THE EFFECTS OF DIETARY DOCOSAHEXAENOCIC ACID (22:6n-3) AND ARACHIDONIC ACID (20:4n-6) ON GROWTH, SURVIVAL AND STRESS RESISTANCE IN BLACK SEA BASS (*Centropristis striata*) LARVAE

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ABSTRACT

The black sea bass is an important commercial and recreational fishery species on the U.S. Atlantic coast. Decreasing commercial catch and high market value make the black sea bass a promising candidate for aquaculture. Knowledge of the nutritional requirements of the larval stages is fundamental to achieve large scale production of fingerlings or growout farms. Essential fatty acids, especially 22:6n-3 (Docosahexaenoic acid, DHA), 20:5n-3 (Eicosapentaenoic acid, EPA) and 20:4n-6 Arachidonic acid (ArA) are known to have important affects on larval growth and survival in marine finfish. Little or no published information is available on the nutritional requirements of larval black sea bass. The objectives of this study were to determine the effects of dietary DHA to ArA ratio on survival, growth, and stress resistance of black sea bass larvae raised from first feeding to metamorphic stages.

Yolksac larvae (day 0 post-hatching = d0ph) were stocked into 15-L aquaria (44 larvae/L) under 34 ppt and 20°C. Larvae were fed enriched rotifers and *Artemia* nauplii containing two levels of DHA (0 and 10%) in conjunction with three levels of ARA (0, 3 and 6%) in a 2 x 3 factorial design. Five replicate aquaria were assigned to each treatment. Larvae were fed enriched rotifers (*Brachionus sp.*) from d2ph to d17ph, and *Artemia* from d18 to d24ph, when all larvae had reached the metamorphic stage. Larvae were sampled weekly to monitor growth as (notochord length (NL), dry weight, wet weight) and survival. On d24ph, a hypersaline (60 ppt) stress test (median survival time at 50% survival) was administered. Rotifers, *Artemia* and samples of larvae were collected from each treatment to determine fatty acid composition.

On d24ph, larvae fed treatment 10:6 (DHA:ARA) showed significantly ($P < 0.05$) higher survival (16.7%), than larvae fed 0:0 (DHA:ARA) (7.0%). NL and dry weight were also significantly ($P < 0.05$) greater at the 10:6 (DHA:ARA) treatment level (8.65 mm, 2.14 mg) than in the 0:0 (DHA:ARA) (7.7 mm, 1.65 mg) treatment. During hypersaline challenge, no significant differences ($P > 0.05$) were shown between larvae fed 10% DHA (25.6 min) and larvae fed 0% DHA (18.2 min.). On d24ph, levels of ArA in black sea bass larvae were higher than levels provided in their diets, while DHA levels were higher in larvae fed 10% DHA than in larvae fed 0% DHA. EPA (20:5n-3) levels were also higher in the larvae fed 10% DHA than in larvae fed 0% DHA. These results suggest conversion of EPA to DHA by black sea bass larvae, but at rates insufficient to produce optimum growth and survival.

To summarize, black sea bass larvae fed prey containing 10% DHA showed better growth and survival than those fed 0% DHA. Increased ARA within the range of 0-6% improved growth and survival from the first feeding through metamorphic stages.

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DEDICATION

To my wife Laura, and to my children Jeremy and Kaitlyn who's love, support and patience keeps me focused on achieving my goals. To my family and friends for their endless support and encouragement. I love every one of you far more than words on a page can express. Thanks!

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INTRODUCTION

Overview

Decreasing yields of commercial fisheries worldwide has fueled an increasing interest in the field of aquaculture. Aquaculture simply defined is the rearing of aquatic organisms primarily as food. Today, fish is the only important food source that is still primarily gathered from the wild rather then farmed, with marine capture historically accounting for >80% of the world's fish supply (Tidwell and Allen 2001). To provide relief for the declining marine fisheries, aquaculture production must increase to meet the worldwide demand for seafood.

Marine finfish production is poised as a key growth area for aquaculture in the United States as governmental agencies recognize the need to better utilize ocean resources (Lee and Ostrowski 2001). In marine fish larviculture, the first feeding of early larval stages, appears to be the major bottleneck for large scale production (Dhert et al. 2001). Unlike freshwater fish, marine finfish produce large numbers of very small eggs that generate vulnerable, free-living and rapidly developing larvae (Sargent et al. 1999). In nature, a marine fish larval diet naturally consists of zooplankton and phytoplankton, to achieve optimum nutritional value. These requirements are difficult to meet in the culture environment. The stress encountered in artificial surroundings can sometimes cause feeding problems in the larvae, as they have an underdeveloped gastrointestinal system and are not able to utilize artificially prepared feeds (De Silva and Anderson 1995). Providing the optimum nutritional needs through secondary cultures of microalgae, rotifers and brine shrimp for the larvae is a necessity for successful marine finfish larviculture.

To reduce mortality at first feeding, it is important to identify the optimal live feed. Depending on the mouth size of fish larvae, either copepod nauplii, *Artemia* nauplii or rotifers are provided as the first feed to the larvae (Lee and Ostrowski 2001). The use of rotifers and *Artemia* nauplii is widespread because of their availability and simple methods of production (Strottrup 1992).

Essential fatty acid requirements in larval marine finfish

Fatty acids are long chain carboxylic acids obtained from fats and oils by hydrolysis (Carey 2000). These organic compounds typically contain 4-30 carbons, with most containing 10-22 (Hammond 1993). Most naturally occurring fatty acids contain a single COOH group and a straight unbranched carbon chain, which may in turn contain no double bond (saturated fatty acid), a single double bond (mono-unsaturated fatty acid), or more than one double bond (polyunsaturated fatty acids, PUFA). Those fatty acids that contain two or more double bonds and are generally greater than or equal to 20 carbons are regarded as highly unsaturated fatty acids, HUFA. In general, the unsaturated fatty acids are more chemically reactive than corresponding saturated fatty acids. Highly unsaturated fatty acids posses a higher degree fluidity and therefore a lower melting point compared to a saturated molecule capable of more stable bonding.

Depending on the species of fish to be cultured, different amounts of PUFA are required for better larval survival, fast growth, and normal development (Lee and Ostrowski 2001). It is believed that the optimal dietary formulations for first-feeding larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of pre-feeding fish (Rainuzzo et al. 1997).

With the exception of the land snail (*Capea nemoralis*), animals are not known to be capable of de novo synthesis of fatty acids with double bonds in the n-6 (linoleic series) and n-3 (linolenic series) positions; plants however, are able to synthesize these fatty acids de novo (Tacon 1990). The three long-chain fatty acids that are essential for many marine species are docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ArA, 20:4n-6) (Copeman et al. 2002). In view of the seeming inability of animals to synthesize n-6 and n-3 series fatty acids de novo they must be supplied with these fatty acids in a ready-made form within the diet (Tacon 1990).

Live foods that are commonly used for first-feeding of marine larvae, such as rotifers and *Artemia* sp., are naturally low in these essential fatty acids. In the early 1980's it was shown that the nutritional value of *Artemia* and rotifers to marine fish larvae could be enhanced by the presence or supplementation of essential fatty acids, especially EPA (20:5n-3) and DHA (22:6n-3) of the n-3 fatty acid series (Watanabe 1983). Prior to being fed to the larvae, rotifers and *Artemia* can be fed formulated micronized diets or microalgae such as *Nannochloropsis oculata* (EPA) and *Isochrysis galbana* (DHA) containing high concentrations of essential fatty acids. It has been demonstrated that enrichment of live foods with lipids rich in n-3 and n-6 fatty acids prior to feeding to fish larvae is necessary for optimal growth, survival and resistance to handling stress (Watanabe 1982; Takeuchi et al. 1990, Koven et al. 2001).

Role of fatty acids in larval nutrition

For many years, the nutritional aspects of essential fatty acids in fish have been extensively studied with considerable attention focused on the n-3 PUFA requirements of marine fish, particularly EPA and DHA (Ibeas et al. 1997). The essentiality of these fatty acids is based on their roles as necessary components of cell membranes, as a major energy source for absorbtion of fat soluble vitamins (A,D, E, and K) and as precursors for prostaglandin hormones. Most marine fish studied to date are usually unable to convert 18:3n-3 (linolenic acid) to the more highly unsaturated 20:5n-3 (EPA) and 22:6n-3 (DHA) (Bessonart et al. 1999). Marine fish species whose diets are rich in 22:6n-3 and 20:5n-3 have lost the ability to chain elongate and further desaturate 18:3n-3 to the corresponding PUFA (Tacon 1990). This metabolic insufficiency has been identified as a relative deficiency in one of two enzymes in the conversion pathway from 18:3n-3 to EPA, i.e. the C18 to C20 elongase multienzyme complex or the Δ5 fatty acid desaturase (Bell and Sargent 2002). To compensate, commercial preparations routinely used to enrich the live food (rotifers and *Artemia* nauplii) of the larval stages of cultured marine teleosts contain abundant amounts of DHA, together with relatively lower levels of EPA (Koven et al. 2001).

In all of the marine larval fish species examined to date, DHA has been demonstrated to be superior to EPA in providing vitality to the larvae (Watanabe 1993). Given that DHA is naturally found at very 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 et al. 2002). Elevated dietary EPA relative to DHA has been shown to have a negative impact on larval neural function and thus growth and

survival (Copeman et al. 2002). Mourente et al. (1991) demonstrated that the most significant aspect of lipid metabolism in the developing brain of juvenile turbot was the accumulation of DHA. In this study, the accumulation of DHA was specific, with no other fatty acid increasing in percentage in total lipid. Increased mortalities were shown in juvenile turbot (*Scophthalmus maximus*) (Bell et al. 1985) when the 22:6n-3/20:5n-3 ratio was decreased from 13.8 to 2.2, without changing the percentage of total n-3 PUFA. Turbot larvae tended to exhibit lower pigmentation success with lower DHA/EPA ratio in the total lipid fraction of the larvae (Rainuzzo et al. 1997). In starved gilthead seabream (*Sparus aurata*) larvae, Koven et al. (1989) found that DHA 22:6n-3 was highly conserved and this may attest to its particular importance during the larval growth stage.

Generally the n-6 series fatty acids are 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 1980). Marine carnivorous fish are faced with the same inability to chain elongate and further desaturate linoleic acid (18:2n-6) to the more highly unsaturated n-6 fatty acids such as 20:4n-6 (ArA) as they do with converting 18:3n-3 to 20:5n-3 (EPA) and 22:6n-3 (DHA). Despite this inability, n-6 series fatty acids have received little attention in feeding studies in marine fish nutrition.

Arachidonic acid is known to be the primary precursor fatty acid of eicosanoid production in fish (Bessonart et al. 1999). The eicosanoids are a range of highly active C20 compounds formed in small or even trace amounts by virtually every tissue in the body and are produced in response to stressful situations, both at cellular and whole body level (Sargent et al. 1999). Eicosanoid C20 fatty acid derived metabolites such as prostaglandins, thromboxanes, and leukotrienes are involved in various areas of cellular

regulation including control of fluid electrolyte fluxes, cardiovascular system, reproductive function and the neural system. Series-2 prostaglandins derived from arachidonic acid have long been used to induce spawning of fish (Bell and Sargent 2002). Prostaglandins have been identified in the testes of flounder and bluefin tuna and in the semen of chum salmon (Bell et al. 1986). A recent study by Furuita et al. (2002) showed that addition of ArA to broodstock diets improved the egg quality of Japanese flounder, however, excess arachidonic acid may have negative effects on their reproduction.

Among the n-6 PUFA, several studies have pointed out the importance of ArA in fish metabolism. For the juvenile turbot, Castell et al. (1994) showed that diets containing ArA as the only HUFA resulted in higher growth and survival than any mixtures of ArA/DHA or DHA alone. Bessonart et al. (1999) demonstrated increasing levels of dietary ArA significantly improved larval growth of the gilthead seabream. Koven et al. (2001) demonstrated improved survival of the larval gilthead seabream when provided with dietary ArA prior to exposure to handling stress. However, levels of ArA that are too high can produce negative effects such as poor larval pigmentation in flatfish species as seen in the yellowtail flounder *Limanda ferruginea* (Copeman et al. 2002), turbot *Scophthalmus maximus* (Estevez et al. 1999) and halibut *Hippoglossus hippoglossus* (McEvoy et al. 1999).

Conclusion

Essential fatty acid enrichment of live feed diets has become a routine aspect of larval marine fish aquaculture. It has been demonstrated that proper development, growth and survival of larvae can be greatly improved when the proper proportion of

EPA, DHA of the n-3 and ArA of the n-6 series fatty acids are provided. In general these HUFAs are believed to be responsible for the key metabolic functions of the essential fatty acids (Tacon 1990). Marine fish fed diets containing higher levels of these more highly unsaturated fatty acids, as opposed to their precursors 18:3n-3 (linolenic acid) or 18:2n-6 (linoleic acid) have had a higher rate of survival throughout larval developement (Ostrowski and Divakaran 1990).

In marine fish both EPA and DHA have been considered essential due to their requirement for good growth and the fact that all marine fish studied to date are barely able to convert 18:3n-3 to EPA and DHA if at all (Bell and Sargent 2002). Despite the relatively small amount of ArA represented in the tissue, studies have suggested that it is also essential for certain marine fish (Koven et al. 2001).

Black Sea Bass

The black sea bass *Centropristis striata* is the target fish for this study. The black sea bass is a commercially and recreationally important marine finfish (family Serranidae) found from the Gulf of Maine to Florida. It is a generalized carnivore with a diet consisting of various motile organisms, mostly epibenthic fauna consisting of decapod crustaceans and polycheate worms (Sedberry 1988). Similar feeding habits have been observed in other serranids (Hood 1994). Cupka et al. (1973) suggested that the presence of sessile organisms, such as colonial tunicates, in their stomach contents indicate some grazing activity.

Off the North Carolina coast, peak spawning occurs in March to May. Moving northward, spawning occurs progressively later sometimes until October (Shepherd

2000). The black sea bass are capable of multiple spawning events each season. Eggs are released in a broadcast fashion where they remain buoyant and pelagic along the continental shelf (Shepherd 2000). They do not remain pelagic for long, as larvae no longer than 13 mm were found in ichthyoplankton surveys (Kendall 1972). It is presumed that once the fish reaches this size they assume a demersal or estuarine lifestyle (Kendall 1972).

Currently there is little known about the nutritional needs of *C. striata* larvae. The objective of this study was to determine the DHA:ArA ratio that will produce optimal survival, growth, and stress resistance for culture of black sea bass larvae. Based on the results, we hope to recommend levels of ArA supplementation for commercially available enrichment products (such as Algamac 2000) which typically lack ArA, to improve overall larval performance.

The null hypotheses to be tested:

1. Varying the amount of docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ArA) in the live feed enrichment of the rotifer (*Brachionus rotundiformis*) will have no effect on growth of black sea bass (*Centropristis striata*) larvae.
2. Varying the amount of DHA/ArA in the live feed enrichment of *B. rotundiformis* will have no effect on the survival of *C. striata* larvae.
3. Varying the amount of DHA/ArA in the live feed enrichment of *B. rotundiformis* will have no effect on stress resistance of *C. striata* larvae.

Objectives

The objective of this study was to determine the effects of different levels of dietary docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ArA) on the growth, survival and stress resistance of black sea bass larvae from first feeding to metamorphic stages.

METHODS

Experimental Animals

This study was conducted at the University of North Carolina Wilmington Aquaculture Facility, Wrightsville Beach, NC. Adult black sea bass (mean wt. = 0.927 kg; range = 0.320-1.93 kg) were collected by a commercial fisherman in traps set at approximately 14-m depth off Carolina Beach, NC in November 2000 (Copeland et al. 2003). The fish were held outdoors in six cylindrical tanks (diam.=1.83 m, depth = 0.81 m, volume = 2,134 L) under 34 ppt salinity and a controlled photothermal cycle. Seawater (34 ppt) was pumped from the Atlantic Intracoastal Waterway adjacent to Wrightsville Beach. Filtered (1 μm) U.V.-treated seawater was used for larval rearing experiments. In May of 2003 adult females at the post-vitellogenic stage of ovarian development were hormonally induced to spawn using leuteinizing hormone releasing hormone – analogue (LHRH-a) (Berlinsky et al. 1996; Watanabe et al. 2003) at a dose of 75 μg/kg body weight. Following volitional spawning, eggs were siphoned from the egg collectors and transferred to a separatory funnel in seawater where floating (viable) eggs were separated from sinking (non-viable) eggs. Floating fertilized eggs were placed in 125-L incubators, where they were quantified and their embryonic development was monitored through hatching, approximately 52 hrs post fertilization. Newly-hatched yolksac larvae (day 0) were transferred to a 15-L hatching cone where they were counted before distribution to the rearing aquaria.

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 an opaque black curtain to eliminate extraneous light. Each water bath contained eight cylindrical black plastic aquaria (15-L working volume) where 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 40-W fluorescent bulbs supplying full spectrum lighting simulating natural light.

Experimental Design

To study the effects of dietary arachidonic acid on black sea bass larval growth, survival and stress resistance, larvae were fed live prey organisms, including rotifers and *Artemia* that were enriched in emulsions containing different proportions of DHA:ArA. Two levels of DHA (0 and 10%) were used in conjunction with three levels of ArA (0, 3 and 6%) in a 2 x 3 factorial design. Five replicate aquaria were maintained per treatment.

To begin the experiment, yolksac stage larval black sea bass (1 day post hatch) were stocked using volumetric methods into aquaria under 34 ppt and 19 ° C and at a density of 40 larvae/L. Temperature was raised 1 °C per day until an optimum larval rearing temperature of 22 °C was reached (Copeland 2004).

Live Prey Enrichment

The DHA/ARA emulsions were formulated by combining different ratios of DHAsco and ARAsco 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, USA) (Appendix A). Emulsions were sealed and refrigerated for daily use. DHAsco (DHA-rich Single Cell Oil) and ARAsco (ARA rich Single Cell Oil) 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 ppt and at temperatures of 22-25 °C. Rotifers were fed a diet of preserved algae, *Nannochloropsis oculata* (Reed Mariculture, San Jose, California). *Artemia* were hatched from decapsulated cysts 24 h prior to enrichment.

During enrichment, rotifers and *Artemia* were held in 3-L beakers in aerated, UV-treated seawater at 34 ppt. Photoperiod and temperature were maintained at 24 L**:** 0 D and 25°C. Dissolved oxygen was maintained above 4 ppm. During enrichment, rotifer density was maintained at 500,000 per L. Enrichment products were added at 0.1 g/500,000 rotifers every 4 hours. Rotifers were enriched for 8 hours before feeding to the larvae.

