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Effects of dietary arachidonic acid on larval performance, fatty acid profiles, stress resistance, and expression of Na^+/K^+ ATPase mRNA in black sea bass *Centropristis striata*

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ABSTRACT

Dietary arachidonic acid (ARA, 20:4n-6) supplementation on larval performance, tissue fatty acid profiles, resistance to hypersaline stress and expression of Na^+/K^+ ATPase mRNA was studied in black sea bass *Centropristis striata* from the first feeding through metamorphosis at 24 days post-hatching (d24ph). Thirty 15-L aquaria were stocked with d1ph yolksac stage larvae at 100 ind/L. Salinity (34 g/L), temperature (22 °C), photoperiod (18L:6D), light intensity (1000 lx), diffused aeration (100 mL/min) and D.O. (>5 mg/L) were held constant. Non-viable microalgae *Nannochloropsis oculata* was maintained at 300,000 cells/mL as background. To evaluate the effects of dietary arachidonic acid (ARA) supplementation, live prey, rotifers *Brachionus rotundiformis* and *Artemia* sp., was enriched with emulsions containing 10% docosahexaenoic acid (DHA, 22:6n-3) and five different levels of ARA (0, 6, 8, 10 and 12% total fatty acids, TFA). In a sixth treatment, live prey was enriched with a premium commercial fatty acid booster (26% DHA, 0% ARA). Rotifers were fed from d2 to d20ph at 10–23 ind/mL, while *Artemia* were fed from d18 to d22ph at 0.5–3 ind/mL. On d24ph, larval fatty acid profiles reflected dietary levels, and no significant ($P>0.05$) differences in larval growth (notochord length, wet and dry wt.), survival (range = 24.3–32.7%), or hypersaline stress resistance (ST-50 = 27.1–31.8 min) among treatments were evident. However, larvae fed diets supplemented with ARA (6–12% TFA) demonstrated a significant ($P<0.05$) increase in Na^+/K^+ ATPase mRNA 24 h after a sublethal salinity (43 g/L) challenge, whereas larvae fed 0% ARA and the commercial diet (devoid of ARA) showed no significant increase. The results suggested that dietary supplementation with ARA at 6–12% promoted the adaptive physiological responses to hypersalinity stress and hypo-osmoregulatory ability in black sea bass larvae.

1. Introduction

Black sea bass *Centropristis striata* (family Serranidae) is a high-value marine finfish that inhabits continental shelf waters from Maine to northern FL that supports important commercial and recreational fisheries (Mercer, 1989). This species is overfished in the area south of Cape Hatteras (NCDMF, 2009). A high demand placed on the commercial fishery to supply both local and niche markets has stimulated an interest in developing techniques for aquaculture of black sea bass. Protocols for controlled breeding of black sea bass are well developed (Berlinsky et al., 2004; Denson et al., 2007; Howell et al., 2003; Watanabe et al., 2003, in press). A constraint to the artificial propagation of this species is reliable larviculture techniques to provide healthy fingerlings to grow-out operations. A number of studies have investigated the optimum environmental parameters for production of black sea bass fingerlings, including temperature, photoperiod, salinity and light intensity (Berlinsky et al., 2004; Copeland and Watanabe, 2006). Little or no experimental data is available on larval nutrition.

Available live feed organisms for marine fish larvae, rotifers and *Artemia*, are generally deficient in essential fatty acids and must be enriched with these components before feeding to the larvae. Essential fatty acids (EFAs) are a class of lipids that the fish is unable to synthesize *de novo* (Kanazawa, 2003; Tacon, 1990) and are critical to growth and survival. Known EFAs include docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6). DHA is necessary for developing neural tissues, optical tissues and membrane structure (Furuita et al., 1998; Kanazawa, 2003; Mourente and Tocher, 1992; Valentine and Valentine, 2004; Voet et al., 1999). If not provided sufficiently in the

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diet, DHA is synthesized from EPA which is synthesized from linolenic acid (LN, 18:3n-3) through a process of desaturation and carbon chain elongation (Tacon, 1990; Voet et al., 1999), while ARA is synthesized from linoleic acid (LL, 18:2n-6). ARA and EPA are precursors to eicosanoids such as prostaglandins, leukotrienes and thromboxanes (Bell and Sargent, 2003; Van Anholt et al., 2004; Voet et al., 1999) that are released during stress and the inflammatory response (Van Anholt et al., 2004). In contrast to freshwater fish, the functional activities of the enzymes Δ5 desaturase and C18–20 elongases necessary for the synthesis of DHA, EPA or ARA (Kanazawa et al., 1979; Tocher and Ghioni, 1999) from their precursor fatty acids LL and LN (Kanazawa, 2003; Lavens et al., 1995; Tacon, 1990) are low in marine fish.

Previous studies have demonstrated the importance of high levels of DHA compared to EPA in the diets of larval marine fish (Rainuzzo et al., 1997; Tocher and Sargent, 1984; Watanabe, 1993; Watanabe et al., 1989). More recent studies have also demonstrated the importance of ARA in larval growth, survival, and stress resistance in different species of marine finfish. Striped bass *Morone saxatilis* fed dietary ARA and subjected to a hypersaline challenge showed elevated tissue ARA and increased hypersaline stress tolerance (Harel et al., 2001). In summer flounder *Paralichthys dentatus* (Willey et al., 2003) and gilthead seabream *Sparus auratus* (Koven et al. 2001), dietary ARA supplementation improved growth, survival and stress resistance. In *S. auratus*, increasing dietary ARA within the range of 0 to 25% TFA increased cortisol levels of larvae exposed to chronic hyperosmotic and hyposmotic stress (Koven et al., 2003). Larvae fed an intermediate level of ARA (12.5%) showed higher survival when subjected to salinity changes than larvae fed the highest or lowest level of ARA. These workers suggested that increasing levels of cortisol may enhance the sodium–potassium pumps in the gills, kidneys and intestines, which control the internal homeostasis of the larvae (Koven et al., 2003; Van Anholt et al., 2004). In black sea bass larvae fed diets containing 10% DHA, increasing ARA within the range of 0–6% improved growth and survival from first feeding through metamorphic stages (Rezek et al., 2010).

Osmoregulation in larval fish is accomplished by Na^+/K^+ ion-transport pumps within cells in the integument until the gills and intestinal osmoregulatory systems develop (Rombough, 2007). The enzyme Na^+/K^+ ATPase (NKA) mediates the active secretory functions of chloride cells in juvenile fish by generating a chemical gradient for ion transport. Osmoregulatory ability can be evaluated by measuring the expression of NKA messenger RNA (mRNA), with a higher mRNA level indicating the elaboration of the ion transfer pump and better osmoregulatory ability (Carrier, 2006; Ostrowski et al., 2011; Scott et al., 2004; Scott and Schulte, 2005). In the silver sea bream *Sparus sarba* increases in NKA mRNA levels during the first 35 days of larval development were reported (Deane et al., 2003), but the role of larval nutrition on elaboration of NKA mRNA has not been investigated. The objectives of this study were to evaluate the effects of dietary ARA supplementation (0–12%) in black sea bass larvae on growth, survival, tissue fatty acid profiles, resistance to hypersaline challenge, and the expression of NKA mRNA as an index of hypo-osmoregulatory ability.

2. Methods

2.1. Experimental animals

This experiment was conducted at the University of North Carolina Wilmington Center for Marine Science Aquaculture Facility, Wrightsville Beach, NC. Wild-caught black sea bass broodstock were maintained in recirculating tanks (vol. = 2134 L) supported by biofilters, ultraviolet sterilizers, heat pumps and fluorescent lights to control water quality, temperature and photoperiod. Seawater (34 g/L) was pumped from the adjacent Atlantic intracoastal waterway and was filtered (1-μm) and UV-sterilized. Volitional spawning was induced in a mature post-vitellogenic stage females using pelleted luteinizing hormone releasing hormone-analog (Berlinsky et al., 2000; Berlinsky et al., 2004; Watanabe et al., 2003, Watanabe et al. in press). Eggs were collected from broodtanks and viable (buoyant) eggs were separated from nonviable (non-buoyant) eggs. Fertilized eggs were incubated in a 125-L tank at 34 g/L and at 18 °C until hatching.

2.2. Experimental system

The experimental system consisted of four temperature controlled water baths (213-L) situated in an indoor laboratory. Water in each bath was circulated through a heater/chiller and temperature was regulated by a digital thermostat. The experimental units were 15-L, cylindrical, black tanks, with eight tanks in each water bath. A light hood over each bath contained two 40-watt full-spectrum fluorescent light bulbs controlled by timers for photoperiod manipulation and was adjusted vertically for surface light intensity.

