or ARA on resistance to salinity stress (Jensen, Madsen and Kristiansen 1998; Koven, 2003; Van Anholt, Koven and Lutzky 2004).

In summary, dietary DHA levels (0-10%) combined with dietary ARA within the range of 0 to 6% had no significant effects on growth, survival and hypersaline stress resistance in larval southern flounder through metamorphic stages. Southern flounder larvae were apparently able to accumulate sufficient ARA from their live prey even though present in these organisms in seemingly small concentrations. Carcass DHA levels in d27ph larvae fed live prey lacking this essential fatty acid indicated that a significant amount of DHA was derived from endogenous yolk reserves, or that this species can synthesize DHA from 18 carbon and 20 carbon n3 precursors at levels that are sufficient for normal growth and survival.

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