During enrichment, *Artemia* density was maintained at 200,000 nauplii per liter. Enrichment products were added at 0.3 g/250,000 nauplii every 8 hours. *Artemia* were enriched for 16 h before feeding to the larvae. Samples of rotifers and *Artemia* were

collected immediately following the prescribed enrichment time and analyzed to verify that their fatty acid compositions reflected those of their respective treatment emulsions.

Feeding

On d3ph, rotifers were added at a density of 10 ind. / mL. Once feeding began, rotifer density was increased to 20 ind. / mL. 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 *Nannochloropsis oculata* was added daily to maintain a density of 300,000 cells / mL. On d18ph, *Artemia* nauplii were added at a density of 0.5 ind. / mL in addition to enriched rotifers. By d20ph, rotifer feeding was reduced and larvae were fed *Artemia* enriched with the prescribed treatments at a density of 2 ind./mL increasing to 3.5 ind. / ml by d22ph.

Environmental Conditions

Light intensity was maintained constant (500 lux) at the water surface by adjusting the height of the hoods. Photoperiod was maintained at 18L **:** 6D. Temperature was maintained at 22 °C. Aeration was supplied at approximately 80-100 mL/min through diffusers (4 cm x 1.3 cm x 1.3 cm) weighted with ceramic insulators and placed at the center of each aquaria.

Salinity, dissolved oxygen, light intensity, pH, and air flow were measured daily in each rearing unit. Once feeding began, 30-55 percent of water in each rearing unit was

exchanged daily. The water surface of each unit was skimmed 3 times daily with a paper towel to remove oil films.

Growth and Survival

To monitor growth and survival, larvae were sampled from each tank on d3, d9, d17 and d24ph. Larvae were sampled volumetrically with a minimum of 10 larvae removed with each sample. Larvae were anesthesized (0.3 ppt 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 (tip of snout to tip of notochord), yolksac length and width, and oil droplet diameter were measured with an ocular micrometer. Wet and dry weights were recorded using a Sartorius (Goettingen, Germany) electrobalance to the nearest 10 μg. Wet weights were recorded by weighing approximately 10 larvae on a microscope slide and then subtracting the weight of the slide. To determine dry weights, larvae were dried at 60°C (approximately 72 h) until they reached a constant weight and re-weighed.

The larval density (number of larvae/L) was determined by dividing the number of larvae sampled by the volume of water collected. Larval survival on each sampling date was calculated as the quotient of larval density and larval density determined at d3ph, expressed as a percentage. On d 24 ph, all remaining larvae were preserved for analysis of carcass fatty acid composition.

Fatty Acid Analysis

To prepare samples of live prey and larvae for fatty acid analysis, 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 U.S.), filled with nitrogen, and then frozen (-25°C) before lipid extraction and fatty acid analysis.

Fatty acid analysis was conducted using a revised Folch et al. (1957) method using a chloroform/methanol mixture (1:1 v/v) to extract lipids from tissue. Lipid was extracted from the samples by hand held homogenization followed by sonication in 5 mL of 1:1 chloroform/methanol. The extract was poured through a Pasteur pipette filter into a 50 mL round bottom flask to remove cell debris. The sample was concentrated under reduced pressure (Buchi R-3000) (temp 35 °C) for 2 minutes. The 1:1 chloroform:methanol solvent (5 mL) was then used to transfer the solution through a Pasteur pipette filter into a pre-weighed 50 mL round bottom flask. The solvent was evaporated under reduced pressure and the sample was weighed to obtain the percent lipid.

To convert lipid fatty acids to their methyl esters (FAMEs) for GC analysis, the lipid was redissolved using 1:1 chloroform:methanol. This solution was transferred to a 5 mL conical vial with a stirring magnet and nitrogen gas was blown over the sample for 5-10 minutes to evaporate the chloroform:methanol solvent. The first phase of the FAME reaction is a base catalyzed hydrolysis of the glycerol esters, which required the addition of 1 mL 0.5M NaOH/MeOH to the sample, which was 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 trifuoride-methanol (BF_3) and heating for an additional 30 minutes.

Once the reaction was complete and allowed to cool, saturated aqueous NaCl (1 mL) and hexane (1 mL) were added to the sample in the reaction vial. The vial was capped and shaken. After the organic layer separated from the aqueous layer it was removed with a pipette. The aqueous layer was extracted again with hexane and then with 50% ether/hexane. Each time the organic layers were filtered through 32 μm silica in a Pasteur pipette into a 50 mL round bottom flask. The solution was concentrated and transferred to a GC vial in 400-600 μL 100% chloroform. Samples were refrigerated in airtight GC vials, under nitrogen gas, to prevent oxidation and deterioration.

FAMEs from all samples were identified and quantified using GC-FID. Helium was used as the carrier gas. The column temperature profile was: 195 °C, hold for 8 min, ramp to 270 at 15 °C / min and hold at 270 °C for 2 min. FAME peaks were integrated using the HP Chemstation software package and individual FAMEs were indentified by comparison of retention times to standards: GLC-84 (Nu Chek Prep U.S.), eicosaentaenoic acid methyl ester, arachidonic acid methyl ester (Sigma-Aldrich U.S.), steariodonic acid methyl ester (Cayman Chemical U.S.).

Stress Resistance

To measure stress resistance, larvae were subjected to a hypersaline (65ppt) challenge on d 24 ph (Dhert et al. 1991). A minimum of 20 larvae were randomly sampled from each rearing unit and transferred to a 100 x 15 mm petri containing 50 mL

of 65 ppt seawater at 22 °C made with Instant Ocean aquarium salt (Aquarium Systems USA and France). Survival was monitored at 5 min. intervals until complete mortality was observed. Stress sensitivity index (ST50) was based on mean survival time, the time at which survival fell to 50%.

Analytical Methods

The effects of DHA and ArA, and their interaction, on notochord length, dry weight and wet weight were expressed statistically as treatment means ± standard error of the mean (SEM) and compared by a two-way analysis of variance. A t-test ($P < 0.05$) was used to compare each mean to the 0:0 (control) treatment level. For survival percentage data, arcsine transformation was performed before analysis. Analyses were performed using the JMP IN version 4 statistical software (SAS Institute Inc.).

RESULTS

Fatty Acid Composition of Enriched Rotifers

The fatty acid composition of the enriched rotifers is shown in Table 1. No significant ($P > 0.05$) treatment or interactive effects were observed in total lipid (mean = 6.3% wet weight). A significant ($P < 0.05$) effect of DHA enrichment on levels of total saturated fatty acids (SFA) was observed, while there was no significant ($P > 0.05$) effect of ArA enrichment or interactive effects. The SFAs were higher ($P < 0.05$) in rotifers enriched with 10% DHA than in rotifers enriched with 0% DHA (Fig. 1). Of the SFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in 16:0 (palmitic acid), 18:0 (stearic acid) and 20:0 (arachidic acid). Significant ($P < 0.05$) effects of DHA and ArA enrichment on the levels of 14:0 (myristic acid) were observed, with no interactive effects. Concentration of 14:0 was higher ($P < 0.05$) in rotifers enriched with10% DHA than in those enriched with 0% DHA (Fig. 2). The concentration of 14:0 was also higher ($P < 0.05$) in rotifers enriched with 6% ArA (than in those enriched with 3% and 0% ArA enriched rotifers.

Significant ($P < 0.05$) effects of both DHA and ArA enrichment on levels of monounsaturated fatty acids (MUFA) were observed, with no interactive effects. MUFA levels were higher ($P < 0.05$) in rotifers enriched with 0% DHA than in those enriched with 10% DHA (Fig. 3). MUFA levels were higher ($P < 0.05$) in rotifers enriched with 0% ArA than in those enriched with 3% and 6% ArA. Of the MUFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in the 16:1 (palmitoleic acid) and 20:1 (eicosenoic acid). Significant ($P < 0.05$) effects of DHA and ArA enrichment on levels of 18:1n-9 (oleic acid) were observed, with no interactive effects.

Concentration of 18:1n-9 was higher ($P < 0.05$) in rotifers enriched with 0% DHA than with 10% DHA (Fig. 4), and higher ($P < 0.05$) in rotifers enriched with 0% ArA than those enriched with 6% ArA.

A significant ($P < 0.05$) effect of DHA enrichment on the levels of n-3 polyunsaturated fatty acids (PUFA) was observed, while there were no significant ($P > 0.05$) ArA or interactive effects. The n-3 PUFAs were higher ($P < 0.05$) in rotifers enriched with 10% DHA than in rotifers enriched with 0% DHA (Fig. 5). Of the n-3 PUFA detected, no significant ($P > 0.05$) treatment or interactive effects were found for 20:5n-3 (eicosapentaenoic acid) or 22:5n-3 (docosapentaenoic acid). Significant ($P < 0.05$) effects of DHA enrichment on 18:3n-3 (linolenic acid) levels were observed, with no significant ($P > 0.05$) ArA or interactive effects. Concentration of 18:3n-3 was higher ($P < 0.05$) in rotifers enriched with 0% DHA than with 10% DHA (Fig. 6). Significant ($P < 0.05$) effects of DHA enrichment on levels of 22:6n-3 (docosahexaenoic acid) were observed, with no significant ($P > 0.05$) effect of ArA enrichment, or interactive effects. Concentration of 22:6n-3 was higher ($P < 0.05$) in rotifers enriched with 10% DHA than those enriched with 0% DHA (undetected) (Fig. 7). A significant ($P < 0.05$) effect of DHA enrichment on the ratio of 22:6n-3/20:5n-3 was observed, with no significant ($P > 0.05$) ArA or interactive effects. The ratio of 22:6n-3/20:5n-3 was higher ($P < 0.05$) in rotifers enriched with 10% DHA than those enriched with 0% DHA (undetected) (Fig. 8).

A significant ($P < 0.05$) effect of ArA enrichment on levels of n-6 PUFAs was observed, with no significant ($P > 0.05$) DHA or interactive effects. The n-6 PUFAs were higher ($P < 0.05$) in rotifers enriched with 6% ArA than in rotifers enriched with 0%

and 3% ArA (Fig. 9). Significant effects of ArA enrichment on levels of 18:2n-6 (linoleic acid) and 20:4n-6 (arachidonic acid) were observed, with no significant ($P > 0.05$) DHA or interactive effects. Concentration of 18:2n-6 was higher ($P < 0.05$) in rotifers enriched with 6% ArA than in rotifers enriched with 0% and 3% ArA (Fig. 10). Concentration of 20:4n-6 (ArA) was higher ($P < 0.05$) in rotifers enriched with 6% ArA than in rotifers enriched with 3% and 0% ArA (Fig. 11).

Significant ($P < 0.05$) DHA, ArA and interactive effects were observed on the ratio of n-3/n-6 PUFAs. The ratio of n-3/n-6 PUFAs was higher in the rotifers enriched with 10% DHA than in those enriched with 0% DHA (Fig. 12). The ratio of n-3/n-6 PUFAs was higher ($P < 0.05$) in rotifers enriched with 0% ArA than in rotifers enriched with 3% and 6% ArA. However, at 10% DHA, the n3/n6 PUFA ratio was considerably higher at 0% ArA than at 3% or 6% ArA, while at 0% DHA, these differences were less pronounced.

A significant ($P < 0.05$) effect of ArA enrichment on the ratio of 20:5n-3/22:4n-6 was observed, with no significant ($P > 0.05$) DHA or interactive effects. The ratio of 20:5n-3/22:4n-6 was higher ($P < 0.05$) in rotifers enriched with 0% ArA than those enriched with 3% and 6% ArA (Fig. 13).

Table 1. Fatty acid composition (% of total FAMES) of enriched rotifers *Brachionus plicatilis*.

Rotifer Treatments (DHA:ARA)						
Fatty acids	0:0	0:3	0:6	10:0	10:3	10:6
14:0	1.5 ± 0.07	1.35 ± 0.12	1.5 ± 0.09	3.9 ± 0.19*	4.0 ± 0.24*	4.5 ± 0.18*
16:0	15.9 ± 0.24	15.8 ± 0.19	16.1 ± 0.51	15.9 ± 0.24	16.5 ± 1.0	17.3 ± 0.91
16:1	5.2 ± 0.18	4.7 ± 0.29	4.7 ± 0.14	5.4 ± 0.39	5.6 ± 0.67	6.2 ± 0.42
18:0	11.8 ± 0.3	11.0 ± 0.27	11.0 ± 0.37	9.9 ± 0.33	10.2 ± 0.33	10.0 ± 0.37
18:1n-9	48.4 ± 1.6	47.8 ± 1.7	43.9 ± 0.76	40.8 ± 2.14*	37.8 ± 0.9*	32.4 ± 1.5*
18:2n-6	3.5 ± 0.13	3.7 ± 0.16	4.7 ± 0.16*	3.3 ± 0.18	4.0 ± 0.21	4.7 ± 0.37*
18:3n-3	3.3 ± 0.11	3.2 ± 0.11	4.1 ± 0.07*	3.3 ± 0.18	3.0 ± 0.07	2.5 ± 0.12*
20:0	2.1 ± 0.45	1.3 ± 0.16	2.3 ± 0.09	1.6 ± 0.28	0.95 ± 0.61	1.2 ± 0.23
20:1s	2.6 ± 0.36	3.2 ± 0.48	3.0 ± 0.04	2.1 ± 0.62	0.93 ± 0.93	3.8 ± 1.0
20:4n-6	1.0 ± 0.07	3.6 ± 0.24*	5.1 ± 0.54*	1.1 ± 0.1	4.2 ± 0.23*	5.9 ± 0.51*
20:5n-3	3.5 ± 0.48	3.2 ± 0.63	2.6 ± 0.41	3.9 ± 0.65	3.4 ± 0.25	3.7 ± 0.58
22:5n-3	1.2 ± 0.12	1.1 ± 0.25	1.0 ± 0.14	1.5 ± 0.22	1.3 ± 0.05	1.3 ± 0.15
22:6n-3	Undetected	Undetected	Undetected	7.5 ± 0.96*	8.2 ± 0.65*	6.4 ± 1.0*
∑Saturates	31.2 ± 0.72	29.5 ± 0.28	31.0 ± 0.45	31.1 ± 0.29	31.6 ± 0.71	33.1 ± 0.91
∑Monounsaturates	56.1 ± 1.2	55.7 ± 1.2	51.5 ± 0.86	48.3 ± 1.9*	44.3 ± 0.12*	42.4 ± 0.94*
∑n-3 Polyunsaturates	8.1 ± 0.49	7.4 ± 0.77	7.7 ± 0.48	16.2 ± 1.6*	15.8 ± 0.62*	17.9 ± 1.6*
∑n-6 Polyunsaturates	4.6 ± 0.13	7.3 ± 0.22*	9.7 ± 0.41*	4.4 ± 0.22	8.2 ± 0.07*	10.5 ± 0.46*
n-3/n-6 Polyunsaturates	1.8 ± 0.12	1.0 ± 0.08*	0.19 ± 0.03*	3.7 ± 0.33*	1.9 ± 0.07	1.3 ± 0.13
22:6n-3/20:5n-3	0	0	0	2.0 ± 0.16*	2.4 ± 0.28*	1.7 ± 0.22*
22:6n-3/20:4n-6	0	0	0	6.8 ± 0.28*	1.9 ± 0.06*	1.1 ± 0.08*
20:5n-3/20:4n-6	3.3 ± 0.26	0.87 ± 0.12*	0.51 ± 0.04*	3.5 ± 0.39	0.82 ± 0.08*	0.63 ± 0.07*
Total Lipid (% wet tissue wt)	6.0 ± 0.35	6.7 ± 0.25	6.05 ± 0.28	6.2 ± 0.24	5.8 ± 0.48	6.7 ± 0.38

Asterisk (*) indicates mean values (± SEM, N = 3) within rows that are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control

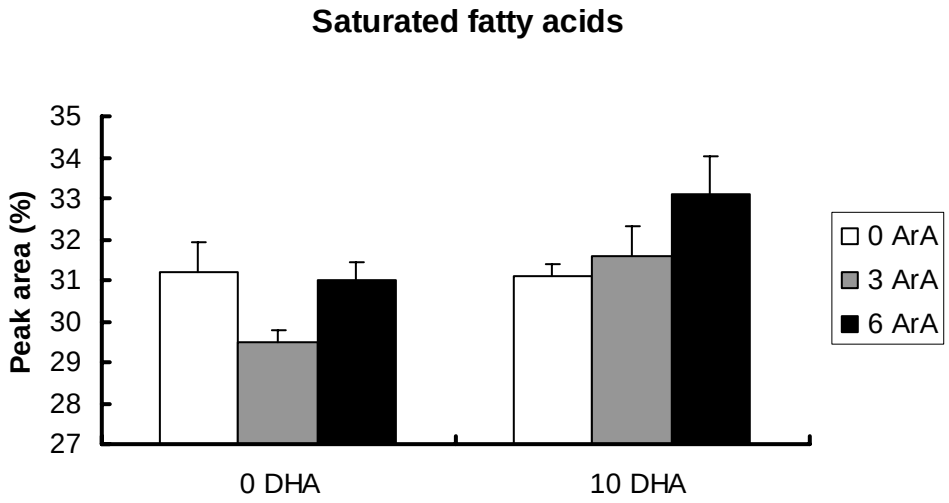

Figure 1. Percentage of total saturated fatty acids (SFAs) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

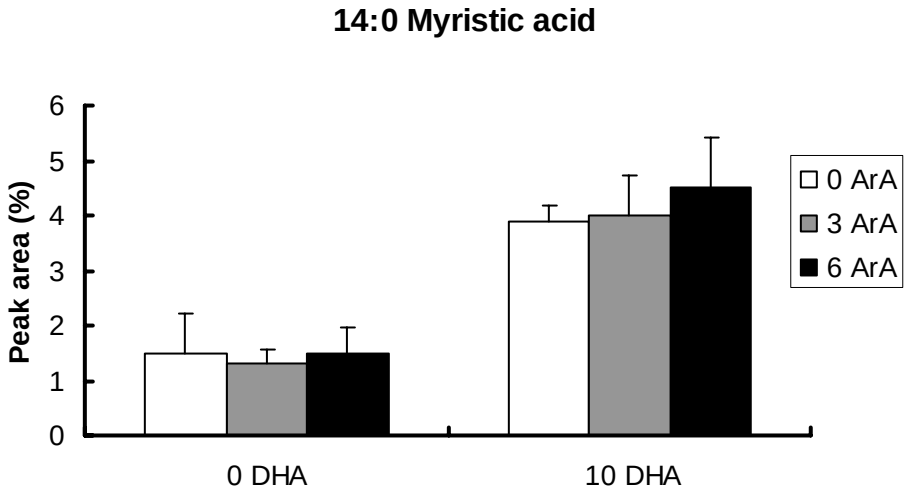

Figure 2. Percentage of total FAMES of 14:0 (myristic acid) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects (Two-way ANOVA).