2.3. Experimental design

To begin the experiment, newly hatched yolk-sac larvae (approximately 52 h post-fertilization) were distributed from the incubator to each experimental tank at a density of 100 ind/L. To determine the effects of dietary ARA, black sea bass larvae were fed live prey organisms, rotifers and *Artemia*, enriched to contain 10% DHA and five different levels of ARA: 0, 6, 8, 10 and 12% total fatty acids (TFA). In addition, one group of larvae were fed live prey enriched with a popular commercial fatty acid booster for marine finfish larvae (Algamac 2000, Aquafauna Bio-Marine, Hawthorne, CA, USA), containing 26% DHA, 10.6% docosapentaenoic acid (DPAn-6, 22:5n6), 0.6% EPA and 0% ARA, to establish performance of larvae using current industry standard procedures and to evaluate retro conversion of DPAn-6 to ARA in live prey and in black sea bass larvae. Larvae were reared through metamorphosis to 24 days post-hatching (d24ph).

2.4. Experimental conditions

Environmental conditions, including salinity (35 g/L), temperature (22 °C) (Berlinsky et al., 2000), pH (7.3–8.0), photoperiod (18L:6D), light intensity (1000 lx) (Copeland and Watanabe, 2006), diffused aeration (100 mL/min) and dissolved oxygen (>5 mg/L) were monitored daily in each tank and maintained at prescribed levels throughout the experiment. Background micro-algae *Nannochloropsis oculata* non-viable paste (Reed Mariculture, Campbell, CA, USA) was added daily to maintain a density of 300,000 cells/mL (Berlinsky et al. 2004). To maintain water quality, 5 L (33.3% of the tank volume) was siphoned daily and replaced with filtered and sterilized seawater. Surface oils were removed daily using a paper towel.

2.5. Feeds

S-type rotifers *Brachionus rotundiformis* were grown at the UNCW Aquaculture Facility on non-viable microalgae *N. oculata* paste (Reed Mariculture, Campbell, CA, USA) (Bentley et al., 2008). Rotifers were enriched with the treatment emulsions for 8 h before feeding to the fish larvae. *Artemia* metanauplii hatched from decapsulated cysts were enriched with the treatment emulsions for 16 h before feeding to the larvae. Prey fatty acid profiles were identified by gas chromatography (Tables 1 and 2).

Rotifers were introduced to the rearing tanks on d2ph at a density of 10/mL until first feeding on d3ph, increasing to 15/mL by d3ph and then to a maximum of 23/mL by d18ph. *Artemia* were co-fed with rotifers on d18ph at a density of 0.5/mL. On d20ph, *Artemia* density was increased to 2/mL, and rotifer feeding ceased. On d22ph, *Artemia* density was increased to 3.0/mL for the remainder of the experiment.

Table 1
Total lipid and fatty acid composition (% of total fatty acids) (means ± SEM, $N = 3$) of enriched rotifers *Brachionus rotundiformis*.

Enriched diets received by larvae (% of total fatty acids as ARA and Algamac 2000)						
Fatty acids	0%	6%	8%	10%	12%	Algamac
14:0	3.76 ± 0.05 a	4.54 ± 0.19 a	3.67 ± 0.024 a	4.31 ± 0.17 a	3.87 ± 0.20 a	5.66 ± 0.20 b
16:0	15.5 ± 0.71 a	15.5 ± 0.76 a	15.8 ± 1.37 a	17.4 ± 0.99 ab	16.7 ± 0.25 a	21.7 ± 1.26 b
16:1n-7	8.46 ± 0.33	10.0 ± 0.84	8.14 ± 0.70	7.60 ± 0.59	8.39 ± 0.24	10.2 ± 0.59
18:0	2.86 ± 0.14 b	2.13 ± 0.03 b	3.38 ± 0.23 ab	4.28 ± 0.45 a	4.02 ± 0.30 ab	2.93 ± 0.17 b
18:1n-9	32.5 ± 0.74 a	23.1 ± 1.88 b	20.9 ± 0.25 bc	18.4 ± 0.50 cd	15.4 ± 0.28 d	5.53 ± 0.32 e
18:2n-6	8.71 ± 0.69 a	8.24 ± 0.21 a	7.79 ± 0.18 a	8.43 ± 0.31 a	7.66 ± 0.38 a	3.28 ± 0.24 b
18:3n-3	3.12 ± 0.06 a	2.56 ± 0.05 bc	2.67 ± 0.08 b	2.28 ± 0.03 c	2.51 ± 0.08 bc	4.26 ± 0.11 d
18:4n-3	0	0.53 ± 0.27 ab	0.90 ± 0.02 ab	1.21 ± 0.05 ab	1.29 ± 0.10 b	0.45 ± 0.23 a
20:1	1.95 ± 0.06	1.26 ± 0.22	1.77 ± 0.27	1.74 ± 0.14	1.63 ± 0.26	1.81 ± 0.11
20:4n-6	1.62 ± 0.21 a	8.27 ± 0.27 b	9.54 ± 0.09 b	11.6 ± 0.64 c	14.3 ± 0.26 d	2.88 ± 0.04 a
20:5n-3	7.74 ± 0.64	9.12 ± 1.00	7.37 ± 0.63	6.83 ± 0.33	7.75 ± 0.51	8.83 ± 0.59
22:5n-3	3.19 ± 0.09 bc	2.97 ± 0.07 bc	2.90 ± 0.07 c	3.36 ± 0.14 ab	2.91 ± 0.09 c	3.71 ± 0.09 a
22:5n-6	0	0	0	0	0	6.05 ± 0.69
22:6n-3	9.72 ± 0.62 a	10.8 ± 0.54 a	9.95 ± 0.34 a	10.2 ± 0.51 a	10.1 ± 0.12 a	19.8 ± 2.04 b
∑ Saturates	22.2 ± 0.78 a	22.2 ± 0.90 a	22.9 ± 1.82 a	26.0 ± 1.51 ab	24.6 ± 0.51 ab	30.3 ± 1.44 b
∑ Monounsaturates	43.0 ± 0.74 a	34.3 ± 0.85 b	30.8 ± 1.08 c	27.7 ± 0.60 cd	25.4 ± 0.29 de	23.6 ± 0.32 e
∑ n-3 Polyunsaturates	23.8 ± 1.03 a	25.9 ± 0.52 a	23.8 ± 0.45 a	23.9 ± 0.63 a	24.6 ± 0.52 a	37.1 ± 1.82 b
∑ n-6 Polyunsaturates	10.3 ± 0.61 a	16.5 ± 0.48 b	17.3 ± 0.14 bc	20.1 ± 0.93 cd	21.9 ± 0.60 d	12.2 ± 0.52 a
n-3/n-6 Polyunsaturates	2.31 ± 0.14 a	1.58 ± 0.08 b	1.37 ± 0.03 bc	1.19 ± 0.04 c	1.12 ± 0.05 c	3.03 ± 0.07 d
DHA/EPA	1.27 ± 0.12 a	1.23 ± 0.21 a	1.38 ± 0.17 ab	1.50 ± 0.12 ab	1.32 ± 0.11 ab	2.29 ± 0.38 b
DHA/ARA	6.16 ± .070 a	1.30 ± 0.03 b	1.04 ± 0.03 b	0.88 ± 0.01 b	0.71 ± 0.01 b	6.86 ± 0.64 a
EPA/ARA	4.83 ± 0.22 a	1.11 ± 0.16 c	0.77 ± 0.07 c	0.59 ± 0.05 c	0.54 ± 0.04 c	3.07 ± 0.24 b
Total lipid (%)	9.63 ± 0.84	9.27 ± 0.36	10.1 ± 0.48	9.93 ± 0.85	10.1 ± 0.14	9.43 ± 0.79
Total lipid (mg/g DW)	552.6 ± 53.71	541.2 ± 20.87	589.8 ± 29.85	579.3 ± 50.23	589.3 ± 9.24	553.5 ± 46.96

Mean values within rows that are not followed by the same letter are significantly ($P<0.05$) different.
Total lipid is reported as % and mg/g dry weight (DW).

2.6. Live prey enrichment

The DHA/ARA emulsions were formulated by using different proportions of DHAsco and ARAsco (Advanced Bionutrition, Columbia, MD, USA). DHAsco is a DHA-rich single cell oil extracted from the heterotrophically grown algae *Crypthecodinium cohnii*. ARAsco is an ARA-rich single cell oil extracted from the fungus *Mortierella alpina*. These triglyceride oils were mixed with olive oil and water with 5% lecithin, 1% ascorbic acid and 1% Tween-80 (with oil weight) (Advanced Bionutrition, Columbia, MD, USA). Rotifers were enriched ($0.1\ g/0.5\times10^6$) every 4 h and were harvested after 8 h. *Artemia* were enriched ($0.3\ g/0.25\times10^6$) every 8 h and were harvested after 16 h.