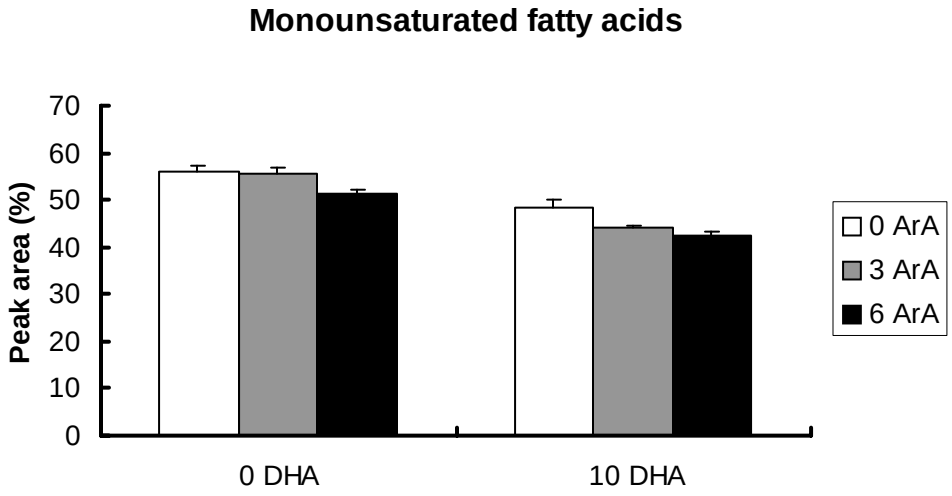

Figure 3. Percentage of total FAMES of monounsaturated fatty acids (MUFAs) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects (Two-way ANOVA).

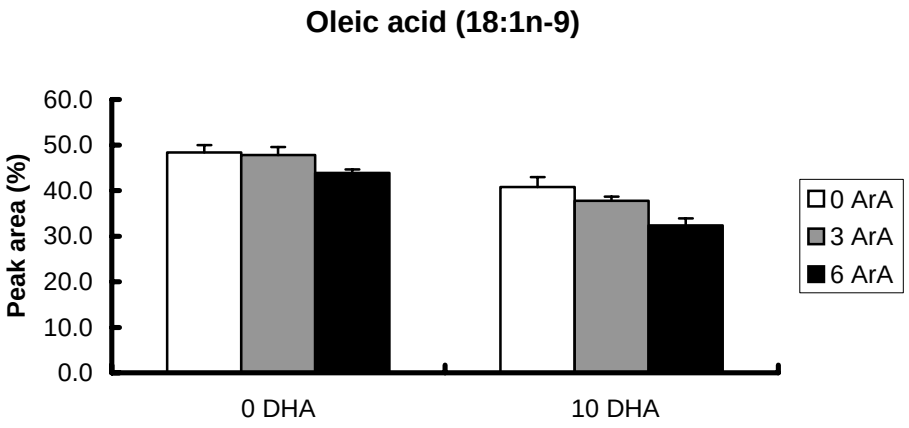

Figure 4. Percentage of total FAMES of oleic acid (18:1n-9) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects (Two-way ANOVA).

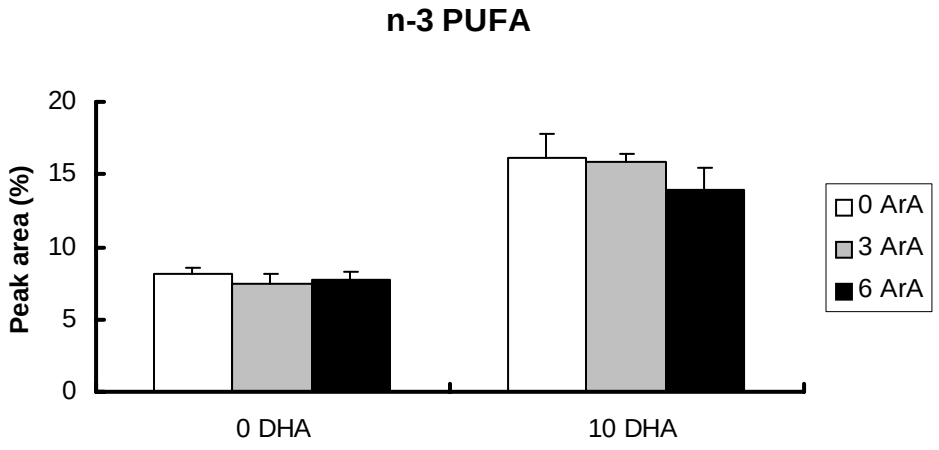

Figure 5. Percentage of total FAMES of n-3 polyunsaturated fatty acids (PUFA) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

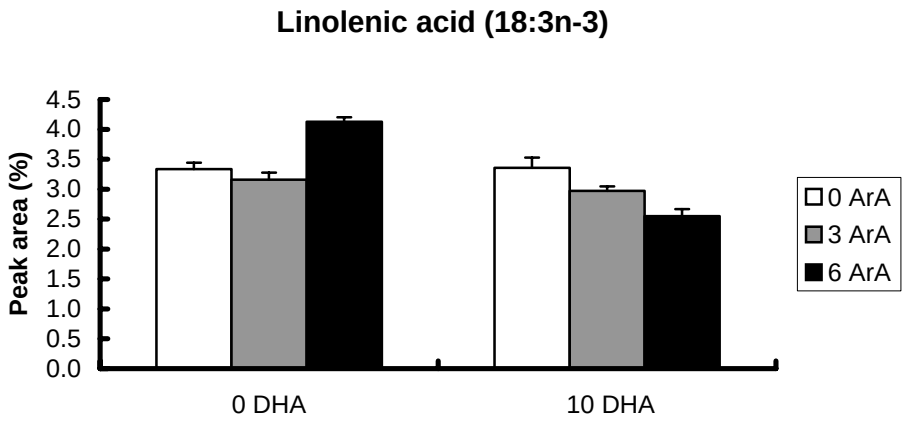

Figure 6. Percentage of total FAMES of linolenic acid (18:3n-3) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

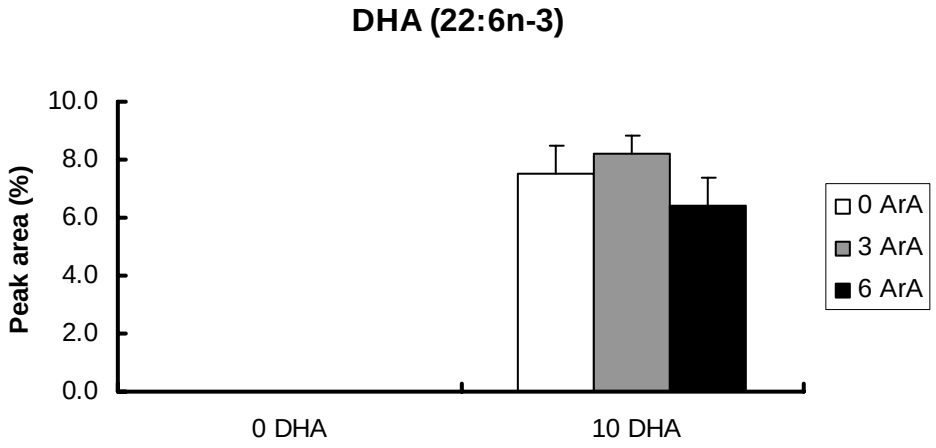

Figure 7. Percentage of total FAMES of 22:6n-3 (DHA) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

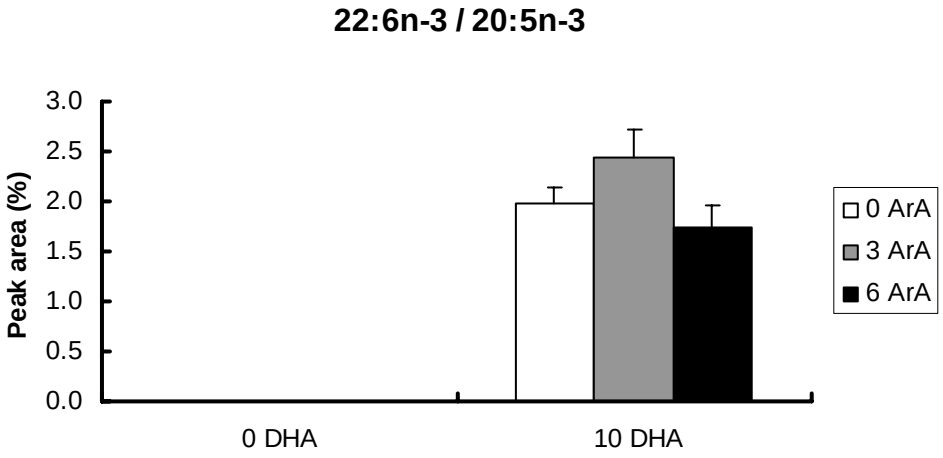

Figure 8. Ratio of 22:6n-3 (DHA) to 20:5n-3 ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

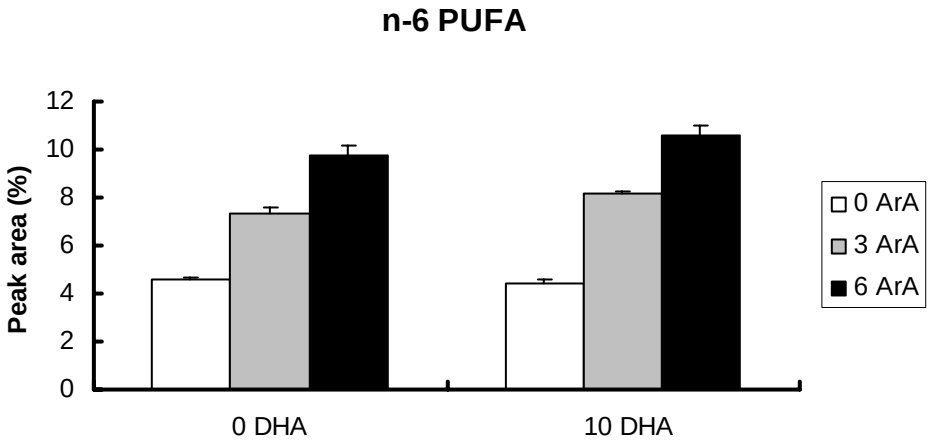

Figure 9. Percentage of total FAMES of n-6 series polyunsaturated fatty acids ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no DHA or interactive effects (Two-way ANOVA).

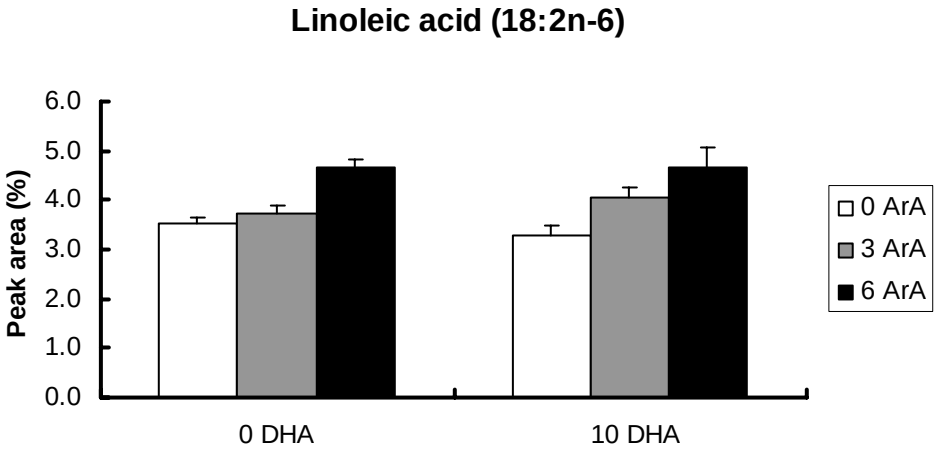

Figure 10. Percentage of total FAMES of linoleic acid (18:2n-6) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no DHA or interactive effects (Two-way ANOVA).

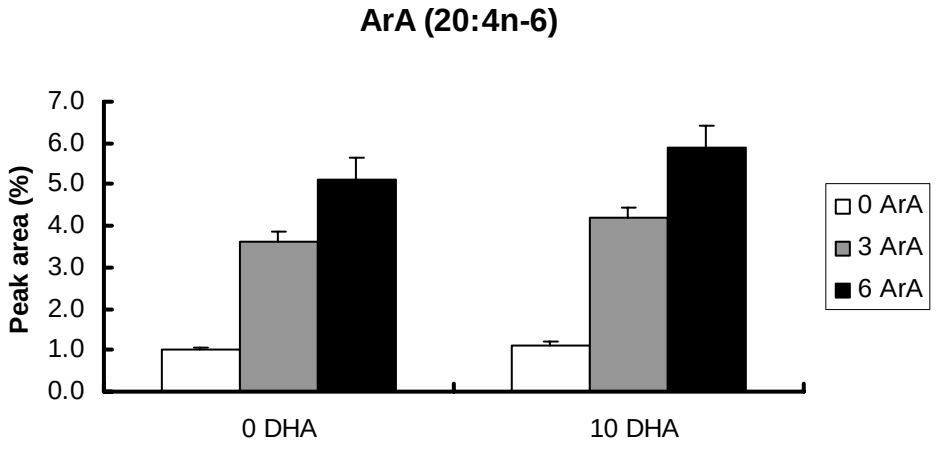

Figure 11. Percentage of total FAMES of 20:4n-6 (ArA) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no DHA or interactive effects (Two-way ANOVA).

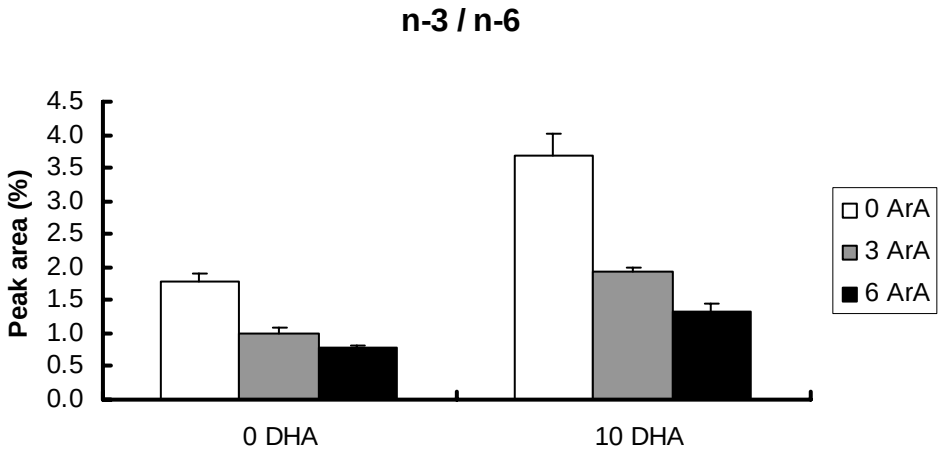

Figure 12. Percentage of total FAMES of the n-3 / n-6 ratio ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects (Two-way ANOVA).

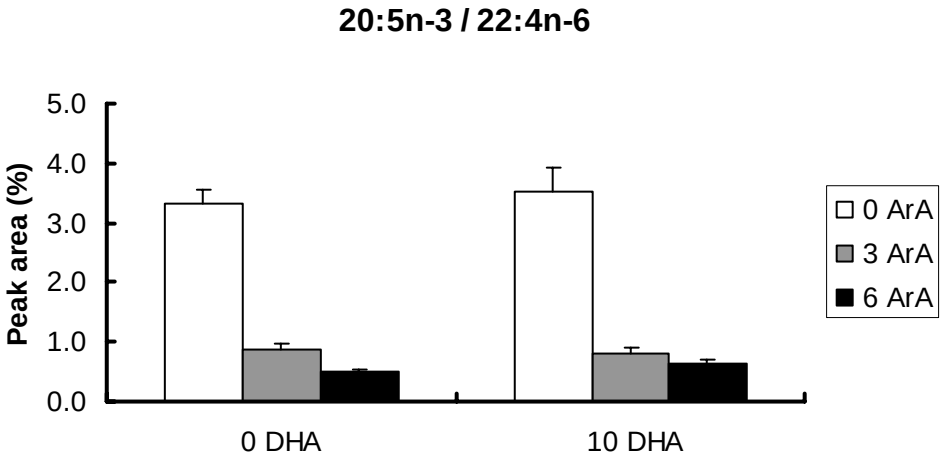

Figure 13. Ratio of 20:5n-3 (EPA) to 22:4n-6 (ArA) ($\bar{X}$ ± SEM, N = 3) in rotifers enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no DHA or interactive effects (Two-way ANOVA).

Fatty Acid Composition of Enriched *Artemia*

The fatty acid composition of the enriched *Artemia* is shown in Table 2. No significant ($P > 0.05$) treatment or interactive effects were observed in total lipid (mean = 4.2% wet weight). No significant ($P > 0.05$) treatment or interactive effects were observed in levels of saturated fatty acids (SFAs) (mean = 20.4%). Of the SFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in 16:0 (palmitic acid). A significant ($P < 0.05$) effect of DHA enrichment on levels of 14:0 (myristic acid) and 18:0 (stearic acid) was observed, with no significant ($P > 0.05$) ArA or interactive effects. Concentration of 14:0 was higher ($P < 0.05$) in *Artemia* enriched with 10% DHA than in those enriched with 0% DHA (Fig. 14). Concentration of 18:0 was higher ($P < 0.05$) in *Artemia* enriched with 0% DHA than in those enriched with 10% DHA (Fig.15).

No significant ($P < 0.05$) treatment or interactive effects of DHA and ArA enrichment were observed in levels of monounsaturated fatty acids (MUFAs). Of the MUFAs detected, 16:1 (palmitoleic acid) and 18:1n-9 (oleic acid), no significant ($P > 0.05$) treatment or interactive effects were observed.

No significant ($P < 0.05$) treatment or interactive effects of DHA and ArA enrichment were observed in levels of total n-3 PUFA (mean = 35.6%). Of the n-3 PUFA detected, no significant ($P > 0.05$) treatment or interactive effects were observed in 18:3n-3 (linolenic acid), 18:4n-3 (stearidonic acid), and 20:5n-3 (eicosapentaenoic acid, EPA). A significant effect of DHA enrichment on DHA levels was observed, with no significant ArA or interactive effects. Concentration of 22:6n-3 was higher ($P < 0.05$) in

Artemia enriched with 10% DHA than in those enriched with 0% DHA (undetected) (Fig. 16).

A significant ($P < 0.05$) effect of DHA enrichment on the ratio of 22:6n-3/20:5n-3 was observed, with no significant ($P > 0.05$) ArA or interactive effects. The ratio of 22:6n-3/20:5n-3 was higher ($P < 0.05$) in *Artemia* enriched with 10% DHA than those enriched with 0% DHA (undetected) (Fig. 17).