2.7. Sampling and data collection

Using volumetric methods, sampling was conducted on d4, d10, d17 and d24ph. A target sample size of ten larvae was collected from each replicate tank, and the volume sampled was measured to determine the density of larvae in each sample. At each sampling, growth was determined in anesthetized (0.3–0.5 g/L 2-phenoxyethanol) larvae from notochord length using a microscope fitted with an ocular

Table 2
Total lipid and fatty acid composition (% of total fatty acids) (means ± SEM, $N = 3$) of enriched *Artemia* nauplii.

Enriched diets received by larvae (% of total fatty acids as ARA and Algamac 2000)						
Fatty acids	0%	6%	8%	10%	12%	Algamac
14:0	1.48 ± 0.06 a	1.49 ± 0.09 a	1.68 ± 0.15 a	1.66 ± 0.08 a	1.42 ± 0.09 a	3.09 ± 0.14 b
16:0	10.2 ± 0.19 a	10.5 ± 0.08 a	10.8 ± 0.39 a	10.5 ± 0.28 a	10.4 ± 0.01 a	15.7 ± 0.55 b
16:1n-7	3.13 ± 0.01 a	2.77 ± 0.20 ab	2.62 ± 0.14 ab	2.57 ± 0.19 ab	2.46 ± 0.06 b	3.98 ± 0.11 c
18:0	5.08 ± 0.29	5.55 ± 0.15	5.39 ± 0.14	5.42 ± 0.17	5.99 ± 0.23	nd
18:1n-9	34.9 ± 2.15 ab	28.9 ± 1.58 bc	26.5 ± 1.06 cd	24.1 ± 0.51 cd	20.9 ± 0.67 d	40.2 ± 0.94 a
18:2n-6	7.41 ± 0.48 a	6.64 ± 0.03 a	6.64 ± 0.05 a	6.62 ± 0.02 a	6.49 ± 0.07 a	4.40 ± 0.14 b
18:3n-3	22.4 ± 1.48 a	22.0 ± 1.65 a	22.2 ± 1.53 a	22.6 ± 1.12 a	24.7 ± 1.45 a	4.49 ± 0.21 b
18:4n-3	4.33 ± 0.30	3.98 ± 0.34	4.27 ± 0.35	4.44 ± 0.45	4.31 ± 0.27	4.98 ± 0.34
20:1	1.61 ± 0.22	1.65 ± 0.31	1.05 ± 0.05	1.11 ± 0.03	1.19 ± 0.05	1.15 ± 0.02
20:4n-6	0	5.9 ± 0.41 a	7.52 ± 0.73 a	9.09 ± 1.04 ab	10.9 ± 0.73 b	1.74 ± 0.29 c
20:5n-3	1.79 ± 0.09 a	1.80 ± 0.07 a	1.80 ± 0.08 a	1.63 ± 0.15 a	1.79 ± 0.13 a	3.66 ± 0.45 b
22:5n-6	0	0	0	0	0	3.92 ± 0.52
22:6n-3	5.06 ± 0.30 a	6.55 ± 0.49 a	6.46 ± 0.68 a	6.60 ± 0.54 a	5.80 ± 0.51 a	10.9 ± 1.23 b
∑ Saturates	16.8 ± 0.19	17.5 ± 0.13	17.8 ± 0.67	17.5 ± 0.31	17.8 ± 0.20	18.8 ± 0.69
∑ Monounsaturates	39.7 ± 2.20 a	33.3 ± 1.15 b	30.1 ± 1.00 bc	27.8 ± 0.45 cd	24.5 ± 0.59 d	48.9 ± 0.71 e
∑ n-3 Polyunsaturates	33.6 ± 1.39 a	34.3 ± 1.51 a	34.7 ± 1.55 a	35.3 ± 1.04 a	36.6 ± 1.31 a	24.0 ± 1.16 b
∑ n-6 Polyunsaturates	7.41 ± 0.48 a	12.6 ± 0.39 bc	14.2 ± 0.69 c	15.7 ± 1.02 cd	17.4 ± 0.75 d	9.75 ± 0.49 ab
n-3/n-6 Polyunsaturates	4.57 ± 0.32 a	2.75 ± 0.20 b	2.47 ± 0.18 b	2.27 ± 0.19 b	2.12 ± 0.15 b	2.46 ± 0.18 b
DHA/EPA	2.83 ± 0.12	3.64 ± 0.29	3.58 ± 0.29	4.05 ± 0.21	3.27 ± 0.37	3.05 ± 0.46
DHA/ARA	0	1.10 ± 0.03 a	0.85 ± 0.02 a	0.73 ± 0.03 a	0.53 ± 0.03 a	6.59 ± 1.17 b
EPA/ARA	0	0.31 ± 0.02 a	0.24 ± 0.02 a	0.18 ± 0.01 a	0.17 ± 0.01 a	2.14 ± 0.09 b
Total lipid (%)	5.03 ± 0.20 a	4.93 ± 0.39 a	8.06 ± 0.92 b	7.1 ± 0.15 ab	6.90 ± 0.23 ab	8.14 ± 0.28 b
Total lipid (mg/g DW)	373.5 ± 15.5 a	377.2 ± 39.86 a	600.4 ± 67.9 b	528.7 ± 12.7 ab	514.8 ± 19.2 ab	606.6 ± 23.1 b

Mean values within rows that are not followed by the same letter are significantly ($P<0.05$) different.
Total lipid is reported as % and mg/g dry weight (DW).

micrometer. Larval wet and dry weights (DW) were measured to the nearest 10 μg using a Sartorius (Sartorius AG, Goettingen, Germany) electronic balance. Survival was determined from larval densities at each sampling as a percentage of initial density.

2.8. Carcass analysis

Approximately 200 mg (wet weight) of black sea bass larvae was sampled from each replicate tank on d24ph. Excess water was removed and larvae were stored (−20 °C) under nitrogen gas in cryogenic vials (Fisher scientific, USA) until lipid extraction. Using a revised Folch et al. (1957) method, lipids were extracted from whole larval tissues in 1:1 chloroform/methanol by homogenization and sonication. Samples were then filtered and solvent was removed using a rotary evaporator (Buchi labortechnik AG, Meierseggstrasse, Switzerland). The extract was then dissolved in 1:1 chloroform/methanol, filtered and transferred into a pre-weighed 50 mL round bottom flask. The solvent was roto-evaporated to dryness and the remaining lipid was weighed to determine percent total lipid.

Lipids were converted into fatty acid methyl esters (FAMEs), first by dissolving in 900 μL of 0.5 M NaOH/MeOH and heating (between 70 and 100 °C) for 30 min. After the 30 min reaction, 1.5 mL of boron trifluoride-methanol (14% BF_3 in MeOH from Sigma Aldrich, St Louis, MO, USA) was added, followed by another reaction period of 30 min at 70–100 °C.

Isolation of FAMEs at room temperature (25 °C) was accomplished by the addition of 1 mL of saturated NaCl and 1 mL of 100% hexane. After agitation, the layers were allowed to separate and, using a Pasteur pipette, the organic layer was drawn off and passed through a silica column (0.5×2.0 cm, 35–60 μ SiO_2 in a glass Pasteur pipette with a glass wool plug). The aqueous layer was extracted an additional time with 1 mL hexane and passed through the SiO_2 column and then the column was rinsed with 1 mL of 50% ether/hexane two times to remove the entire sample. FAMEs were roto-evaporated and dissolved in 400 μL of 100% hexane for GC analysis. FAMEs were analyzed using a Hewlett Packard 6890 gas chromatography flame-ionization-detector (GC-FID) (Hewlett Packard, USA) (Rezek et al., 2010). Using helium as the carrier gas, injected samples passed through the column in approximately 15 min. The column was initially heated to 195 °C and held for 8 min, ramped at a rate of 15 °C/min and held at 270 °C for the remaining 2 min. FAME peaks were identified using the known standards GLC-84, docosapentaenoic acid methyl ester n-3, docosapentaenoic acid methyl ester n-6 (Nu Check Prep, Elysian, MN, USA), eicosapentaenoic acid methyl ester, arachidonic acid methyl ester (Sigma Aldrich, St Louis, MO, USA) and stearidonic acid methyl ester (Cayman Chemical, USA).

2.9. Expression of Na^+/K^+ ATPase mRNA

On d25ph black sea bass larvae were subjected to a less-than-lethal hyperosmotic salinity challenge of 43 g/L. Larvae were sampled before increasing the salinity (0 h) and after 24 h. Due to the small size of the larvae, ten larvae were pooled for each sample and stored at −20 °C in RNAlater (Ambion, Woodward, Texas, USA) to preserve the integrity of RNA until extraction.

2.10. RNA extraction and reverse transcription

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, California, USA). The protocol for animal tissues was modified by using Trizol (Invitrogen, Carlsbad, California, USA) instead of Buffer RLT (Qiagen, Valencia, California, USA). RNA was bound to a column with 70% ethanol and then eluted with 30 μL of nuclease-free water. Maximum RNA yield was obtained by eluting a second time with the 30 μL eluant. Concentration of RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, California). To normalize every sample, 4 μg of total RNA was used for first strand cDNA synthesis.

2.11. Sequencing of Na^+/K^+ ATPase

Gill tissue excised from juvenile fish was used to obtain partial sequence of the black sea bass Na^+/K^+ ATPase (NKA). The same protocols for RNA extraction and reverse transcription were followed. Degenerate primers (forward: 5′-ATG CAN GTN GCN CAY ATG TGG-3′ and reverse: 5′-GGN GGR TCD ATC ATN GCC AT-3′) were designed from known NKA sequences obtained through GenBank (PubMed) and aligned using ClustalW (EMBL-EBI, European Bioinformatics Institute). Restriction fragment length polymorphisms produced through traditional PCR, using degenerate primers, were verified with electrophoresis and purified using a Wizard SV Gel & Cleanup Kit (Promega, Madison, Wisconsin, USA). A sequencing reaction was used with this product in the presence of Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Foster City, California, USA). A sephadex column was used to clean up the sequencing reaction product. The eluant was then speed-vac'd to dryness and resuspended in 10 μL of Hi-Dye (Applied Biosystems, Foster City, California, USA) then transferred to a sequencing plate. Forward and reverse sequences were obtained from a capillary DNA sequencer, ABI 3000 (Applied Biosystems, Foster City, California, USA). They were checked and assembled using Vector NTI (Invitrogen, Carlsbad, California, USA) to obtain partial sequence of NKA. This segment of the NKA gene was verified using BLAST (PubMed) and the partial sequence was stored in GenBank (accession number: DQ492680).

2.12. Quantitative PCR

Quantitative real-time PCR was accomplished using qPCR primers (forward: 5′-AGC CTA TGG ACG ACG AGA TGA-3′ and reverse: 5′-CCA GCT CAA CAT AGG CGT TCT-3′) designed in Primer Express (Applied Biosystems, Foster City, California, USA) from the partial sequence of the black sea bass NKA (GenBank accession number: DQ492680). All samples were analyzed in the presence of SYBR green fluorescent Dye (Applied Biosystems, Foster City, California, USA) using an Agilent 7500 quantitative PCR system (Agilent, Palo Alto, California, USA). For the relative quantitation of NKA among samples the same standard was used for both sample periods. This standard consisted of a full-strength (1.0) and a dilution series of 0.5, 0.25, and 0.125. The standard was used to show differences in linearity between the log concentration and the critical threshold cycle number of each sample. Transcript abundance for all samples was reported relative to the standard.

2.13. Resistance to hypersaline stress

A hypersaline stress test was administered to a sample of larvae from each replicate tank on d24ph. A stress sensitivity index, the median survival time index (ST-50) was used (Dhert et al., 1990). This is the time at which larval survival falls to 50% following exposure of a group of 20 larvae to an acute hypersaline challenge of 55 g/L seawater. Mean survival time (MST), or the mean time to death for sampled larvae, was also calculated as an index of stress resistance (Watanabe et al., 1985).

2.14. Data analysis

All data were expressed as mean ± standard error of the mean (SEM). After homogeneity of variances was determined, treatment means were compared by one-way ANOVA. A Welch ANOVA was used when the assumptions of ANOVA were not met. Multiple comparisons among means were performed using Tukey's test. Survival data was arcsine transformed for statistical analysis (Sokal and Rohlf, 1995). A

significance level of $P \leq 0.05$ was used in all tests. Statistical software JMP IN version 4 (SAS Institute Inc., Cary, North Carolina, USA) was used to perform computational analyses.

3. Results

3.1. Fatty acid composition of enriched rotifers

No significant ($P>0.05$) treatment effects were observed in total lipid ($\overline{X} = 9.74\%$ or 567.7 mg/g dry wt.) in enriched rotifers (Table 1). Saturated fatty acids (SFAs) showed no significant ($P>0.05$) differences among the 0–12% ARA treatments ($\overline{X} = 22.2$–26.0%). Rotifers enriched with Algamac 2000 (ALG) had higher ($P<0.05$) total SFAs ($\overline{X} = 30.3$) than the 0, 6 and 8% ARA treatments (Table 1). Rotifers enriched with Algamac 2000 (ALG) had higher ($P<0.05$) total SFAs (e.g. 14:0 and 16:0) ($\overline{X} = 30.3$) than the 0, 6 and 8% ARA treatments ($\overline{X} = 22.4$) (Table 1). A significant ($P<0.05$) trend toward decreasing MUFAs (i.e., 18:1n-9 oleic acid) with increasing dietary ARA was observed from 43.0 in 0% ARA to 25.4 in 12% ARA (Table 1) and 23.6 in ALG (Table 1).

No significant ($P>0.05$) treatment effects were observed in n-3 polyunsaturated fatty acids (PUFAs) among the 0–12% ARA treatments ($\overline{X} = 24.4$) (Table 1). The concentration of n-3 PUFAs (e.g. 18:3n-3, 22:5n-3 and 22:6n-3) was higher ($P<0.05$) in the ALG treatment ($\overline{X} = 37.1$) than in the 0–12% ARA treatments (Table 1).

A significant ($P<0.05$) trend toward increasing levels of n-6 PUFAs with increasing dietary ARA was observed from 10.3 at 0% ARA to 21.9 at 12% ARA (Table 1). The ALG treatment ($\overline{X} = 12.2$) had significantly ($P<0.05$) less n-6 PUFAs than the 6, 8, 10 and 12% ARA treatments ($\overline{X} = 16.5$–21.9), but was not significantly ($P>0.05$) different from the 0% ARA treatment ($\overline{X} = 10.3$). A significant ($P<0.05$) trend toward increasing 20:4n-6 with increasing dietary ARA was observed from 1.62 at 0% ARA to 14.3 at 12% ARA (Table 1). The level of 20:4n-6 in the ALG treatment ($\overline{X} = 2.88$) did not differ ($P>0.05$) from 0% ARA, but was lower than the 6–12% ARA treatments. Docosapentaenoic acid (22:5n-6), was detected in rotifers enriched with ALG ($\overline{X} = 6.05$), but was not found in the ARA treatments (Table 1).

The ratio of n-3/n-6 PUFA was significantly ($P<0.05$) higher in the ALG ($\overline{X} = 3.03$) than 0–12% ARA treatments ($\overline{X} = 1.51$) (Table 1). Among the ARA treatments, a trend ($P<0.05$) toward lower n-3/n-6 PUFA ratio was observed from 2.31 at 0% ARA to 1.12 at 12% ARA. Both ALG ($\overline{X} = 6.86$) and 0% ARA ($\overline{X} = 6.16$) had higher ($P<0.05$) ratios of 22:6n-3/20:4n-6 (DHA/ARA) than the 6, 8, 10 and 12% ARA treatments ($\overline{X} = 0.98$). The ratio of 20:5n-3/20:4n-6 (EPA/ARA) was higher ($P<0.05$) in the 0% ARA ($\overline{X} = 4.83$) than in the 6–12% ARA treatments ($\overline{X} = 0.54$–1.11) (Table 1). ALG had a higher ($P<0.05$) ratio of 20:5n-3/20:4n-6 ($\overline{X} = 3.07$) than the 6–12% ARA treatments.

3.2. Fatty acid composition of enriched Artemia

Significant ($P<0.05$) treatment effects were observed in percent total lipid in enriched *Artemia* (Table 2), with generally higher values in the 8% and ALG treatments (8.06–8.14%, 600.4–606.6 mg/g DW), than in the 0 and 6% ARA treatments (4.93–5.03%, 373.5–377.2 mg/g DW). No significant ($P<0.05$) treatment effects were observed in SFAs ($\overline{X} = 17.7$) (Table 2).

A significant ($P<0.05$) trend toward decreasing MUFAs (e.g. 18:1n-9) with increasing dietary ARA was observed from 39.7 at 0% ARA to 24.5 at 12% ARA (Table 2). MUFAs were significantly ($P<0.05$) higher in ALG ($\overline{X} = 48.9$) than in the ARA treatments (Table 2).