A significant ($P < 0.05$) effect of ArA enrichment on levels of n-6 PUFAs was observed, while there were no significant ($P > 0.05$) DHA or interactive effects. The n-6 PUFAs were higher ($P < 0.05$) in *Artemia* enriched with 6% ArA than in those enriched with 3% and 0% ArA (Fig. 18). Of the n-6 PUFA detected, no significant ($P > 0.05$) treatment or interactive effects were found in 18:2n-6 (linoleic acid). A significant effect of ArA enrichment on levels of 20:4n-6 (arachidonic acid, ArA) was observed with no significant ($P > 0.05$) DHA or interactive effects. Concentration of 20:4n-6 was higher ($P < 0.05$) in *Artemia* enriched with 6% ArA than in those enriched with 3% and 0% ArA (Fig. 19).

A significant ($P < 0.05$) effect of ArA enrichment on the ratio of n-3/n-6 PUFAs was observed, with no significant ($P > 0.05$) DHA or interactive effects. The ratio of n-3/n-6 PUFAs was higher ($P < 0.05$) in *Artemia* enriched with 0% ArA than those enriched in 3% and 6% ArA (Fig. 20).

A significant ($P < 0.05$) effect of ArA enrichment on the ratio of 20:5n-3/20:4n-6 was observed, with no significant ($P > 0.05$) DHA or interactive effects. The ratio of 20:5n-3/22:4n-6 was higher ($P < 0.05$) in *Artemia* enriched with 0% ArA than those enriched with 3% and 6% ArA (Fig. 21).

Table 2. Fatty acid composition (% of total FAMES) of enriched Artemia nauplii.

Artemia Treatments (DHA:ARA)						
Fatty acids	0:0	0:3	0:6	10:0	10:3	10:6
14:0	0.68 ± 0.23	0.88 ± 0.10	0.91 ± 0.09	2.2 ± 0.13*	1.6 ± 0.23*	1.9 ± 0.08*
16:0	11.9 ± 0.25	12.0 ± 0.38	12.0 ± 0.26	12.7 ± 0.86	11.7 ± .71	12.7 ± 0.41
16:1	3.6 ± 0.16	3.4 ± 0.25	3.6 ± 0.16	3.7 ± 0.20	3.3 ± .20	3.7 ± 0.27
18:0	7.0 ± 0.35	6.8 ± 0.30	6.9 ± 0.36	6.5 ± 0.17	6.5 ± 0.20	6.4 ± 0.20
18:1n-9	33.3 ± 5.7	35.4 ± 5.5	33.3 ± 3.6	34.3 ± 2.9	31.3 ± 3.5	28.7 ± 1.2
18:2n-6	5.5 ± 0.31	5.7 ± 0.23	5.8 ± 0.37	5.2 ± 0.38	5.5 ± 0.47	5.8 ± 0.26
18:3n-3	29.8 ± 3.7	27.3 ± 4.6	27.3 ± 3.6	25.4 ± 2.4	26.0 ± 3.6	27.3 ± 1.6
18:4n-3	4.5 ± 0.74	4.2 ± 0.74	4.2 ± 0.52	3.9 ± 0.40	3.9 ± 0.42	3.9 ± 0.38
20:4n-6	1.0 ± 0.11	2.1 ± 0.29*	3.5 ± 0.65*	0.23 ± 0.23	2.8 ± 0.38*	3.9 ± 0.22*
20:5n-3	2.2 ± 0.37	2.1 ± 0.36	2.2 ± 0.26	2.4 ± 0.26	2.7 ± 0.11	2.5 ± 0.28
22:6n-3	Undetected	Undetected	Undetected	3.5 ± 0.92*	4.4 ± 1.1*	3.1 ± 0.40*
∑Saturates	20.0 ± 0.39	20.0 ± 0.51	19.8 ± 0.21	21.4 ± 0.78	19.9 ± 0.66	21.0 ± 0.55
∑Monounsaturates	36.9 ± 5.5	38.8 ± 5.38	36.9 ± 3.5	38.0 ± 2.7	34.7 ± 3.3	32.3 ± 1.1
∑n-3 Polyunsaturates	36.5 ± 4.8	33.6 ± 5.7	34.0 ± 4.3	35.2 ± 2.8	37.1 ± 3.1	36.9 ± 1.8
∑n-6 Polyunsaturates	6.6 ± 0.41	7.8 ± 0.17*	9.3 ± 0.57*	5.4 ± 0.22	8.3 ± 0.18*	9.70 ± 0.39*
n-3/n-6 Polyunsaturates	5.5 ± 0.45	4.3 ± 0.82	3.7 ± 0.72	6.5 ± 0.70	4.4 ± 0.37	3.8 ± 0.33
22:6n-3/20:5n-3	0	0	0	1.4 ± 0.36	1.6 ± 0.44	1.3 ± 0.27
22:6n-3/20:4n-6	0	0	0	2.5 ± 1.2	1.5 ± 0.21	0.79 ± 0.06
20:5n-3/20:4n-6	2.1 ± 0.18	1.1 ± 0.35	0.72 ± 0.25	4.5 ± 0.71	1.1 ± 0.20	0.66 ± 0.11
Total Lipid (% wet tissue wt.)	3.7 ± 0.24	3.6 ± 0.61	4.2 ± 0.26	3.8 ± 0.77	4.9 ± 081	4.81 ± 0.64

Asterisk (*) indicates mean values (mean ± SEM, N=3) within rows that are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control

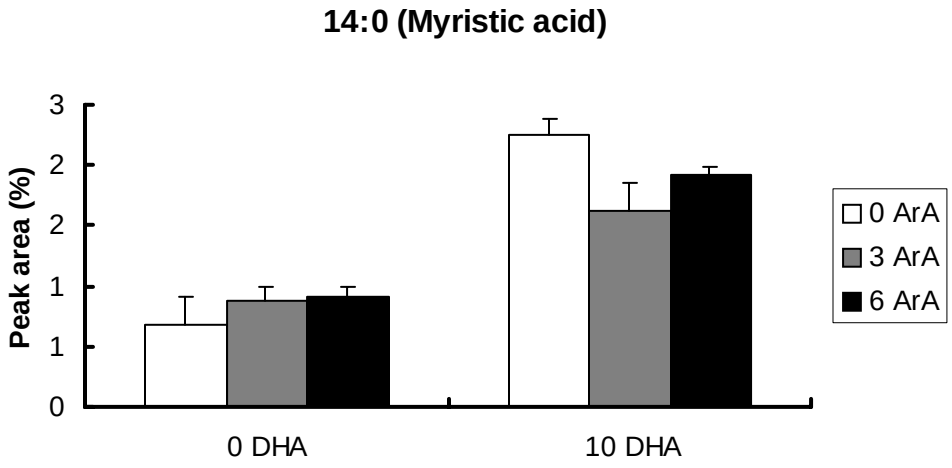

Figure 14. Percentage of total FAMES of 14:0 (myristic acid) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

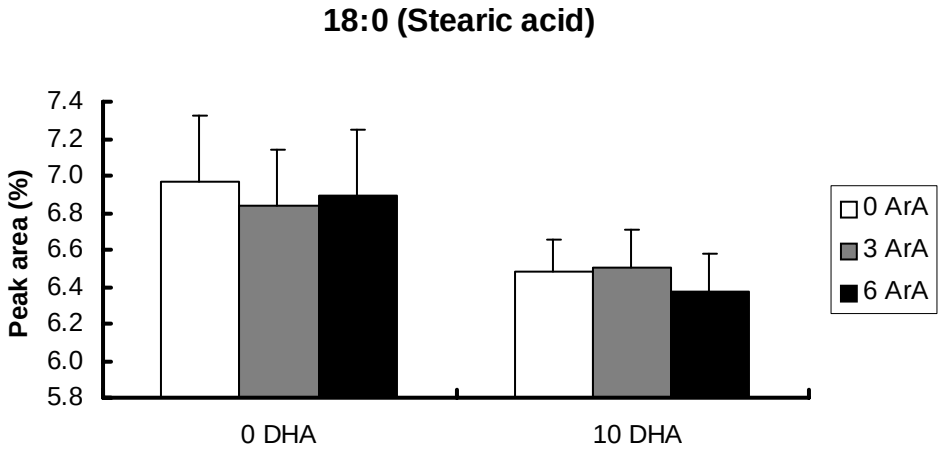

Figure 15. Percentage of total FAMES of 18:0 (stearic acid) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

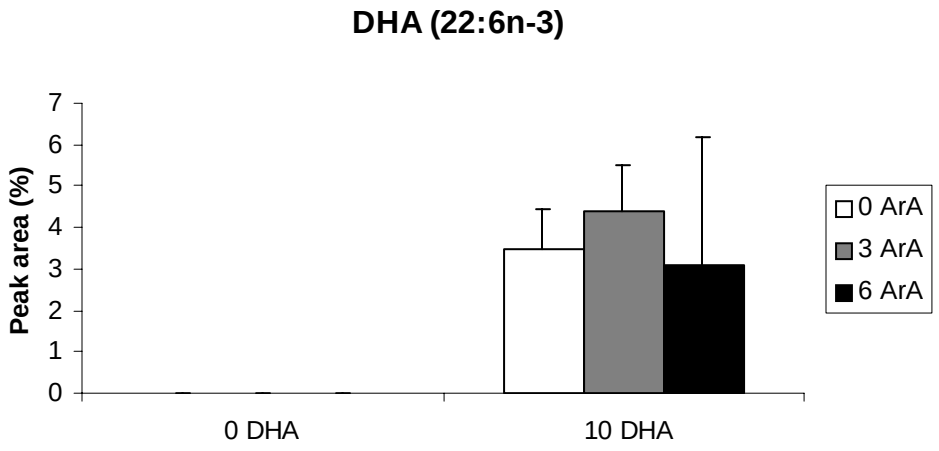

Figure 16. Percentage of total FAMES of 22:6n-3 (docosahexaenoic acid) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$)were observed, with no significant ArA or interactive effects (Two-way ANOVA).

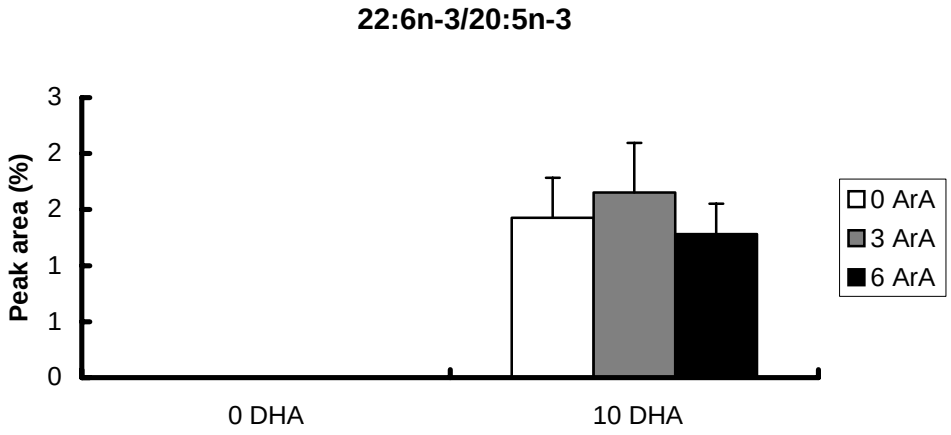

Figure 17. The ratio of 22:6n-3 (DHA) to 20:5n-3 (EPA) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA).

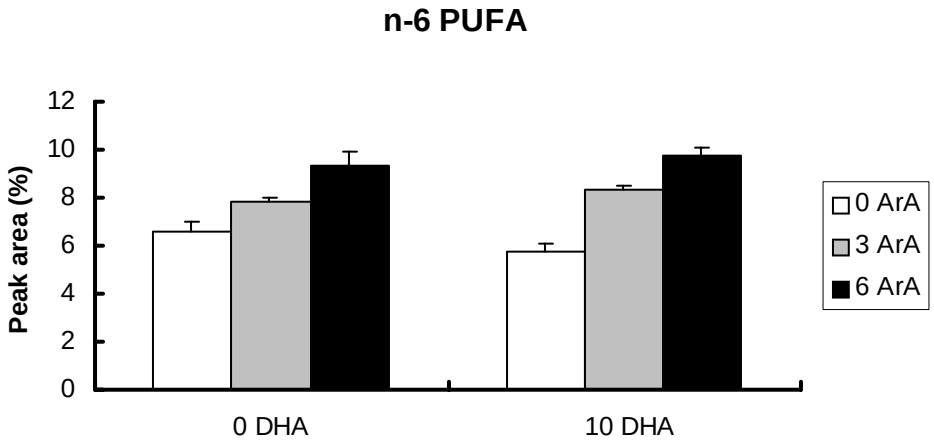

Figure 18. Percentage of total FAMES of total n-6 polyunsaturated fatty acids (PUFA) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no DHA or interactive effects (Two-way ANOVA).

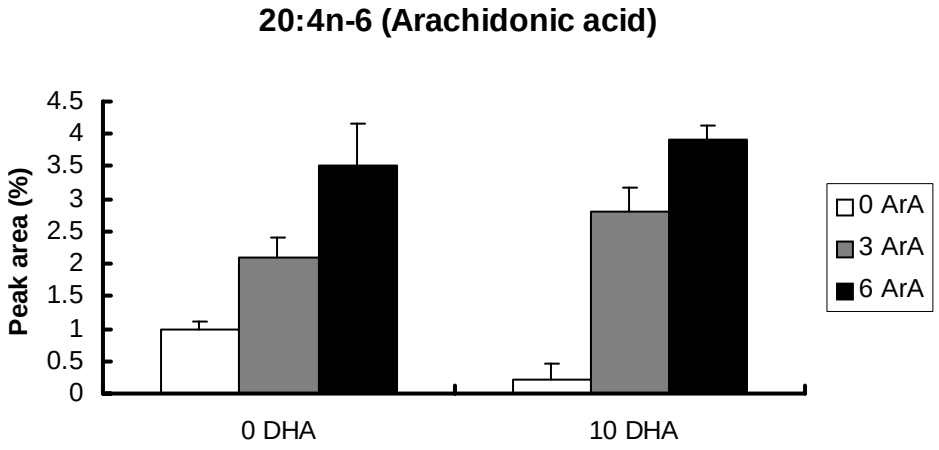

Figure 19. Percentage of total FAMES of 20:4n-6 (ArA) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no significant DHA or interactive effects (Two-way ANOVA).

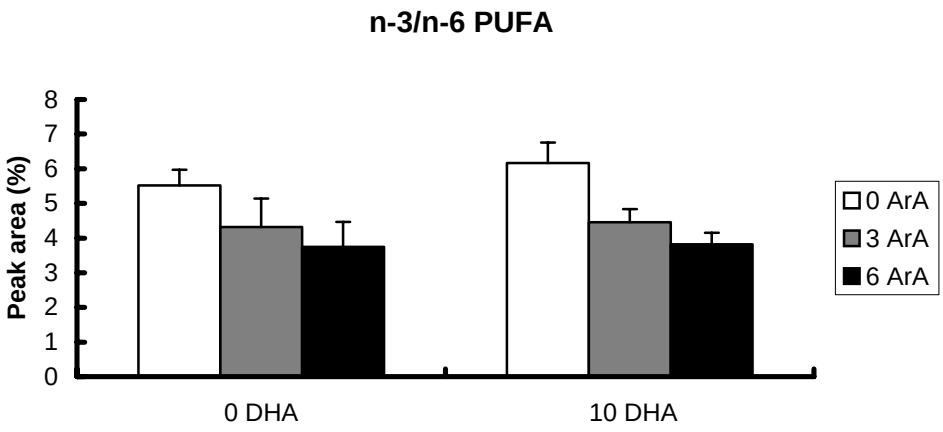

Figure 20. Percentage of total FAMES of ratio of total n-3/n-6 polyunsaturated fatty acids (PUFA) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). A significant effect of ArA ($P < 0.05$) was observed, with no DHA or interactive effects (Two-way ANOVA).

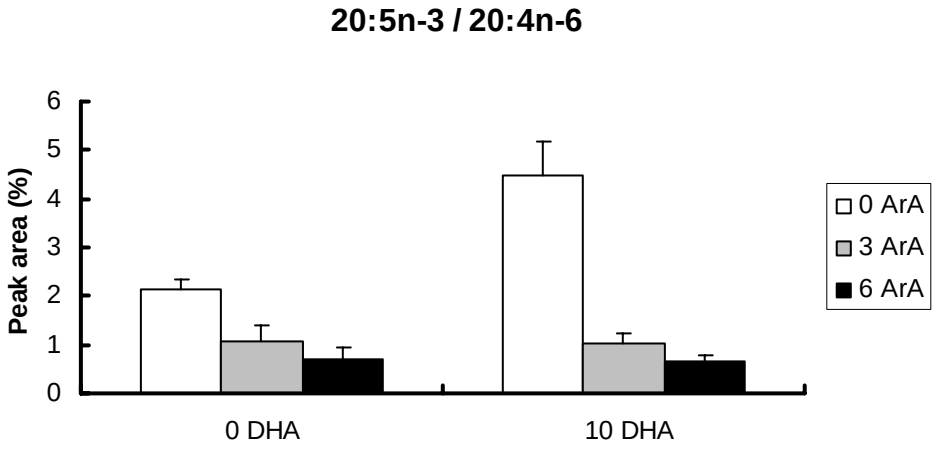

Figure 21. Ratio of 20:5n-3 (EPA) to 20:4n-6 (ArA) ($\bar{X}$ ± SEM, N = 3) in *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). A significant effect of ArA ($P < 0.05$) was observed, with no DHA or interactive effects (Two-way ANOVA).

Fatty acid composition of d24ph larvae

The fatty acid composition of larvae on d24ph is shown in Table 3. No significant ($P > 0.05$) treatment or interactive effects were observed in total lipid (mean = 4.4% wet wt.). No significant ($P > 0.05$) treatment or interactive effects were observed in levels of saturated fatty acids (SFAs). Of the SFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in 20:0 (arachidic acid) (mean = 1.0%). Significant ($P < 0.05$) effects of DHA on the levels of 14:0 (myristic acid) and 16:0 (palmitic acid) were observed, while there was no significant ($P > 0.05$) ArA or interactive effects. Concentration of 14:0 was higher ($P < 0.05$) in larvae fed 10% DHA than in larvae fed 0% DHA (Fig. 31). Concentration of 16:0 was higher ($P < 0.05$) in larvae fed 10% DHA than in larvae fed 0% DHA (Fig. 32). Significant effects of DHA and ArA on levels of 18:0 (stearic acid) were observed, with no interactive effects. Concentration of 18:0 was higher ($P < 0.05$) in larvae fed 0% DHA than in larvae fed 10% DHA (Fig. 33). Concentration of 18:0 was also higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 6% and 3% ArA.