The n-3 PUFA concentration of *Artemia* was lower ($P<0.05$) in the ALG ($\overline{X} = 24.0$) than in the ARA treatments ($\overline{X} = 34.9$) due to lower 18:3n-3 in the ALG ($\overline{X} = 4.49$) than in ARA treatments ($\overline{X} = 22.8$). The concentrations of 20:5n-3 and 22:6n-3 were higher ($P<0.05$) in the ALG ($\overline{X} = 3.66$ and 10.9) than in the ARA ($\overline{X} = 1.76$ and 6.09) treatments. No significant ($P>0.05$) treatment effects were observed on the ratios of 22:6n-3/20:5n-3 ($\overline{X} = 3.40$) (Table 2).

A significant ($P<0.05$) trend toward increasing n-6 PUFAs in *Artemia* with increasing dietary ARA was observed, from 7.41 at 0% ARA to 17.4 at 12% ARA (Table 2). The ALG treatments ($\overline{X} = 9.75$) did not differ significantly ($P>0.05$) from the 0 and 6% ARA treatments. A significant ($P<0.05$) trend toward increasing 20:4n-6 in *Artemia* with increasing dietary ARA was observed, from 0 at 0% ARA to 10.9 at 12% ARA (Table 2). Levels of 20:4n-6 in *Artemia* were higher ($P<0.05$) in the 6, 8, 10 and 12% ARA treatments than in the ALG treatment ($\overline{X} = 1.74$). Docosapentaenoic acid 22:5n-6 was present in *Artemia* enriched with ALG ($\overline{X} = 3.92$), but was not detected in the ARA treatments (Table 2).

The ratio of n-3/n-6 PUFAs in *Artemia* was higher ($P<0.05$) in the 0% ARA treatment ($\overline{X} = 4.57$) than in the 6–12% ARA and ALG treatments ($\overline{X} = 2.12$–2.75) (Table 2). ALG had a higher ($P<0.05$) ratio of 22:6n-3/20:4n-6 ($\overline{X} = 6.59$) than the ARA treatments ($\overline{X} = 0.81$). The ratio of 20:5n-3/20:4n-6 was higher ($P<0.05$) in the ALG treatment ($\overline{X} = 2.14$) than in the ARA treatments ($\overline{X} = 0.22$) (Table 2).

3.3. Fatty acid composition of black sea bass larvae

No significant ($P<0.05$) treatment effects on total lipid ($\overline{X} = 5.48\%$, or 318.5 mg/g dry wt.) or in SFAs ($\overline{X} = 22.8$) in black sea bass larvae were observed (Table 3). A significant ($P<0.05$) trend of decreasing MUFAs with increasing dietary ARA was observed from 31.2 at 0% ARA to 22.4 in 12% ARA. The ALG treatment ($\overline{X} = 18.1$) was significantly ($P<0.05$) lower in MUFAs than the ARA treatments. Concentrations of 18:1n-9 decreased with increasing dietary ARA from 26.3 at 0% ARA to 18.3 at 12% ARA and were higher than in the ALG treatment ($\overline{X} = 13.3$).

Significant ($P<0.05$) treatment effects were observed in the concentrations of n-3 PUFAs (Table 3), which were 9% higher ($P<0.05$) in the ALG treatment ($\overline{X} = 43.9$) than in the ARA treatments ($\overline{X} = 34.9$), with DHA 22:6n-3, linolenic 18:3n-3, and EPA 20:5n-3 accounting for approximately 5, 3, and 1%, respectively, of this difference. Larvae fed prey enriched with ALG contained higher ($P<0.05$) levels of 20:5n-3 ($\overline{X} = 4.76$) than the 0–12% ARA treatments ($\overline{X} = 3.07$). The concentration of 22:5n-3 was higher ($P<0.05$) in the 0, 8 and 10% ARA ($\overline{X} = 1.29$) treatments than in the ALG ($\overline{X} = 0.91$) treatment. The concentration of 22:6n-3 was higher ($P<0.05$) in the ALG ($\overline{X} = 12.6$) than in the ARA treatments ($\overline{X} = 7.45$). Larvae fed 12% ARA had a higher ($P<0.05$) ratio of 22:6n-3/20:5n-3 ($\overline{X} = 2.71$) than larvae fed 0, 6 and 8% ARA ($\overline{X} = 2.11$). Larvae fed ALG (3.48) and 6–12% ARA ($\overline{X} = 0.75$–1.01) showed a lower ($P>0.05$) ratio of 22:6n-3/20:4n-6 than those fed 0% ARA ($\overline{X} = 4.92$) (Table 3).

A trend ($P<0.05$) toward increasing n-6 PUFAs with increasing dietary ARA was observed from 8.42 at 0% ARA to 15.9 at 12% ARA (Table 3). The ALG treatment (11.5%) was lower ($P<0.05$) than 6–12% ARA, but higher ($P<0.05$) than 0% ARA. A trend toward increasing 20:4n-6 with increasing dietary ARA was observed from 1.64 at 0% ARA to 10.2 at 12% ARA (Fig. 1). There was higher ($P<0.05$) 20:4n-6 detected in the ALG ($\overline{X} = 3.61$) than in the 0% ARA treatment ($\overline{X} = 1.64$), but less ($P<0.05$) than in the 6–12% ARA treatment ($\overline{X} = 6.73$–10.2) (Fig. 1). Docosapentaenoic acid 22:5n-6 was only detected in the ALG treatment ($\overline{X} = 3.16$) (Table 3).

The ratio of n-3/n-6 PUFA was higher ($P<0.05$) in the 0% ARA treatment ($\overline{X} = 4.20$) than in the 6–12% ARA ($\overline{X} = 2.72$–2.21) and ALG treatments ($\overline{X} = 3.83$) (Table 3). The ratio of n-3/n-6 PUFA in ALG treatment was significantly ($P<0.05$) higher than in the 6–12% ARA treatments (Fig. 2). Larvae in the ALG treatment had a higher ($P<0.05$) ratio of 22:6n-3/20:4n-6 ($\overline{X}$ 3.48) than the 6–12% ARA treatments ($\overline{X} = 0.86$). The 0% ARA treatment had a higher ($P<0.05$) ratio of 22:6n-3/20:4n-6 ($\overline{X} = 4.92$) than the ALG treatment or ARA treatments (Table 3). Larvae in the ALG treatment had a higher

Table 3
Total lipid and fatty acid composition (% of total fatty acids) (means ± SEM, $N = 3$) of black sea bass *Centropristis striata* larvae (day 24 post-hatching).

Enriched diets received by larvae (% of total fatty acids as ARA and Algamac 2000)						
Fatty acids	0%	6%	8%	10%	12%	Algamac
14:0	0.79 ± 0.01 ab	0.75 ± 0.02 b	0.71 ± 0.02 b	1.03 ± 0.12 a	0.73 ± 0.02 b	0.83 ± 0.05 ab
16:0	13.8 ± 0.27 ab	13.4 ± 0.31 b	13.2 ± 0.05 b	14.2 ± 0.39 ab	13.5 ± 0.08 b	14.9 ± 0.34 a
16:1n-7	2.55 ± 0.02 b	2.52 ± 0.06 b	2.29 ± 0.01 b	2.93 ± 0.32 ab	2.31 ± 0.10 b	2.96 ± 0.12 a
18:0	8.49 ± 0.27	7.79 ± 0.12	8.41 ± 0.04	7.63 ± 0.48	8.60 ± 0.28	8.01 ± 0.61
18:1n-9	26.3 ± 0.28 a	23.0 ± 0.17 b	21.4 ± 0.25 c	19.2 ± 0.36 d	18.3 ± 0.12 d	13.3 ± 0.41 e
18:2n-6	6.77 ± 0.08 a	6.39 ± 0.06 b	5.97 ± 0.01 c	6.09 ± 0.12 bc	5.73 ± 0.08 c	4.53 ± 0.09 d
18:3n-3	19.0 ± 0.40 a	21.2 ± 0.42 ab	19.3 ± 0.31 a	19.5 ± 0.66 a	20.1 ± 0.35 ab	22.5 ± 1.17 b
18:4n-3	4.33 ± 0.30	3.98 ± 0.34	4.27 ± 0.35	4.44 ± 0.45	4.31 ± 0.27	4.98 ± 0.34
20:1	2.33 ± 0.09	1.71 ± 0.31	2.19 ± 0.11	1.61 ± 0.32	1.85 ± 0.17	1.91 ± 0.22
20:4n-6	1.64 ± 0.04 a	6.73 ± 0.18 c	7.87 ± 0.09 d	9.32 ± 0.23 e	10.2 ± 0.19 f	3.61 ± 0.02 b
20:5n-3	3.89 ± 0.09 b	3.25 ± 0.13 c	3.15 ± 0.04 c	3.06 ± 0.02 c	2.84 ± 0.13 c	4.76 ± 0.25 a
22:5n-3	1.28 ± 0.04 a	1.12 ± 0.09 ab	1.26 ± 0.03 a	1.33 ± 0.09 a	1.17 ± 0.07 ab	0.91 ± 0.05 b
22:5n-6	0	0	0	0	0	3.16 ± 0.11
22:6n-3	8.07 ± 0.52 a	6.79 ± 0.21 a	6.72 ± 0.11 a	8.06 ± 0.42 a	7.64 ± 0.31 a	12.6 ± 0.57 b
∑ Saturates	23.0 ± 0.54	21.9 ± 0.36	22.3 ± 0.07	22.9 ± 0.51	22.8 ± 0.23	23.8 ± 0.88
∑ Monounsaturates	31.2 ± 0.22 a	27.3 ± 0.19 b	25.9 ± 0.34 c	23.8 ± 0.37 d	22.4 ± 0.16 d	18.1 ± 0.46 e
∑ n-3 Polyunsaturates	35.4 ± 0.02 a	35.7 ± 0.41 a	33.7 ± 0.32 a	35.0 ± 0.59 a	35.1 ± 0.32 a	43.9 ± 1.18 b
∑ n-6 Polyunsaturates	8.42 ± 0.05 a	13.1 ± 0.21 c	13.8 ± 0.09 d	15.4 ± 0.21 e	15.9 ± 0.13 e	11.5 ± 0.11 b
n-3/n-6 Polyunsaturates	4.20 ± 0.03 a	2.72 ± 0.04 c	2.44 ± 0.03 d	2.27 ± 0.03 d	2.21 ± 0.03 d	3.83 ± 0.12 b
DHA/EPA	2.09 ± 0.18 a	2.10 ± 0.10 ab	2.13 ± 0.05 ab	2.63 ± 0.13 bc	2.71 ± 0.21 c	2.67 ± 0.25 abc
DHA/ARA	4.92 ± 0.33 a	1.01 ± 0.02 c	0.85 ± 0.01 c	0.86 ± 0.03 c	0.75 ± 0.02 c	3.48 ± 0.16 b
EPA/ARA	2.37 ± 0.06 a	0.48 ± 0.01 c	0.41 ± 0.01 cd	0.33 ± 0.01 cd	0.28 ± 0.02 d	1.32 ± 0.07 b
Total lipid (%)	5.58 ± 0.19	6.13 ± 0.09	5.11 ± 0.31	5.70 ± 0.32	5.25 ± 0.36	5.13 ± 0.33
Total lipid (mg/g DW)	349.6 ± 10.71	344.2 ± 4.57	290.9 ± 18.7	305.1 ± 16.5	327.6 ± 22.2	293.5 ± 18.6

Mean values within rows that are not followed by the same letter are significantly ($P<0.05$) different.
Total lipid is reported as % and mg/g dry weight (DW).

($P<0.05$) ratio of 20:5n-3/20:4n-6 ($\overline{X} = 1.32$) than in the 6–12% ARA treatments ($\overline{X} = 0.37$). The 0% ARA treatment had a higher ($P<0.05$) ratio of 20:5n-3/20:4n-6 ($\overline{X} = 2.37$) than the ALG treatment (Table 3).

3.4. Growth

On d4ph, notochord length averaged 2.94 mm among treatments, with no significant ($P>0.05$) differences (Table 4). Mean notochord length among treatments steadily increased to 4.45, 5.05 and 6.56 mm on d10, d17 and d24ph, respectively, with no significant ($P>0.05$) differences.

On d4ph, wet weights averaged 0.11 mg among treatments, with no significant ($P>0.05$) differences. Mean wet weights steadily increased to 0.35, 1.13 and 3.60 mg on d10, d17 and d24ph, respectively, with no significant ($P>0.05$) differences.

Dry weight data for d4ph was inaccurate and was not used for statistical comparison. On d10ph, dry weights averaged 0.08 mg among treatments, with no significant ($P>0.05$) differences. Mean dry weights steadily increased to 0.18 and 0.63 mg on d17 and d24ph, respectively, with no significant ($P>0.05$) differences.

3.5. Survival and resistance to hypersaline stress

Survival declined sharply by d4ph to a mean of 38.7% among treatments, with no significant ($P>0.05$) differences (Table 5). After this, survival was relatively stable, with mean values among treatments of 29.5%, 27.3% and 29.3% on d10, d17 and d24ph, respectively, with no significant ($P>0.05$) differences. Black sea bass

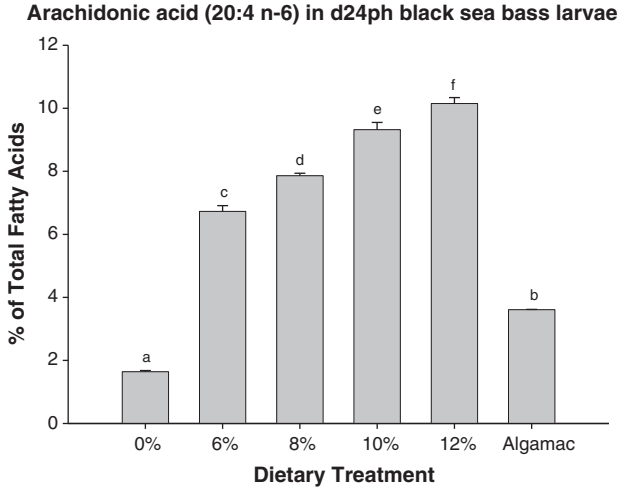

Fig. 1. Percentage of arachidonic acid, 20:4n-6 ($\overline{X} \pm$ SEM, $N = 3$) in black sea bass larvae fed live prey enriched with five different oil emulsions and a commercial diet, Algamac 2000. Each oil emulsion contained 10% DHA and different levels of ARA (0, 6, 8, 10, 12% of total fatty acids). Treatment means not associated with the same letters are significantly different ($P<0.05$).

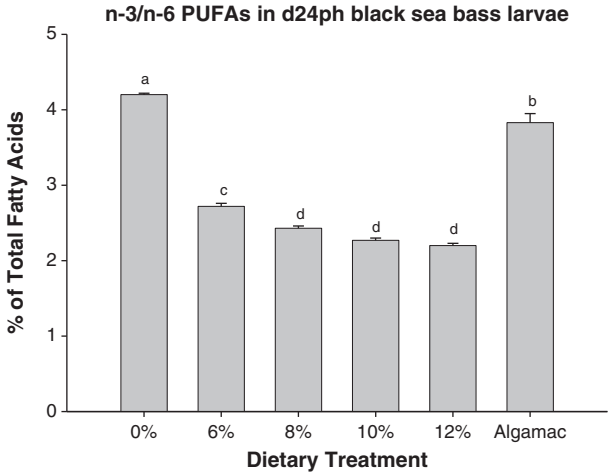

Fig. 2. Percentage of n-3/n-6 polyunsaturated acids, PUFAs ($\overline{X} \pm$ SEM, $N = 3$) in black sea bass larvae fed live prey enriched with five different oil emulsions and a commercial diet, Algamac 2000. Each oil emulsion contained 10% DHA and different levels of ARA (0, 6, 8, 10, 12% of total fatty acids). Treatment means not associated with the same letters are significantly different ($P<0.05$).

Table 4
Growth in notochord length (NL, mm), wet weight (WW, mg), and dry weight (DW, mg) of black sea bass *Centropristis striata* larvae fed live prey enriched with different levels of arachidonic acid 20:4n-6 (0–12% of total fatty acids) or Algamac 2000. Values are means ± SEM ($N = 5$). No significant ($P > 0.05$) treatment differences were observed for any parameter on any sampling date.