Significant ($P < 0.05$) effects of both DHA and ArA on levels of monounsaturated fatty acids (MUFAs) were observed, with no interactive effects. MUFA levels were higher ($P < 0.05$) larvae fed 0% DHA than in larvae fed 10% DHA (Fig. 34). MUFA levels were higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 6% and 3% ArA. Of the MUFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in 16:1 (palmitoleic acid) and 20:1 (Eicosenoic acid). Significant ($P < 0.05$) effects of DHA and ArA on levels of 18:1n-9 (oleic acid) were observed with no interactive effects. Concentration of 18:1n-9 was higher ($P < 0.05$) in larvae fed 0% DHA than in

larvae fed 10% DHA (Fig. 35). The concentration of 18:1n-9 was also higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 3% and 6% ArA.

Significant ($P < 0.05$) effects of both DHA and ArA on levels of n-3 polyunsaturated fatty acids (PUFAs) were observed, with no interactive effects. The n-3 PUFAs were higher ($P < 0.05$) in larvae fed 10% DHA than in larvae fed 0% DHA (Fig. 36). The n-3 PUFAs levels were also higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 6% and 3% ArA. Of the n-3 PUFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in 22:5n-3 (docosapentaenoic acid). Significant ($P < 0.05$) effects of DHA on levels of 18:3n-3 (linolenic acid) and 22:6n-3 (docosahexaenoic acid) were observed, with no significant ($P > 0.05$) effect of ArA or interactive effects. Concentration of 18:3n-3 was higher ($P < 0.05$) in larvae fed 0% DHA than in larvae fed 10% DHA (Fig. 37). Concentration of 22:6n-3 was higher ($P < 0.05$) in larvae fed 10% DHA than in larvae fed 0% DHA (Fig. 38). Significant ($P < 0.05$) effects of both DHA and ArA on levels of 18:4n-3 (stearidonic acid) and 20:5n-3 (eicosapentanoic acid) were observed, with no interactive effects. Concentration of 18:4n-3 was higher ($P < 0.05$) in larvae fed 0% DHA than in larvae fed 10% DHA (Fig. 39). Concentration of 18:4n-3 was also higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 6% and 3% ArA. Concentration of 20:5n-3 (EPA) was higher ($P < 0.05$) in larvae fed 10% DHA than in larvae fed 0% DHA (Fig. 40). Concentration of 20:5n-3 was also higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 3% and 6% ArA. A significant ($P < 0.05$) effect of DHA and ArA enrichment on the ratio of 22:6n-3 / 20:5n-3 was observed, with no interactive effects. The ratio of 22:6n-3/20:5n-3 was higher ($P < 0.05$) in larvae fed 10% DHA than those fed 0% DHA (Fig. 41). The ratio of 22:6n-

3/20:5n-3 was higher ($P < 0.05$) in larvae fed 6% and 3% ArA than in larvae fed 0% ArA.

A significant ($P < 0.05$) effect of ArA on levels of n-6 PUFAs was observed, with no significant ($P > 0.05$) DHA or interactive effects. The n-6 PUFAs were higher ($P < 0.05$) in larvae fed 6% ArA than in larvae fed 3% and 0% ArA (Fig. 42). Of the n-6 PUFAs detected, no significant ($P > 0.05$) treatment or interactive effects were found in 18:2n-6 (linoleic acid). A significant ($P < 0.05$) effect of ArA on levels of 20:4n-6 (arachidonic acid, ArA) was observed, with no significant ($P > 0.05$) DHA or interactive effects. Concentration of 20:4n-6 was higher ($P < 0.05$) in larvae fed 6% ArA than in larvae fed 3% and 0% ArA (Fig. 43).

A significant ($P < 0.05$) effect of ArA on the ratio of n-3/n-6 PUFAs was observed, with no significant ($P > 0.05$) DHA or interactive effects. The ratio of n-3/n-6 PUFAs was higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 3% and 6% ArA (Fig. 44).

A significant ($P < 0.05$) effect of ArA enrichment on the ratio of 20:5n-3/20:4n-6 was observed, with no significant ($P > 0.05$) DHA or interactive effects. The ratio of 20:5n-3/22:4n-6 was higher ($P < 0.05$) in larvae fed 0% ArA than in larvae fed 3% and 6% ArA (Fig. 45).

Table 3. Fatty acid composition (% of total FAMES) of black sea bass larvae at d24ph.

Enriched diets received by larvae (DHA:ARA)						
Fatty acids	0:0	0:3	0:6	10:0	10:3	10:6
14:0	0.13 ± 0.13	0.39 ± 0.23	0.21 ± 0.13	0.60 ± 0.21	0.76 ± 0.02	0.56 ± 0.17
16:0	13.4 ± 0.23	13.8 ± 0.20	13.7 ± 0.27	14.1 ± 0.45	14.35 ± 0.32	14.4 ± 0.32
16:1	2.7 ± 0.27	2.7 ± 0.32	2.5 ± 0.16	2.9 ± 0.11	2.7 ± 0.10	2.6 ± 0.11
18:0	8.1 ± 0.15	7.5 ± 0.15*	7.5 ± 0.06*	7.2 ± 0.12*	6.6 ± 0.07*	6.7 ± 0.03*
18:1n-9	28.4 ± 0.21	27.0 ± 0.19	26.1 ± 0.58*	25.1 ± 0.68*	23.9 ± 0.52*	22.6 ± 0.57*
18:2n-6	7.4 ± 0.18	7.7 ± 0.06	7.7 ± 0.31	7.7 ± 0.37	8.0 ± 0.26	8.1 ± 0.28
18:3n-3	22.6 ± 0.12	21.8 ± 0.50	22.1 ± 0.84	22.2 ± 0.50	20.2 ± 0.33	20.7 ± 0.99
18:4n-3	3.4 ± 0.11	3.2 ± 0.12	3.0 ± 0.12	3.2 ± 0.18	2.9 ± 0.07	2.8 ± 0.12*
20:0	1.3 ± 0.03	1.01 ± 0.32	0.86 ± 0.27	1.1 ± 0.06	1.1 ± 0.09	0.85 ± 0.24
20:1s	2.6 ± 0.02	2.5 ± 0.04	2.6 ± 0.25	2.5 ± 0.06	2.3 ± 0.03	3.1 ± 0.35
20:4n-6	2.0 ± 0.02	3.9 ± 0.26*	6.3 ± 0.25*	1.8 ± 0.07	4.5 ± 0.12*	5.8 ± 0.31*
20:5n-3	4.8 ± 0.09	4.7 ± 0.13	4.2 ± 0.13	5.5 ± 0.20*	5.1 ± 0.17	4.7 ± 0.13
22:5n-3	1.0 ± 0.05	0.75 ± 0.17	0.87 ± 0.04	0.69 ± 0.18	0.88 ± 0.03	1.1 ± 0.11
22:6n-3	2.0 ± 0.09	2.9 ± 0.13	2.2 ± 0.37	5.2 ± 0.24*	6.5 ± 0.37*	5.7 ± 0.76*
∑Saturates	22.9 ± 0.27	22.7 ± 0.50	22.3 ± 0.38	23.0 ± 0.28	22.8 ± 0.36	22.6 ± 0.40
∑Monounsaturates	33.8 ± 0.25	32.3 ± 0.21*	31.3 ± 0.58*	30.5 ± 0.59*	28.9 ± 0.60*	28.4 ± 0.65*
∑n-3 Polyunsaturates	33.8 ± .031	33.4 ± 0.73	32.4 ± 1.0	36.8 ± 1.1	35.7 ± 0.80	35.0 ± 0.45
∑n-6 Polyunsaturates	9.4 ± 0.19	11.6 ± 0.25*	13.9 ± 0.32*	9.5 ± 0.34	12.6 ± 0.18*	14.0 ± 0.52*
n-3/n-6 Polyunsaturates	3.6 ± 0.10	2.9 ± 0.10*	2.3 ± 0.12*	3.9 ± 0.24	2.8 ± 0.10*	2.5 ± 0.11*
22:6n-3/20:5n-3	0.41 ± 0.01	0.61 ± 0.02	0.53 ± 0.08	0.93 ± 0.042*	1.3 ± 0.05*	1.2 ± 0.14*
22:6n-3/20:4n-6	0.97 ± 0.05	0.73 ± 0.07	0.36 ± 0.06*	2.8 ± 0.12*	1.4 ± 0.06*	0.98 ± 0.08
20:5n-3/20:4n-6	2.4 ± 0.06	1.2 ± 0.07*	0.67 ± 0.02*	3.1 ± 0.07*	1.1 ± 0.03*	0.81 ± 0.03*
Total lipid	4.6 ± 0.8	4 ± 0.72	3.8 ± 0.55	4.4 ± 0.15	5.1 ± 0.21	4.3 ± 0.51
(% wet tissue wt.)						

Asterisk (*) indicates mean values (mean ± SEM, N=4) within rows that are significantly (P < 0.05) different from the 0:0 (DHA:ArA) control.

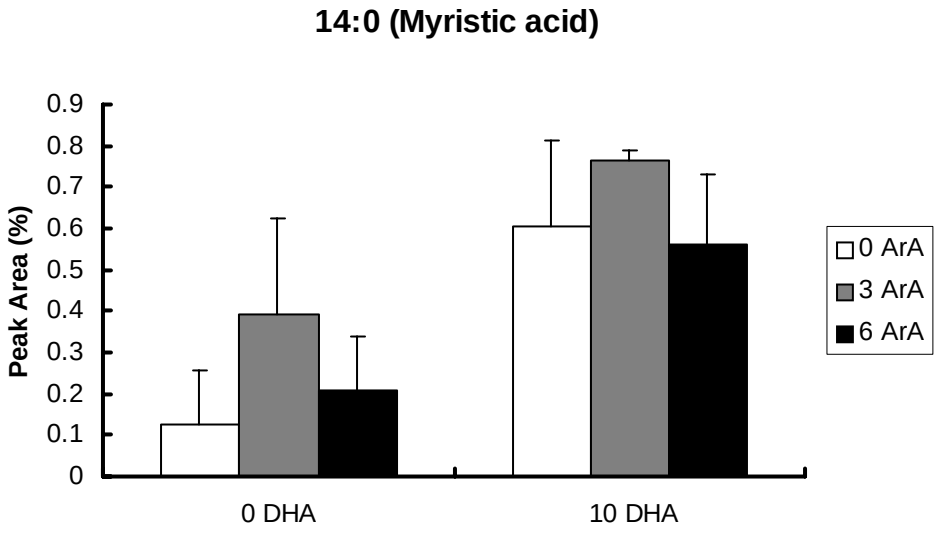

Figure 22. Percentage of 14:0 (myristic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no significant ArA or interactive effects (Two-way ANOVA).

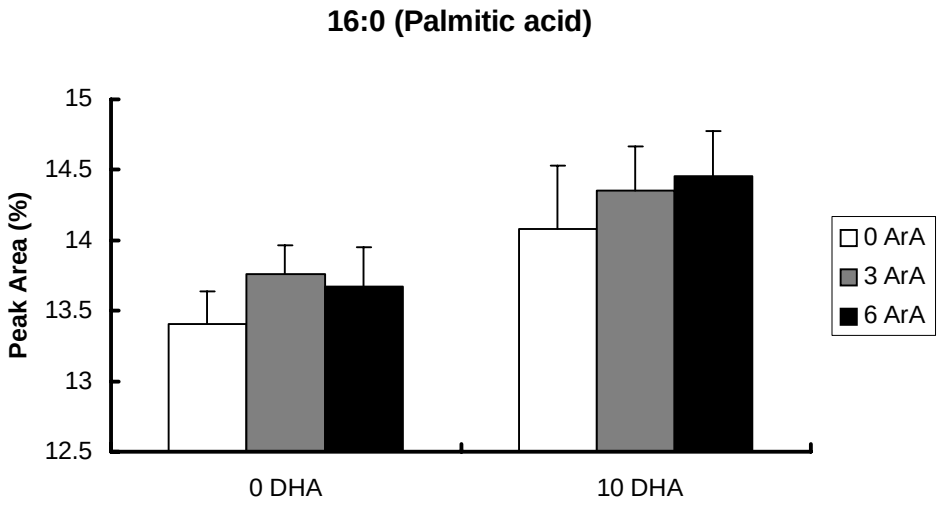

Figure 23. Percentage of 16:0 (palmitic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no significant ArA treatment or interactive effects (Two-way ANOVA).

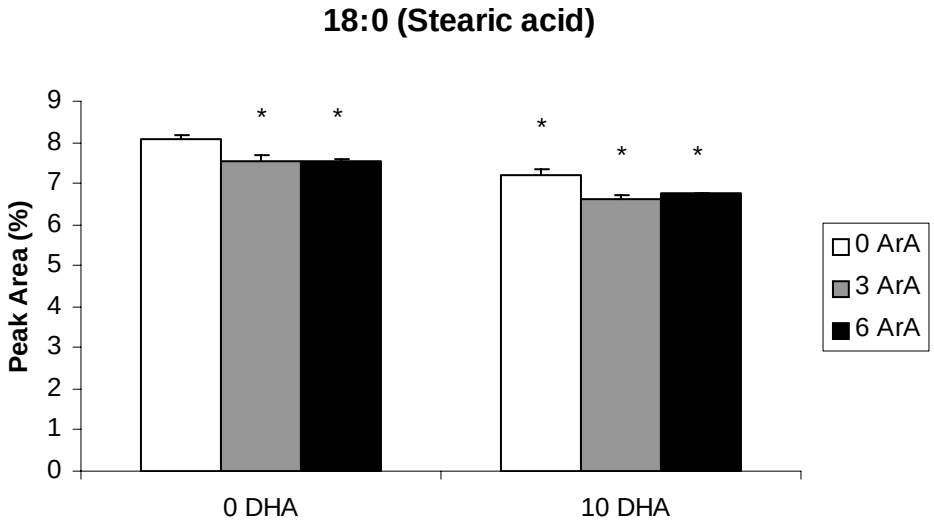

Figure 24. Percentage of 18:0 (stearic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects. Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

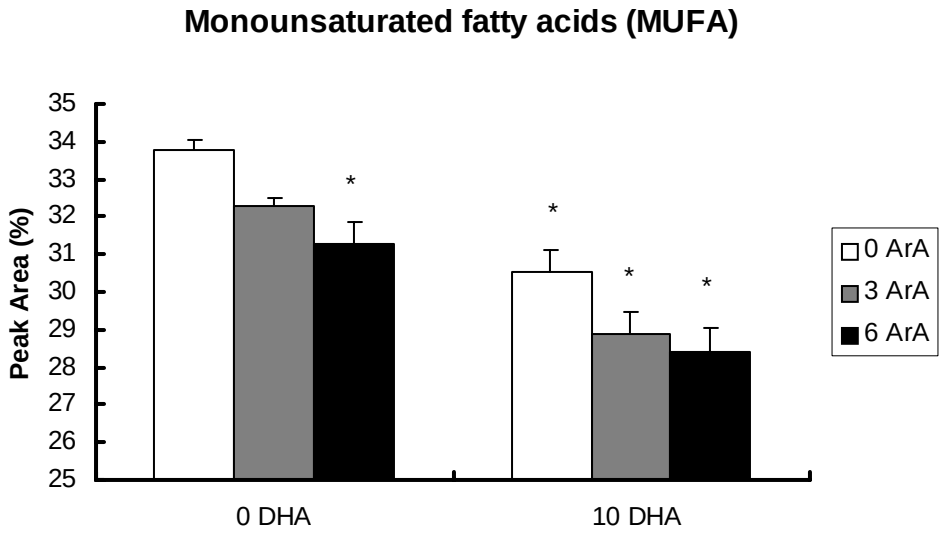

Figure 25. Percentage of total monounsaturated fatty acids (MUFAs) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and artemia enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects. Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

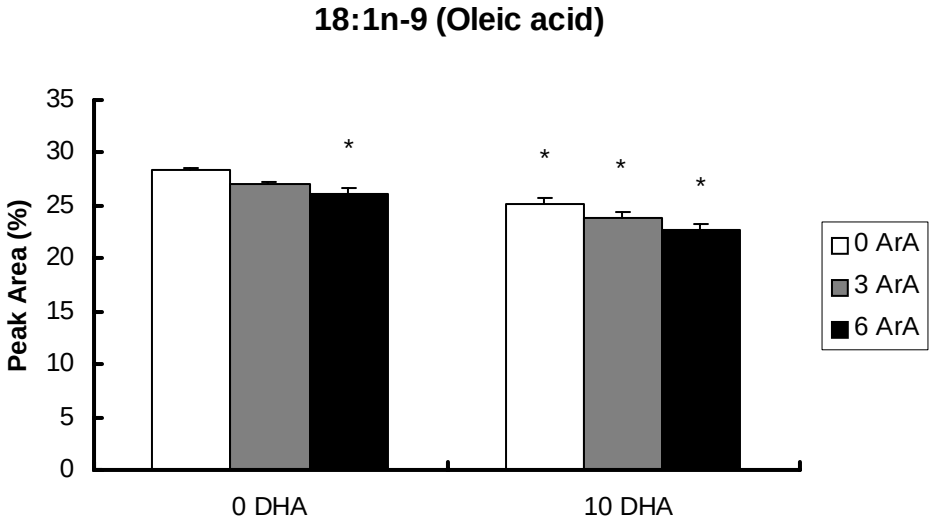

Figure 26. Percentage of 18:1n-9 (oleic acid) ($\overline{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects. Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

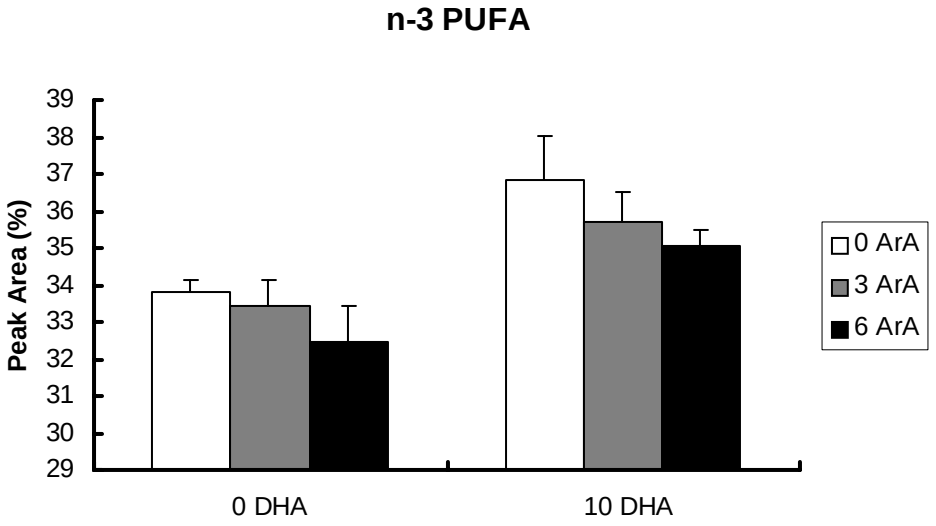

Figure 27. Percentage of n-3 polyunsaturated fatty acids (PUFAs) ($\overline{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects.