Growth							
	Age (dph)	Treatment (% of total fatty acids as ARA and Algamac)					
		0	6	8	10	12	Algamac
NL	4	2.88 ± 0.09	3.03 ± 0.06	2.92 ± 0.08	3.03 ± 0.07	2.98 ± 0.05	2.85 ± 0.09
WW	4	0.14 ± 0.04	0.12 ± 0.02	0.11 ± 0.01	0.16 ± 0.03	0.08 ± 0.01	0.10 ± 0.02
DW	4	No data					
NL	10	4.04 ± 0.05	3.97 ± 0.05	3.92 ± 0.06	3.80 ± 0.05	3.91 ± 0.09	4.06 ± 0.09
WW	10	0.46 ± 0.14	0.31 ± 0.52	0.30 ± 0.05	0.28 ± 0.03	0.33 ± 0.04	0.44 ± 0.04
DW	10	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01
NL	17	5.16 ± 0.09	5.18 ± 0.09	5.21 ± 0.09	4.94 ± 0.08	5.0 ± 0.08	4.83 ± 0.09
WW	17	1.17 ± 0.15	1.24 ± 0.18	1.23 ± 0.17	1.09 ± 0.09	1.02 ± 0.07	1.05 ± 0.10
DW	17	0.20 ± 0.02	0.21 ± 0.03	0.20 ± 0.03	0.17 ± 0.01	0.17 ± 0.02	0.17 ± 0.02
NL	24	6.82 ± 0.16	6.65 ± 0.14	6.60 ± 0.12	6.53 ± 0.11	6.28 ± 0.11	6.52 ± 0.10
WW	24	3.82 ± 0.86	4.17 ± 1.01	3.84 ± 0.64	3.45 ± 0.48	3.10 ± 0.58	3.30 ± 0.44
DW	24	0.63 ± 0.14	0.74 ± 0.17	0.69 ± 0.14	0.66 ± 0.12	0.49 ± 0.08	0.60 ± 0.10

Due to small size, accurate measurements of dry weight could not be obtained on d4ph.

Table 5
Survival (means ± SEM, $N = 5$) of black sea bass *Centropristis striata* larvae fed live prey enriched with different levels of arachidonic acid 20:4n-6 (0–12% of total fatty acids) or Algamac 2000.

Survival (%)						
Age (dph)	Treatment (% of total fatty acids and Algamac 2000)					
	0	6	8	10	12	Algamac
4	31.7 ± 0.12	25.9 ± 0.04	32.2 ± 0.08	56.9 ± 0.12	43.8 ± 0.07	41.9 ± 0.10
10	23.0 ± 0.06	24.9 ± 0.06	27.1 ± 0.05	36.9 ± 0.05	33.4 ± 0.05	31.8 ± 0.08
17	18.2 ± 0.05	27.2 ± 0.06	27.3 ± 0.06	31.7 ± 0.06	25.0 ± 0.03	34.3 ± 0.11
24	24.3 ± 0.08	28.0 ± 0.05	30.1 ± 0.05	32.7 ± 0.08	30.7 ± 0.03	30.2 ± 0.08

Mean values in a row followed by different letters are significantly ($P < 0.05$, ANOVA) different.

larvae subjected to a hypersaline challenge of 55 g/L on d24ph showed no significant ($P > 0.05$) differences in median survival time (ST-50 = 27.1–31.8 min) or mean survival time (MST = 32.5–35.5 min) (Table 6).

3.6. Expression of Na^+/K^+ ATPase mRNA

All quantitative real-time PCR data for Na^+/K^+ ATPase (NKA) mRNA in this study are based on the same standards and are relative to each other. The arbitrary units used do not, however, represent absolute RNA concentrations. The expression of NKA mRNA in d24ph black sea bass larvae before exposure to a sub-lethal salinity of 43 g/L ranged from 0.27 to 0.47 among treatments, with no significant ($P > 0.05$) differences (Fig. 3). The expression of NKA mRNA in larvae after 24 h of exposure to a sub-lethal salinity of 43 g/L ranged from 0.64 to 0.92 among treatments, with no significant ($P > 0.05$) differences (Fig. 3). A significant ($P < 0.05$) increase of NKA after 24 h was observed. Treatments that contained dietary ARA concentrations of 6–12% had significantly ($P < 0.05$) higher levels of NKA mRNA after 24 h than the same ARA treatments at 0 h. In contrast, there were no significant ($P > 0.05$) increases in the levels of NKA mRNA detected in the 0% ARA or ALG treatments after 24 h.

4. Discussion

4.1. Fatty acid composition of enriched rotifers

Total lipid of the rotifers did not differ among treatments, while rotifer fatty acid profiles were significantly affected by the commercial diet Algamac 2000 (ALG) and the experimental emulsions (Table 1). Due to high levels of myristic acid, 14:0 (16.3% TFA) and palmitic acid, 16:0 (42.2% TFA) in ALG, both SFAs were retained in rotifers at significantly higher levels in ALG than in the other treatments.

Rotifers were enriched with experimental emulsions comprising DHAsco (40 g), ARAsco (0–48 g), olive oil (50–2 g), preservatives (10 g) and water (Advanced Bionutrition, Columbia, MD, USA). Since olive oil is high in oleic acid 18:1n-9, the level of MUFAs in rotifers decreased as ARA increased (Bransden et al., 2004).

Total n-3 PUFAs in enriched rotifers were higher in the ALG treatment than in the 0–12% ARA treatments, primarily due to the high level of DHA in ALG (27% TFA, Aquafauna Bio-Marine, Hawthorne, CA, USA) compared to the experimental emulsions (10% TFA). DHA levels in rotifers were, therefore, higher in the ALG (19.8% TFA) than in the 0–12% ARA treatments ($\overline{X} = 10.2\%$ TFA) (Table 1).

Table 6
Hypersaline stress resistance of d24ph black sea bass larvae, *Centropristis striata* fed live prey enriched with different levels of arachidonic acid 20:4n-6 (0–12% of total fatty acids) or Algamac 2000. Larvae were subjected to a hypersaline stress test of 55 g/L. ST-50 and MST data are reported as mean ± SEM ($N = 5$). No significant ($P > 0.05$) treatment differences were observed.

	Treatment (% total fatty acids as ARA and Algamac 2000)					
	0	6	8	10	12	Algamac
ST-50	31.82 ± 1.88	29.32 ± 2.77	27.14 ± 4.28	29.06 ± 3.29	31.61 ± 3.46	28.95 ± 3.76
MST	35.22 ± 1.86	32.98 ± 2.79	33.06 ± 5.67	32.52 ± 3.28	35.51 ± 3.61	32.83 ± 3.85

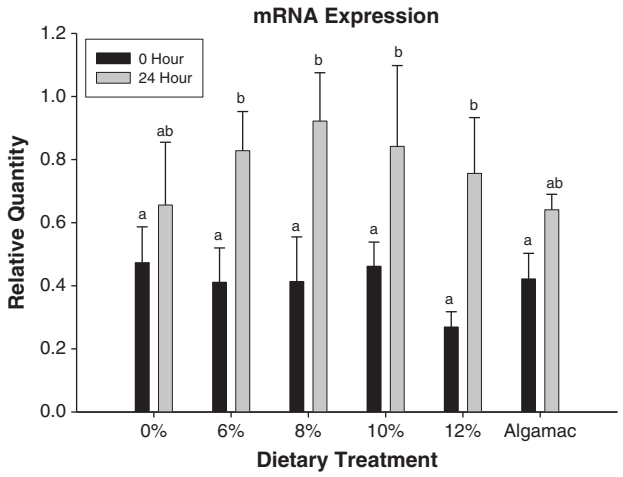

Fig. 3. Expression of Na^+/K^+ ATPase mRNA in black seabass *Centropristis striata* larvae fed live prey enriched with different levels of arachidonic acid 20:4n-6 (0–12% of total fatty acids) or Algamac 2000. Values are means ± SEM ($N = 3$). Larvae were subjected to an increase in salinity of 8 g/L (from 35 to 43 g/L) and were sampled before transfer (0 h, dark bars) and at 24 h (light bars) post-transfer. For each time period (i.e. comparisons among dietary treatments) and for each treatment (i.e., 0 vs 24 h), bars not associated with the same letter are significantly ($P < 0.05$) different.

Concentrations of the n-6 PUFA ARA in enriched rotifers were well correlated to emulsion levels. Docosapentaenoic acid 22:5n6 (DPAn-6) was only found in rotifers enriched with ALG (6.05% TFA), which contained DPAn-6 at 10.6%. Although rotifers may be able to retro convert DPAn-6 to ARA (Barclay and Zeller, 1996), retro conversion was not evident in the present study, since there were no differences in ARA levels between the ALG and 0% ARA treatments. Rotifers were raised on *N. oculata*, which naturally contains ARA (3.94% TFA) (Reed Mariculture, Campbell, CA, USA), causing the levels of ARA in rotifers from all treatments to be slightly higher than emulsion levels (1.62% and 14.3% in the 0% and 12% ARA treatments, respectively).