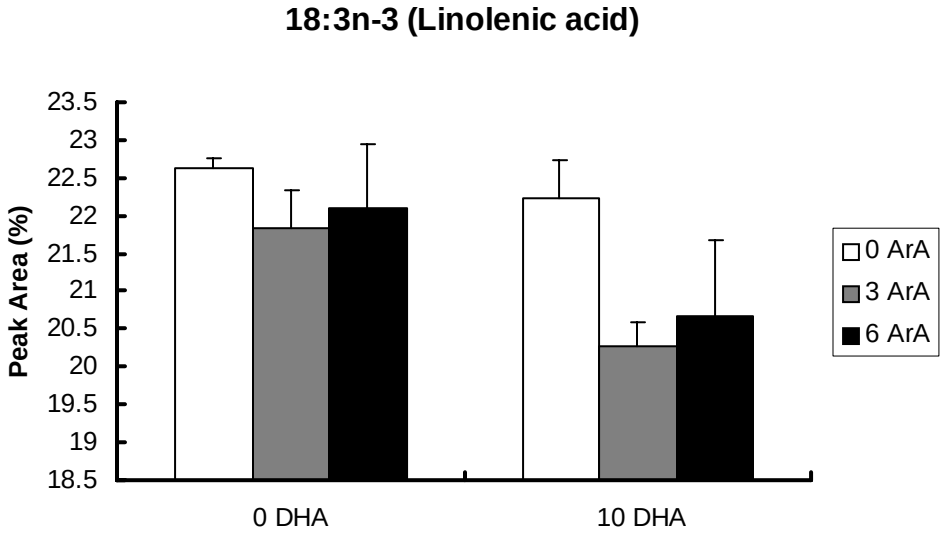

Figure 28. Percentage of 18:3n-3 (linolenic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no significant ArA or interactive effects (Two-way ANOVA).

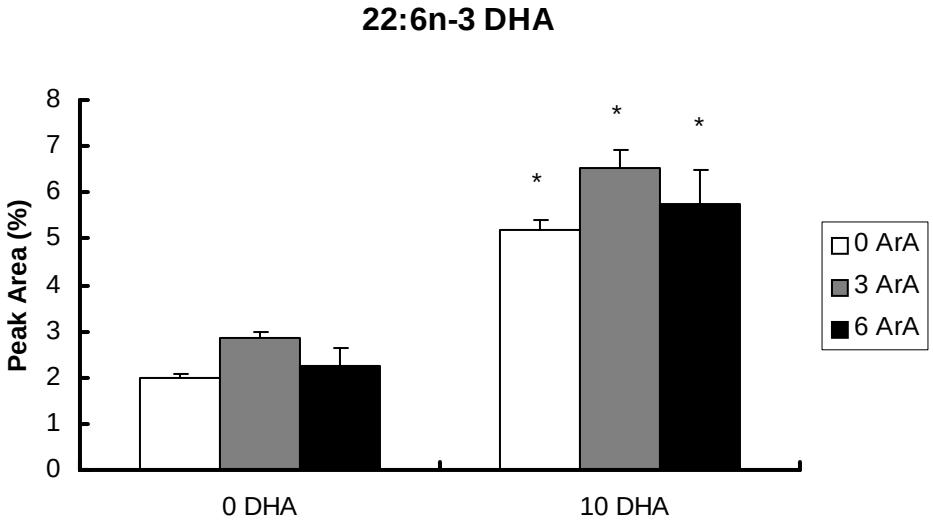

Figure 29. Percentage of 22:6n-3 (docosahexaenoic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no significant ArA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

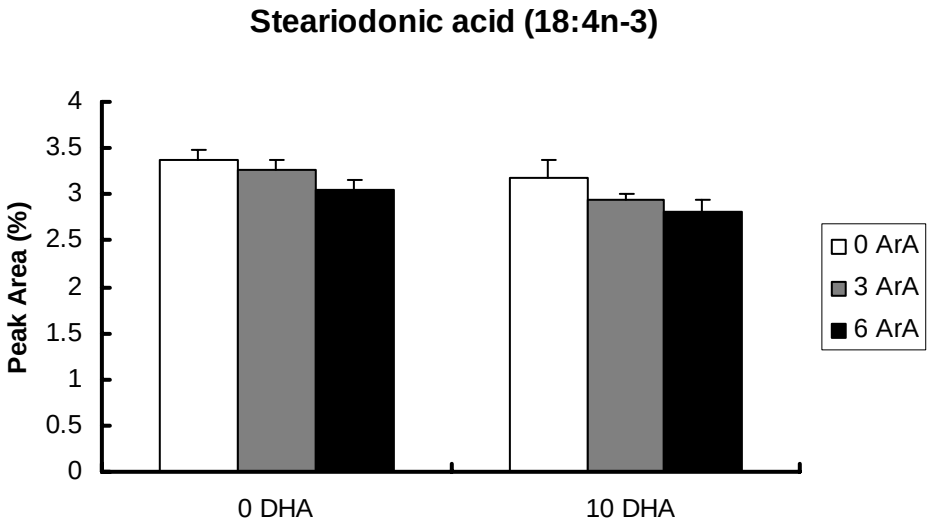

Figure 30. Percentage of 18:4n-3 (stearidonic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects.

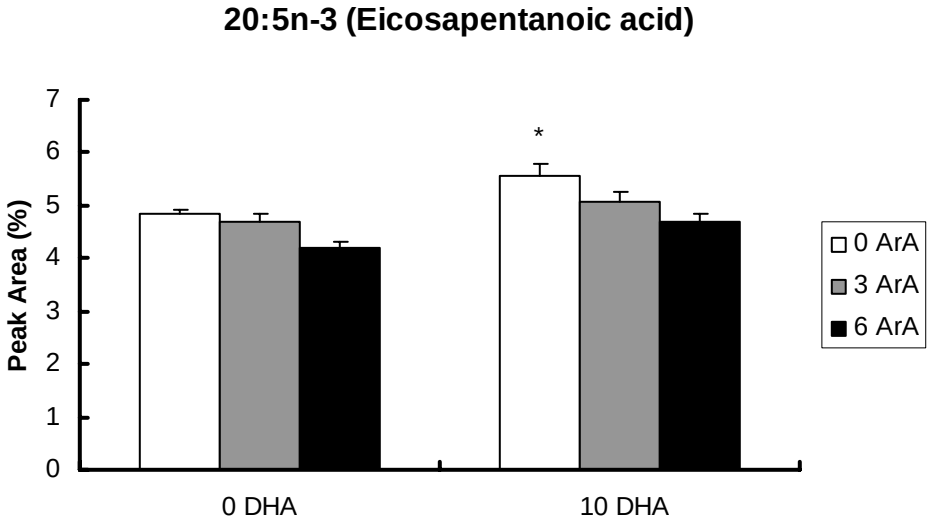

Figure 31. Percentage of 20:5n-3 (eicosapentanoic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects. Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

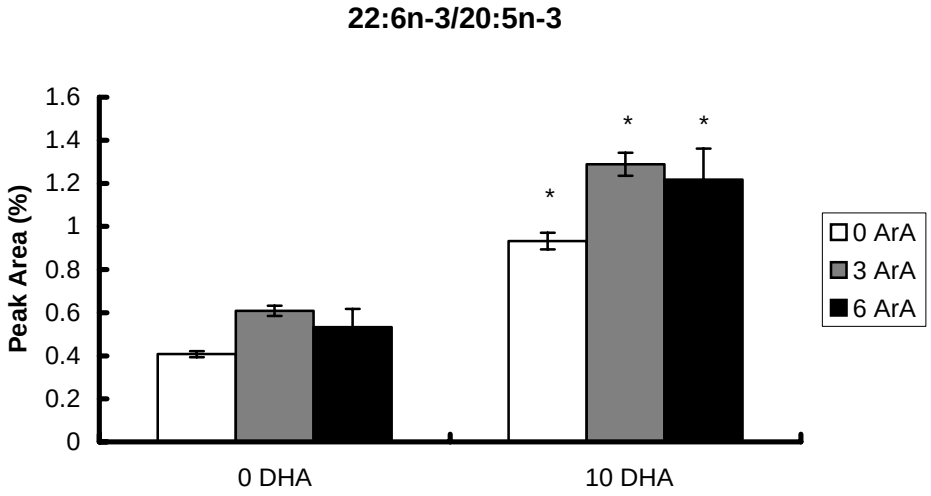

Figure 32. Ratio of 22:6n-3 (DHA) to 20:5n-3 ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) and ArA ($P < 0.05$) were observed, with no interactive effects. Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

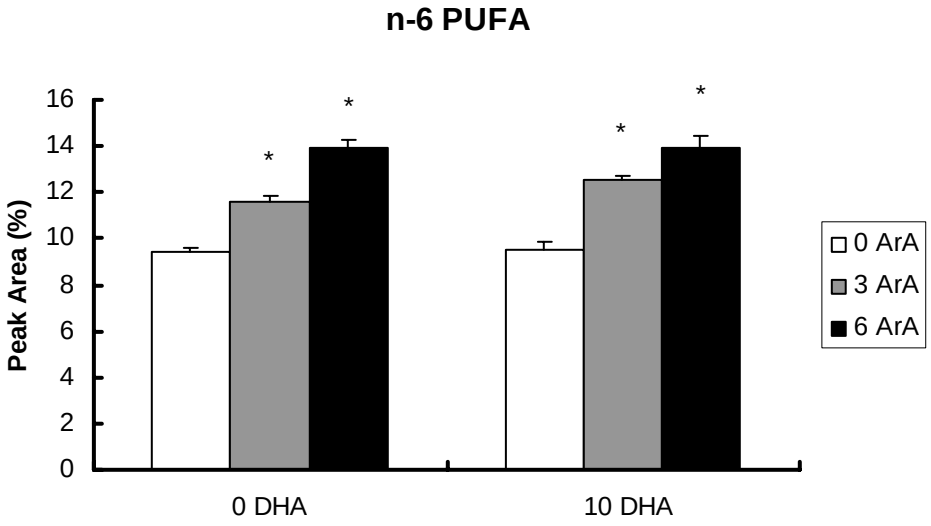

Figure 33. Percentage of n-6 polyunsaturated fatty acids (PUFAs) ($\overline{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no significant DHA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

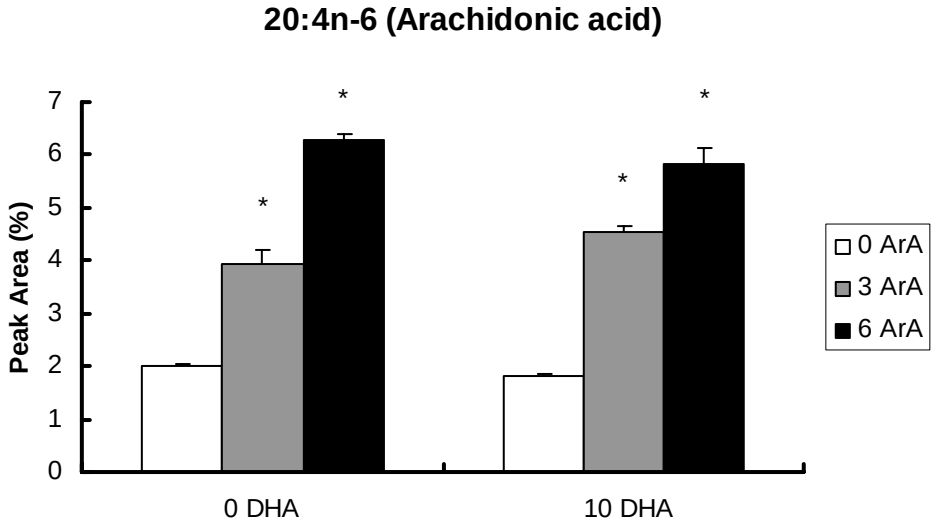

Figure 34. Percentage of 20:4n-6 (arachidonic acid) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no significant DHA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

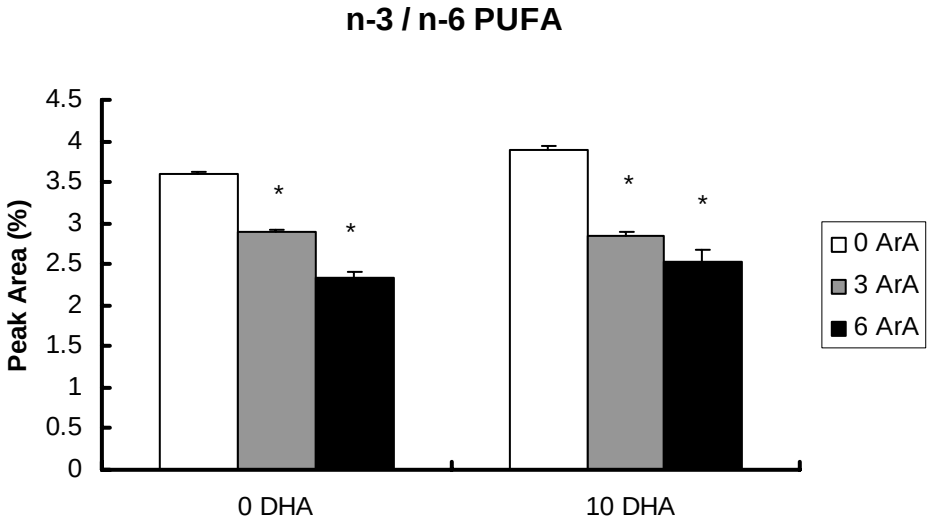

Figure 35. The ratio n-3/n-6 PUFA ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no significant DHA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

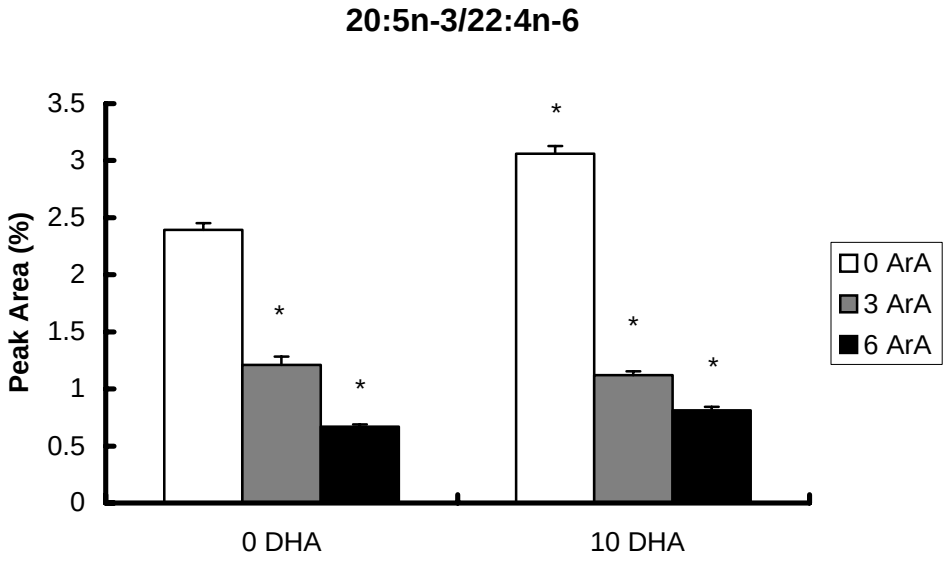

Figure 36. The ratio 20:5n-3 (EPA) to 20:4n-6 (ArA) ($\bar{X}$ ± SEM, N = 4) in black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with six different oil emulsions containing different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of ArA ($P < 0.05$) were observed, with no significant DHA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

Hypersaline Stress Evaluation

No significant ($P > 0.05$) treatment or interactive effects were found in median survival time following hypersaline exposure.

Growth

No significant ($P > 0.05$) treatment or interactive effects were found in notochord length before d17ph (Fig. 37). By d17ph, a significant ($P < 0.05$) effect of DHA on notochord length was observed, while there were no significant ($P > 0.05$) ArA or interactive effects. On d17ph, notochord length was higher ($P < 0.05$) in larvae fed 10% DHA (mean = 5.8 mm) than in larvae fed 0% DHA (mean = 5.6 mm). When all means were compared to the 0:0 (DHA:ArA) control (5.6 mm), notochord length was significantly higher ($P < 0.05$) at the 10:3 (5.9 mm) treatment level (Fig. 38). By d24ph, a significant ($P < 0.05$) effect of DHA on notochord length was observed, with no significant ($P > 0.05$) ArA or interactive effects. On d24ph notochord length was higher ($P < 0.05$) in larvae fed 10% DHA (mean = 8.5 mm) than in larvae fed 0% DHA (mean = 7.8 mm). When all means were compared to the 0:0 (DHA:ArA) control (7.7 mm), notochord length on d24ph was significantly higher ($P < 0.05$) at the 10:0 (8.61 mm) and 10:6 (8.6 mm) treatment levels (Fig. 39).

No significant ($P > 0.05$) treatment or interactive effects were found in larval wet weight before d17ph (Fig. 40). By d17ph, significant ($P < 0.05$) effects of DHA and ArA on larval wet weight were observed, with no interactive effects ($P > 0.05$). On d17ph, larval wet weight was higher ($P < 0.05$) in larvae fed 10% DHA (mean = 3.1 mg) than in

larvae fed 0% DHA (mean = 2.6 mg) (Fig. 41). Also on d17ph, larval wet weight was higher ($P < 0.05$) in larvae fed 6% ArA (mean = 3.2 mg) than in larvae fed 0% or 3% ArA (mean = 2.7 mg). When all means were compared to the 0:0 (DHA:ArA) control (2.4 mg), wet weight was significantly higher ($P < 0.05$) in the 10:6 (3.5 mg) treatment level (Fig. 41). By d24ph no significant ($P > 0.05$) treatment or interactive effects on larval wet weight were observed.

No significant ($P > 0.05$) treatment or interactive effects were found in larval dry weight before d17ph (Fig. 42). By d17ph, a significant ($P < 0.05$) effect of DHA on dry weight was observed, while there were no significant ($P > 0.05$) ArA or interactive effects. On d17ph, dry weight was higher ($P < 0.05$) in larvae fed 10% DHA (mean = 0.55 mg) than in larvae fed 0% DHA (mean = 0.45 mg). When all means were compared to the 0:0 (DHA:ArA) control (0.43 mg), dry weight was higher ($P < 0.05$) at the 10:6 (0.62 mg) treatment level (Fig. 43). By d24ph, no significant ($P > 0.05$) treatment or interactive effects on larval dry weight were observed.

Survival

On d9ph, mean survival for all treatments was 85%, with no significant ($P > 0.05$) treatment effects observed (Fig. 44). On d17ph, mean survival for all treatments declined to an average of 72.4%, with no significant ($P > 0.05$) treatment effects observed. By d24ph, a significant ($P < 0.05$) effect of DHA on survival was observed, while there were no significant ($P > 0.05$) ArA or interactive effects. On d24ph, survival was higher ($P < 0.05$) in larvae fed 10% DHA (mean = 55%) than in larvae fed 0% DHA (mean = 31%).

When all means were compared to the 0:0 (DHA:ArA) control (27%), survival was significantly higher ($P \leq 0.05$) at the 10:6 (62%) treatment level (Fig. 45).

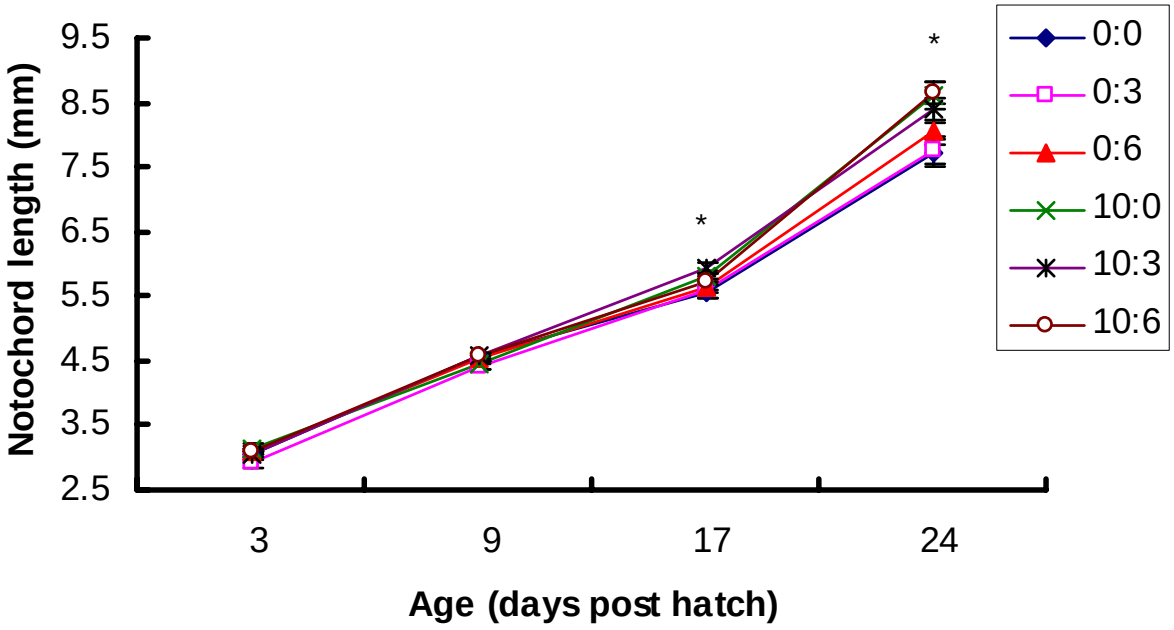

Figure 37. Notochord length (mm) ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae fed rotifers and *Artemia* enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Asterisk (*) indicates significant ($P < 0.05$) treatment effects observed on that sampling date.

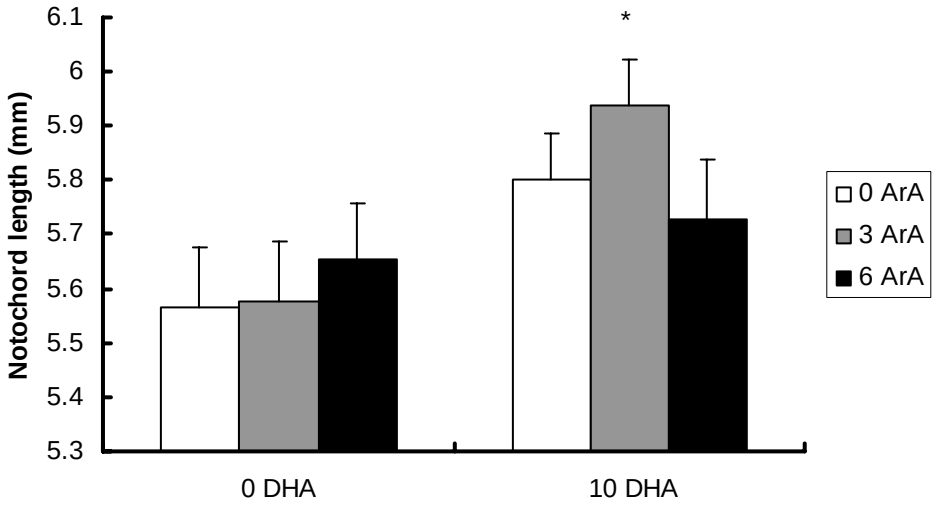

Figure 38. Notochord lengths (mm) ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae (day 17 post hatch) fed rotifers enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

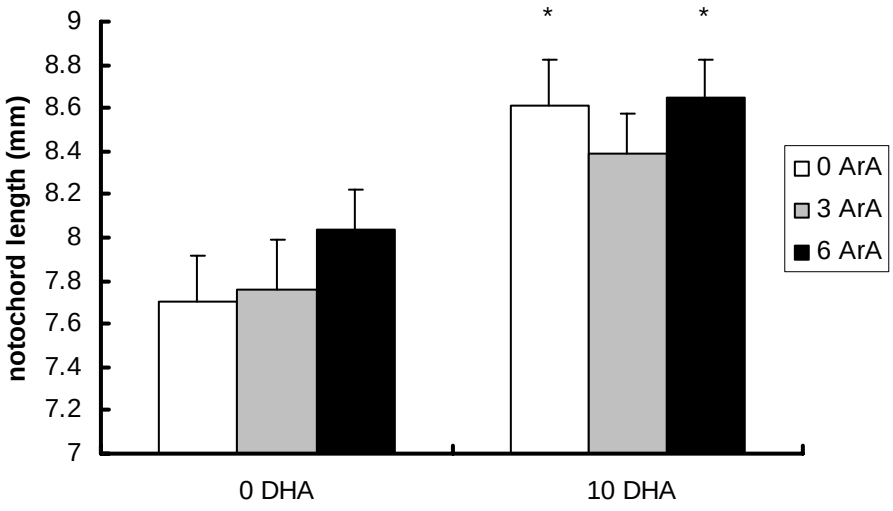

Figure 39. Notochord lengths (mm) ($\bar{X}$ ± SEM, N = 5) of post-metamorphic stage black sea bass larvae (d24ph) fed rotifers and *Artemia* enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no significant ArA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

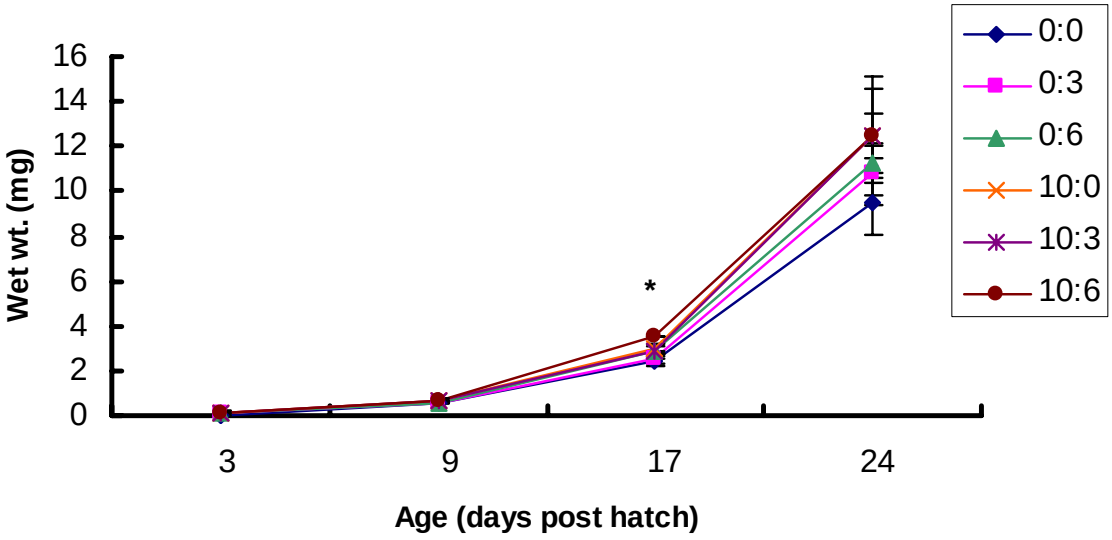

Figure 40. Wet weight (mg) ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae fed rotifers and *Artemia* enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Asterisk (*) indicates significant ($P < 0.05$) treatment effects observed on that sampling date (T-test).

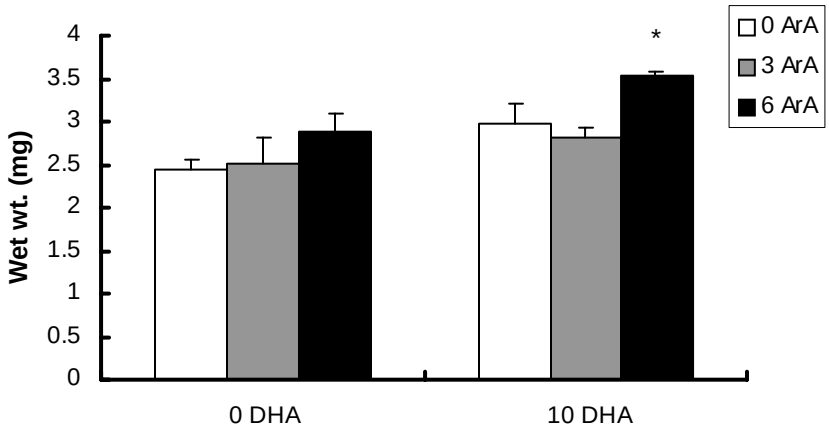

Figure 41. Wet weight (mg) ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae (d17ph) fed rotifers enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA and ArA ($P < 0.05$) were observed, with no interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

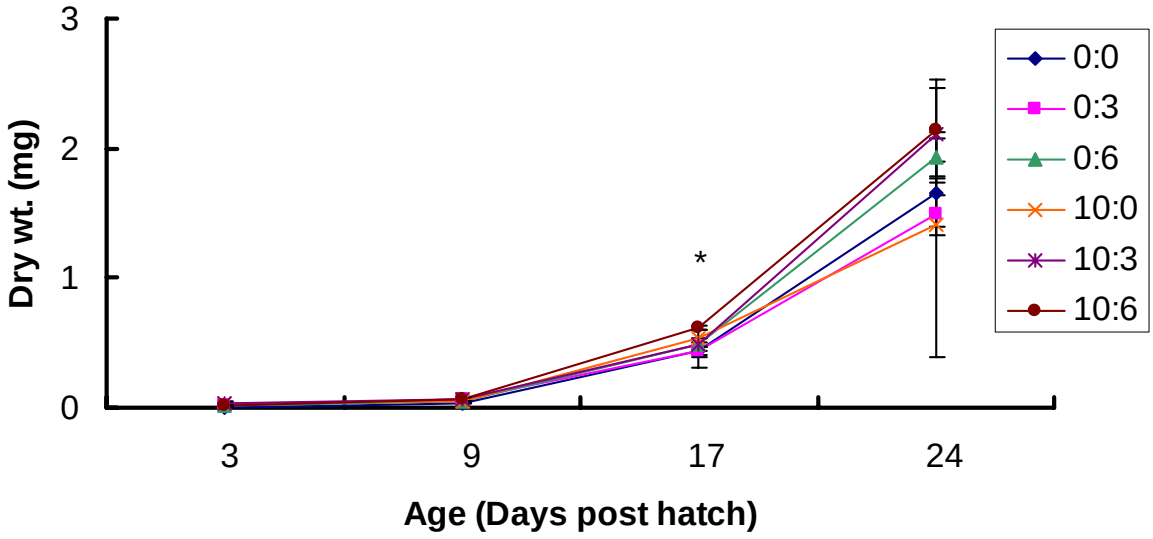

Figure 42. Dry weight (mg) ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae fed rotifers and *Artemia* enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Asterisk (*) indicates significant ($P < 0.05$) treatment effects observed on that sampling date.

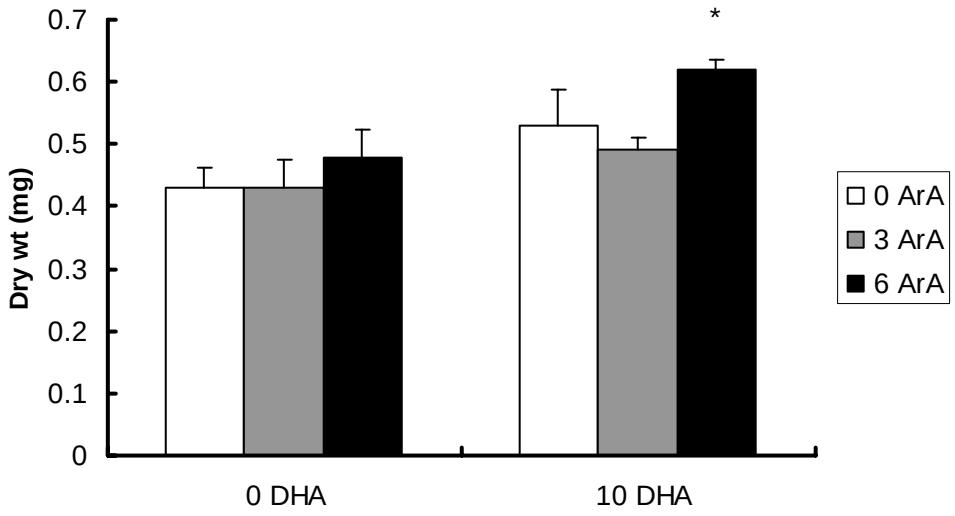

Figure 43. Dry weight (mg) ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae (d17ph) fed rotifers enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no significant ArA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly ($P < 0.05$) different from the 0:0 (DHA:ArA) control (T-test).

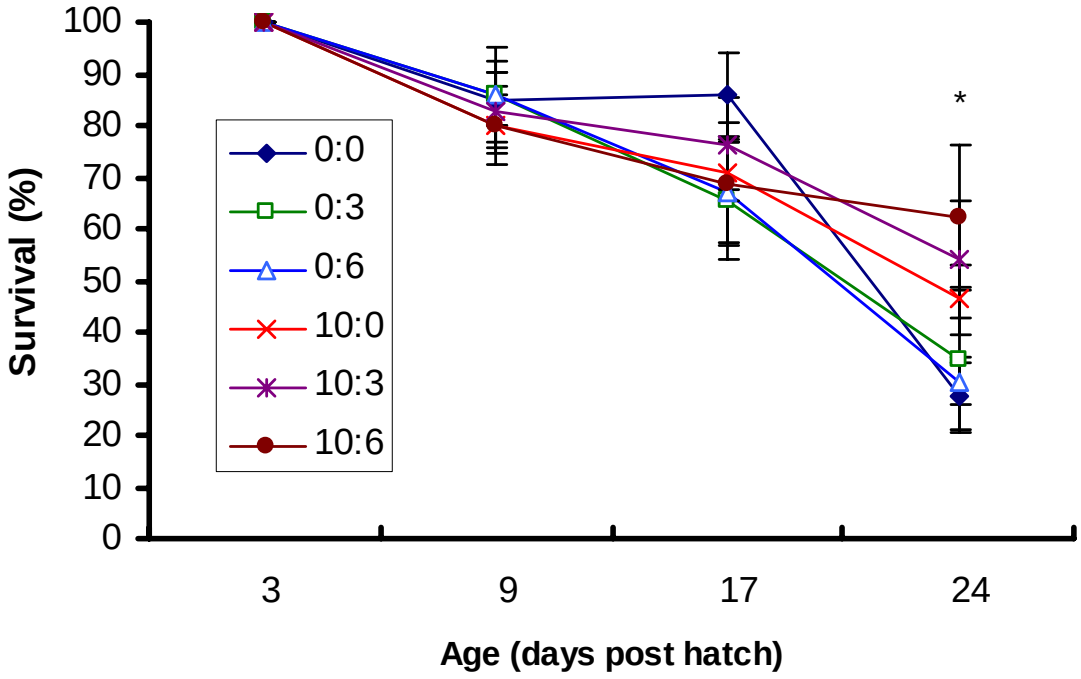

Figure 44. Survival ($\bar{X}$ ± SEM, N = 5) of black sea bass larvae fed rotifers and *Artemia* enriched with different proportions of DHA (0%, 10%) and ArA (0%, 3%, 6%). Asterisk (*) indicates significant ($P < 0.05$) treatment effects observed on that sampling date.

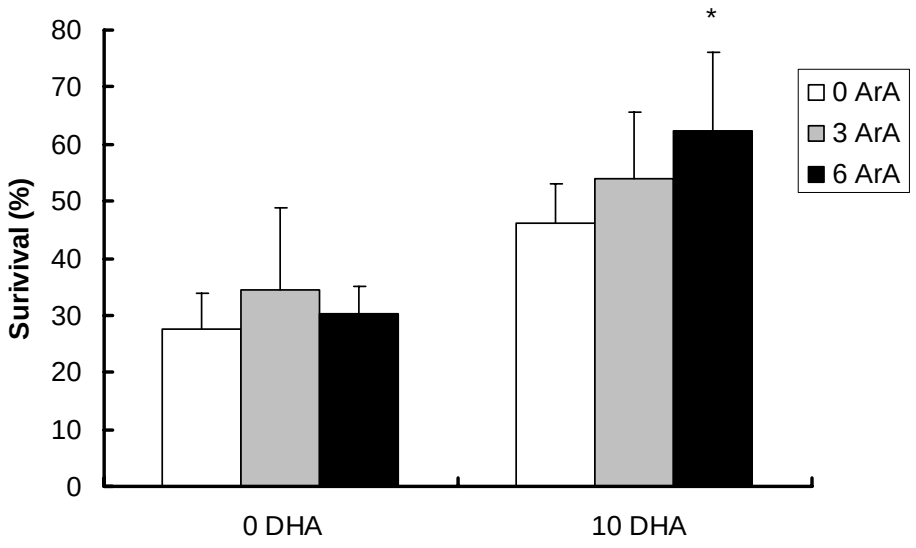

Figure 45. Survival ($\bar{X}$ ± SEM, N = 5) of post-metamorphic black sea bass larvae (d24ph) fed enriched rotifers and *Artemia* with different of DHA (0%, 10%) and ArA (0%, 3%, 6%). Significant effects of DHA ($P < 0.05$) were observed, with no ArA or interactive effects (Two-way ANOVA). Means with an asterisk (*) are significantly different from the 0:0 (DHA:ArA) control (T-test).

DISCUSSION

Fatty Acid Composition of Enriched Rotifers

As expected, no differences were observed in % total lipid of rotifers enriched with the different treatment emulsions, and the essential fatty acid levels found in enriched rotifers reflected those provided in their respective dietary emulsion. Total SFAs were higher in rotifers enriched with 10% DHA than in rotifers enriched with 0% DHA, while sum of MUFAs was higher in rotifers enriched with 0% DHA. The difference in MUFA levels can be attributed to the proportion of olive oil in the enrichment emulsions. Olive oil contains high levels of MUFAs especially 18:1n-9 (oleic acid) which can range from 71 to 80% total fatty acids (Aquilera et al. 2005). Since all emulsions contained 90 g total oil to 100 g water (Appendix A), emulsions containing a lower percentage of DHA and ArA had higher concentrations of olive oil. Therefore, higher MUFAs and lower SFAs.

Of the n-3 fatty acids, the 0% DHA-enriched rotifers contained no detectable DHA, while the 10% DHA-enriched rotifers averaged 7.3% DHA. Other workers have reported PUFA levels in rotifers lower than those provided in their diets (Watanabe 1983). For example, rotifers fed *Nephrochloris salina*, which has a high PUFA content (50.2%), had a PUFA content of 35.7% (Frolov 1991).

While EPA was not supplied in the enrichment emulsions, a small percentage (mean = 3.4%) was found in rotifers in all treatment groups. EPA was probably derived from their diet of *Nannochloropsis oculata,* which is naturally high (31.4%) in EPA content (Reed Mariculture Inc., Campbell CA) .

Of the n-6 PUFAs, ArA levels found in rotifers were very similar to the levels provided in their dietary emulsions. ArA levels in enriched rotifers ranged from 1-5% at treatment levels ranging from 0 to 6%. The small amount of ArA (1%) found in rotifers enriched with 0% ArA can also be attributed to their diet of *Nannochloropsis oculata,* which naturally contains 3.9% ArA (Reed Mariculture Inc., Campbell CA, USA).

Fatty Acid Composition of Enriched *Artemia*

The % total lipid, SFAs and MUFAs in enriched *Artemi*a remained constant among all dietary treatments. Of the n-3 fatty acids, *Artemia* enriched with 0% DHA contained no detectable DHA, while *Artemia* enriched with 10% DHA contained only 3.7% DHA (range = 3.1- 4.4%), considerably lower than in their diet. Unlike rotifers, which could be enriched with DHA to levels closer to those found in their emulsions, the enrichment of *Artemia* is difficult due to their rapid development and catabolism of DHA (Sorgeloos et al. 2001). It is possible that higher levels of DHA and ArA in *Artemia* could be achieved if the enrichment period is extended from 16 hours (as used in this study) to 24 hours, which was found optimum for accumulation of the essential fatty acids (Evjemo et al. 1997). On the other hand, a significant decrease in DHA and EPA levels in *Artemia* after 23 hours of enrichment has been observed, with maximum levels attained after 19 hours (McEvoy et al. 1995). Enrichment success can also depend on the genetic characteristics of the strain of *Artemia* used. After 24 hours of enrichment, 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 et al. 1993). Another

possible explanation of low DHA levels in enriched *Artemia* is oxidation of the enrichment emulsions which were prepared 18 days prior to use.

Of the n-6 PUFA, ArA levels found in enriched *Artemia* ranged from 0.23-3.9% at emulsion levels of 0-6%. The levels of ArA that were detected in the 0:0 enriched *Artemia* are similar to the levels of ArA (0.8% total fatty acids) found in newly hatched unenriched *Artemia franciscana* (Coutteau and Mourente 1997). Other workers have reported that ArA levels in *Artemia* after an 18 hour enrichment period reflected those in their diets (Estevez et al. 1998). It is possible that, the *Artemia* enrichment period (16 hours) used in this study was not adequate for optimal uptake of DHA and ArA from the emulsions. Another possibility is oxidation of the enrichment emulsions during storage, resulting in lower than expected ArA levels in the enriched *Artemia.*

Fatty Acid composition of d24ph Larvae

Total lipid (4.7% wet weight) in d24ph larvae was lower than levels found in all groups of enriched rotifers (5.8% wet weight) and slightly higher than levels in enriched *Artemia* (4.2% wet weight). Saturated and monounsaturated fatty acids, which are primarily catabolized by larvae as a source of metabolic energy (Sargent 1995), reflected levels found in the enriched rotifers and *Artemia* (20.3%).

Of the n-3 PUFAs, levels of linolenic (18:3n-3) acid were slightly lower (21.6%) than provided during the *Artemia* feeding stage (27.2%) and much greater than provided during the rotifer feeding stage (3.2%). High levels of linolenic acid provided during the *Artemia* feeding period are consistent with levels found in unenriched and enriched nauplii (Estevez et al. 1998). Stearidonic (18:4n-3) acid levels in d24ph larvae was lower

(3.1%) than those provided during the *Artemia* feeding stage (4.1%) with 0% provided during the rotifer feeding stage. Stearidonic acid is the second step in the conversion pathway from 18:3n-3 (linolenic acid) to 22:6n-3 (DHA). Limited conversion (chain elongation and desaturation) of stearidonic acid (18:4n-3) to EPA 20:5n-3 has been demonstrated in cultured cell lines of turbot (Ghioni et al. 1999). Therefore, conversion of stearidonic (18:4n-3) acid to EPA (20:5n-3) could potentially be contributing to the EPA content of the black sea bass larvae.

DHA levels in larvae fed 10% DHA on d24ph (5.8% wet weight) were lower than levels provided during the rotifer feeding stage (7.4% wet weight). This may be attributed to feeding larvae enriched *Artemia* after day 18 post hatch, which provided much lower DHA levels (3.4%) to the larvae than targeted. However, DHA levels were detectable (2.4%) in larvae fed 0% DHA. Interestingly, EPA levels were also higher in larvae fed 10% DHA (5.1%) than in larvae fed 0% DHA (4.6%). Since all enrichment emulsions were void of EPA, and levels detected in rotifers and *Artemia* were similar for all treatments, these differences in carcass EPA and DHA levels among treatments suggests conversion of EPA to DHA in black seabass larvae fed 0% DHA.

The ability to convert EPA to DHA has been demonstrated in juvenile turbot, where injection of ^{14}C-20:5n-3 (EPA) resulted in 27% of total radioactivity recovered in 22-carbon fatty acids of triacylglycerols and polar lipids, with the highest percentage in 22:6n-3 (DHA) (Linares and Henderson 1991). Further evidence of EPA to DHA conversion was found in gilthead seabream where fish injected with ^{14}C-20:5n-3 (EPA) and ^{14}C-18:n-3 (Linolenic acid) showed a 10-fold increase in 22:6n-3 (DHA) produced from 20:5n-3 compared to 18:n-3 (Mourente and Tocher 1994). In red seabream and

striped jack, however, larvae fed diets containing varying levels of EPA and DHA showed conversion of 20:5n-3 to 22:5n-3 (DPA) but not to 22:6n-3 (DHA) (Watanabe et al. 1989, Takeuchi et al. 1996).

Of the n-6 PUFAs, carcass ArA levels were higher than levels provided during the rotifer feeding stage and were nearly twice the levels provided during the *Artemia* feeding stage. This suggests that dietary ArA that was indirectly provided to rotifers by *Nannochloropsis* during rotifer culture, was sufficient to allow larvae to accumulate ArA at levels above those provided during the rotifer and *Artemia* feeding stage, even in the 0% ArA diet. Conservation of DHA and ArA was found in larval mahi mahi (*Coryphaena hippurus*) tissue after four days without feed (Ako et al. 1991) demonstrating the essential nature of these fatty acids in first feeding stages of development. In larval gilthead seabream, tissue levels of ArA increased with increasing dietary ArA levels (Bessonart et al. 1999) suggesting that gilthead seabream larval requirements for ArA can be met through dietary manipulations. The ability of black sea bass larvae to retain ArA in the tissue is similar to turbot injected with ^{14}C-20:4n-6 (ArA) (Linares and Henderson 1991) where the percentage of recovery was similar to injected levels with limited conversion of 20:4n-6 to 22:4n-6 or 22:5n-6.

Similar to gilthead seabream and turbot (Mourente and Tocher 1994, Linares and Henderson 1991) the levels of 18:3n-3 and 18:2n-6 in black sea bass larvae on d24ph were higher than levels provided in both rotifers, and *Artemia*, suggesting that these precursors were not converted to the longer chain unsaturated fatty acids, which demonstrates the requirement for 20 and 22 carbon EFAs in their diet.

Stress test

Hypersaline stress evaluation is a commonly used technique for quantifying larval vitality and determining nutritional status in larval and juvenile fish (Dhert et al. 1990, Dhert et al. 1991, Brinkenmeyer and Holt 1998, Gapasin et al. 1998, Furuita et al. 1999, Willey et al. 2003)**.** In this study, no significant differences were found during the hypersaline stress evaluation between larvae fed the 10% DHA treatments and in those fed 0% DHA. These results contradict those found in other marine finfish species such as larval Japanese flounder (*P. olivaceus*), which demonstrated better vitality after exposure to 65 ppt when receiving a high DHA (1.6% dry wt.) diet among treatments ranging from 0% to 2.2% (dry wt.) DHA (Furuita et al. 1999). Red seabream larvae fed rotifers enriched with 20.5% and 21.8% DHA (TFA) survived handling stress much better than larvae fed rotifers containing 0.9% and 2.3% DHA (Watanabe et al. 1989). Larval red sea bream (*Pagrus major*) and juvenile marbled sole (*Limanda yokohamae*) fed diets ranging from 0-2% DHA showed increased resistance to reduced dissolved oxygen and increasing temperature at 2% DHA (Kanazawa 1997). Resistance to air exposure in 23 day old mahi mahi (*Coryphaena hippurus*) was also greater in fish fed diets containing 14.9% DHA (TFA) than in those fed 2.6% DHA (TFA) (Ako et al. 1991, Kraul et al. 1993).

The role that DHA plays in increased stress resistance in larval marine fish is not clear (Weirich and Reigh 2001). In terrestrial mammals a strong correlation was found between mitochondrial DHA levels and the $Na^{+}K^{+}$ ATPase activity in kidney tissue (Turner et al. 2003). It is possible that when marine fish larvae are exposed to hypersaline conditions, cell membrane fluidity is reduced at low DHA levels, affecting

the transport of ions in and out of cells (Watanabe 1993). For marine fish, the active cells in salt secretion across the gills are the chloride cells, which contain Na^+K^+ ATPase and is a large cell filled with many mitochondria containing higher levels of DHA (Bell et al. 1983). Thus, if DHA levels are low in gill tissue, hyperosomotic conditions fish could compromise fish performance (Weirich and Reigh 2001).

ArA is readily incorporated into cell membrane phospholipids and is a precursor to the formation of eicosanoids (e.g. prostaglandins) in fish and mammals (Sargent et al. 1999). Cortisol production is stimulated by prostaglandins (primarily PGE_2) in response to stress (Koven 2003). PGE_2 regulates cortisol release in the hypothalamus-pituitary-adrenal axis in mammals (Lands 1991, Hockings et al. 1993, Nye et al. 1997) and possibly in the hypothalamus-pituitary-interrenal axis in fish (Gutpa et al. 1997). Therefore, it is believed that the stress response in marine fish would improve at increased ArA levels. Cortisol promotes the differentiation of chloride cells and increases the specific activity of Na^+K^+ ATPase in the gills (Koven et al. 2003) allowing adaptation to different salinities (Harel et al. 2001).

In this study, no significant effects on hypersaline stress resistance in black sea bass larvae were observed among dietary ArA levels. In contrast, larval summer flounder fed a diet of 6% ArA and showed increased stress resistance when exposed for two hours to 70 ppt on d18ph and to 80 ppt on d45ph (Willey et al. 2003). In gilthead sea bream, larvae fed dietary ArA (0.59-5.86% dry weight) showed improved survival when subjected to acute handling stress, but not when they were exposed to the chronic stress of daily salinity change (Koven et al. 2003). In that study, ArA appeared to elevate cortisol levels for an extended period of time under conditions of chronic stress, which

contributed to reduced growth and increased mortality. In another study, larval gilthead seabream were exposed to decreased salinity (24 ppt) after feeding them diets containing low (1.5%) or high (6.3% ArA TFA) (Van Anholt et al. 2004). Larvae fed high ArA (6.3% TFA) showed increased Na^+K^+ ATPase activity and cortisol levels, while those fed (1.5% ArA TFA) did not, indicating that higher levels of ArA improved hypoosmotic regulation (Van Anholt et al. 2004). It is possible that these dissimilar results may be related to species-specific differences in osmoregulatory capabilities and response to dietary ArA or to variations in methodology used among studies. For this study, it is possible that a hypersaline water of 65 ppt was too high to allow differences in osmoregulatory abilities to be detected or resolved. In effort to determine dietary DHA and ArA effects on stress resistance, our future studies should include a lower or fluctuating salinity, a Na+ K+ ATPase assay, Na+ K+ ATPase gene expression, or cortisol production analysis.

Growth

In this study, growth in terms of length and weight was higher in larvae fed prey enriched with 10% DHA (vs. 0% DHA), with fastest growth at the 10:6 (DHA:ArA) treatment level on d17 and d24 post hatch. A number of previous studies have demonstrated that larvae fed diets containing higher levels of DHA grew faster than those fed low levels of DHA. DHA is important to the structure and function of cell membranes and is found at high levels in neural tissue and is therefore important for vision and hunting success. In juvenile turbot (*Scophthalmus maximus*), DHA was found

to accumulate in the brain during development (Mourente et al. 1991). Juvenile herring (*Clupea harengus*) (Bell et al., 1995) fed a diet deficient in DHA during the period of rod development showed impaired visual performance. Maximum visual acuity at the first feeding larval stage presumably increases hunting success and growth. For example, red sea bream (*Pagrus major*) 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 has also been demonstrated in larval yellowtail (*Seriola quinqueradiata*) as dietary DHA levels increased from 10.2% to 43.3% (Furuita et al. 1996, Copeman et al. 2002). DHA (9.1% total fatty acids) in the diet of larval striped jack (*Pseudocaranx dentex*) improved growth during the first 12 days post hatching (Takeuchi et al. 1996). European sea bass (*Dicentrarchus labrax*) juveniles fed diets containing 12.4% n-3 PUFA achieved higher growth rates than those fed 9.3% n-3 PUFA, with all diets containing approximately the same DHA/EPA ratio of 1.5:1 (Skalli and Robin 2004).

When growth on d24ph was compared to those fed the control diet of 0:0 (DHA:ArA), larvae fed the 10:0 and 10:3 diets showed no significant differences, However, larvae fed the 10:6 (DHA:ArA) diet showed significantly higher growth. These results are similar to those reported in 21-32 day old gilthead seabream (*Sparus aurata*) where growth was faster in larvae fed 6.3% ArA (TFA) compared to those fed 1.5% ArA (TFA) (Van Anholt et al. 2004). In contrast to these findings, dietary ArA within the range of 0% to 15% TFA had no effect on growth of larval gilthead seabream (*Sparus aurata*) (Koven et al. 2001), or of larval Japanese flounder (*Paralichthys olivaceus*) (Estevez et al. 2001). In larval white bass (*Morone chrysops*), dietary ArA

levels of 0 to 26% TFA also had no effect on growth. On the other hand, in striped bass (*Morone saxatilis*) fastest growth was found at intermediate ArA levels (13% TFA) within a range of 0 to 26% ArA, indicating species specific dietary ArA requirements even among congeneric species (Harel and Place 2003). Differences in ArA requirements among *Morone* species were suggested to reflect freshwater origin of white bass and marine origin of striped bass (Harel and Place 2003). Conversion of lenoleic (18:2n-6) to ArA (20:4n-6) is known to occur in freshwater fish which are more omnivorous in their feeding habits, while carnivorous marine fish are incapable of ArA synthesis suggesting the importance of dietary ArA supplementation in marine finfish diets (Sargent et al. 1995).

Survival

No significant effects of DHA or ArA on overall larval survival were observed until d24ph. In agreement with growth data, survival on day 24 post hatch was higher in larvae fed 10% DHA (55%) than in those fed 0% DHA (31%). Increased survival of larvae fed diets containing higher levels of DHA was reported in red sea bream (21.8% TFA) (Watanabe et al. 1989), yellowtail (43.3% TFA) (Copeman et al. 2002), and larval striped jack (9.1% TFA) (Takeuchi et al. 1996).

When survival on d24ph was compared to those fed the control diet of 0:0 (DHA:ArA) (27%), larvae fed the 10:0 and 10:3 diets (46% - 54%) showed no significant differences. However, larvae fed the 10:6 (DHA:ArA) diet showed significantly better (62%) survival. This increase in survival of black sea bass larvae fed 10% DHA and 6% ArA is similar to what was found in larval summer flounder

(*Paralichthys dentatus*), where larvae fed rotifers and *Artemia* enriched to contain from 0 to 12% ArA showed increased growth and survival to d45ph at ArA levels up to 6%, with no further improvement at higher levels (Willey et al. 2003). Survival of the gilthead seabream (d35ph) fed diets containing from 0 to 5.3% ArA (dry wt) was highest at 1.8% (Bessonart et al. 1999) and 1.7% (Koven et al. 2001) ArA (dry wt). The results of these studies suggest that dietary ArA provided during the rotifer feeding stage significantly improves larval survival later in development during the metamorphic stages (Koven 2001, Willey et al. 2003).

CONCLUSION

In summary, growth was generally highest in black seabass larvae fed the 10:6 DHA:ArA level on d17 and d24ph. Among all treatments, survival on d24ph was significantly higher in larvae fed 10:6 DHA:ArA than in those fed 0:0 DHA:ArA. Based on the hypersaline stress evaluation, d24 larvae fed 10% DHA showed no difference in stress resistance than those fed 0% DHA.

On d24ph, levels of ArA in larvae were higher than levels provided in their diets suggesting accumulation of ArA in larval tissues and indicating a high requirement for this fatty acid. DHA levels were higher in larvae fed 10% DHA than in larvae fed 0% DHA. EPA levels were also higher in larvae fed 10% DHA than in larvae fed 0% DHA. While these results suggest that black seabass larvae were able to convert of EPA to DHA, the rate of conversion was clearly inadequate to produce optimum growth and survival.

Overall, the results of this study indicate the importance of dietary DHA and ArA for growth and survival in black seabass larvae. A DHA level of 10% with increasing ArA within the range of 0 to 6% improved growth and survival. Future studies investigating higher DHA and ArA levels are warranted.

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