4.2. Fatty acid composition of enriched Artemia

In this study, significant treatment differences were found in total lipid, while no differences were observed in saturated fatty acids in *Artemia* (Table 2). As observed in rotifers, MUFAs decreased as ARA increased (Bransden et al., 2004; Copeman et al., 2002). Total n-3 PUFAs in *Artemia* were lower in the PUFA-rich ALG (24.0% TFA) treatment than in the 0–12% ARA treatments ($\overline{X} = 34.9\%$ TFA), because linolenic acid (18:3n-3) was a relatively large contributor in the 0–12% ARA treatments. ALG contained almost 2.5 times more DHA than the 0–12% ARA emulsions, but DHA in *Artemia* enriched with ALG was only 10.9% TFA compared to 6.09% TFA in the 0–12% ARA diets (Table 2). Relatively low DHA in the ALG treatment may be attributed to a retro conversion of DHA to EPA (Navarro et al., 1999). The difficulty of enriching DHA to high levels in *Artemia* was reported by Harel et al. (2002) who found that rotifers retained 3 times more DHA (23.4% TFA) than *Artemia* (7.4% TFA). Koven et al. (2001) observed that following enrichment with ALG, the DHA/ARA ratio in *Artemia* (4.6) was markedly lower than in rotifers (10.1). DHA is a good source of energy and is selectively catabolized into EPA, also making it difficult to bioencapsulate *Artemia* with high levels of DHA (Navarro et al., 1999; Sorgeloos et al., 2001). It is essential that the *Artemia* are enriched when they begin feeding as they reach the Instar II stage (Sorgeloos et al., 2001). In the present study, part of the 18-h *Artemia* enrichment period coincided with the Instar I stage and likely decreased the efficiency of bioencapsulation. By enriching *Artemia* for 24–32 h, DHA levels can be boosted as many as 2–3 times (Faulk et al., 2005; Sorgeloos et al., 2001).

Whereas levels of EPA in rotifers ($\overline{X} = 7.94\%$ TFA) were relatively constant among treatments, EPA in *Artemia* was twice as high in the ALG (3.66% TFA) treatment than in the 0–12% ARA (1.76% TFA) treatments (Table 2), suggesting that *Artemia* catabolized DHA to EPA. Hence, the ratio of DHA/EPA (mean = 3.4) in *Artemia* remained relatively constant among treatments. The DHA/EPA ratio in *Artemia* in all treatments exceeded the recommended minimum of 2:1 for marine finfish larvae (Kanazawa, 2003; Lavens et al., 1995; Lee and Ostrowski, 2001).

Artemia fed 0% ARA contained no detectable ARA (Table 2), consistent with microalgae *N. oculata* as the origin of the ARA found in rotifers. *Artemia* fed ALG contained 1.74% ARA, even though this diet is devoid of ARA (Aquafauna Bio-Marine, Hawthorne, CA, USA), probably due to retro conversion of DPAn-6 (found only in ALG) to ARA by *Artemia* (Barclay and Zeller, 1996; Faulk et al., 2005; Navarro et al., 1999).

4.3. Fatty acid composition of D24ph larvae

In this study, no differences among dietary treatments were observed in whole body tissue lipids or in SFA profiles in black sea bass larvae (Table 3). Clear relationships between dietary MUFAs and PUFAs and whole body tissue levels were observed as reported previously in larvae of Japanese flounder (Furuita et al., 1998), turbot (Estevez et al., 1999), yellowtail flounder *Limanda ferruginea* (Copeman et al., 2002), striped trumpeter *Latris lineata* (Bransden et al., 2004) and yellowtail snapper *Ocyurus chrysurus* (Faulk et al. 2005). Black sea bass larval MUFAs decreased from 31.2 to 22.4% as dietary ARA increased from 0 to 12% (Table 3), while larvae fed ALG contained only 18.1% TFA as MUFAs.

Consistent with dietary ratios, a trend of decreasing EPA/ARA ratios in larval whole body tissues of black sea bass with increasing dietary ARA (Table 3) was observed. Similar observations were made in gilthead seabream (Bessonart et al., 1999), striped bass *M. saxatilis* (Harel et al., 2001), striped trumpeter (Bransden et al., 2004), and yellowtail snapper (Faulk et al., 2005), where increasing dietary ARA caused decreasing EPA/ARA ratios in larval tissues.

Total n-6 PUFAs in larval whole body tissues increased with increasing dietary ARA (Table 3), with tissue ARA levels closely reflecting dietary treatments (Fig. 1). Larvae from the 0% ARA treatment contained 1.64% ARA originating from rotifers fed *N. oculata* (Table 1), while larvae from the ALG treatment contained 3.61% ARA, originating in part to retro conversion of DPAn-6 to ARA in *Artemia* (Navarro et al., 1999) (Table 2). However, higher ARA in tissues of larvae from the ALG treatment (3.61%) than in either rotifers (2.88%) or *Artemia* (1.74%) fed ALG indicated that black sea bass larvae were also able to retro convert DPAn-6 to ARA as reported in gilthead seabream (Koven et al., 2001) and in cobia (Faulk and Holt, 2005).

4.4. Growth

In this study, black sea bass larvae in all treatments grew at the same rates throughout the study under a relatively wide range (0–12% TFA) of dietary ARA levels in feed, in agreement with other studies on early larval stages of marine finfish. Larval turbot fed dietary ARA ranging from 0.8 to 8.4% DW showed no differences in growth after d45ph (Estevez et al., 1999). Striped trumpeter fed dietary ARA ranging from 1.33 to 11.22 mg/g DW also showed no differences in growth through d23ph (Bransden et al. 2004). Although no effects on black sea bass larval growth related to dietary ARA supplementation were observed by d24ph in this study, it is possible that these effects are manifested during later metamorphic stages and under handling and environmental stresses common to hatcheries (Bessonart et al., 1999; Castell et al., 1994; Harel et al., 2001; Willey et al., 2003).

DHA may exert a more important effect than ARA during early larval development in some marine finfish species (Copeman et al.,

2002; Rezek et al. 2010). In yellowtail flounder, larvae fed diets high in ARA (9% TFA) did not grow as well as larvae fed diets deficient in ARA but high in DHA (Copeman et al., 2002). Rezek et al. (2010) showed that black sea bass larvae fed diets deficient in DHA were smaller than larvae fed 10% DHA, but no differences in growth due to increasing ARA within a range of 0–6% TFA were observed. Hence, similar growth of fish fed the 0–12% ARA and commercial (ALG) diets in the present study, was likely due to the relatively high level of DHA in all diets, which ranged from 9.72 to 19.8% and 5.06 to 10.9% TFA in rotifers and *Artemia*, respectively (Tables 1 and 2).

4.5. Survival

In this study, no significant differences in survival to d24ph were observed for black sea bass larvae fed live prey supplemented with ARA within a range of 0–12% (TFA), or with a commercial diet lacking ARA (ALG), indicating that little or no dietary ARA was required to support survival in this species. In larval turbot, dietary ARA from 3 to 15% TFA did not affect survival up to d43ph (Estevez et al. 1999). In the present study, black sea bass larvae fed a 0% ARA diet showed significant tissue levels of ARA (1.64% TFA) derived from *N. oculata*-fed rotifers, and larvae from the ALG treatment also showed significant tissue levels of ARA (3.61% TFA) derived from *Artemia* and from retro conversion of DPAn-6 to ARA in the larvae. Hence, a dietary requirement for ARA was minimized by retro conversion from DPAn-6 in larval tissues (Faulk and Holt, 2005). Larval gilthead sea bream (d35ph) with very low levels of tissue ARA (2.7 mg/g DW) derived from retro conversion of DPAn-6 in ALG to ARA in larval tissues had significantly lower mortalities than larvae fed an ARA-deficient diet (1.0 g/g DW tissue ARA) with high DHA (35.9% TFA), also demonstrating that very low levels of ARA can improve survival and that retro conversion of DPAn-6 to ARA can minimize ARA requirements (Koven et al., 2001).

4.6. Resistance to hypersaline stress

There were no significant differences among dietary treatments on the ability of d24ph black sea bass larvae to tolerate an acute hypersaline (55 g/L) challenge. Larval striped trumpeter (d23ph) also showed no relationship between dietary ARA levels from 1.33 to 11.22 mg/g DW and ability to tolerate acute hypersaline (55 g/L) challenge (Bransden et al., 2004). Contrary to these findings, dietary ARA fed at proper levels improved hypersaline stress resistance in older larvae of some species. For example, d46ph striped bass larvae raised in low salinity water (2–6 g/L) and fed 15.4 mg/g DW ARA showed higher survival during a hypersaline (25 g/L) challenge than larvae fed low (4.7 mg/g DW) or high (19.6 mg/g DW) ARA (Harel et al., 2001). Summer flounder larvae (d45ph) raised at 32 g/L and fed increasing dietary ARA ranging from 0 to 12% TFA showed improved hypersaline (70–80 g/L) tolerance at an intermediate level of ARA (6% TFA) (Willey et al., 2003). Available data again suggests that differences in resistance to an acute hypersaline challenge are manifested during later pre-metamorphic and metamorphic stages of larval development.

The lack of differences in resistance to an acute hypersaline (55 g/L) challenge in this study is consistent with results of a previous study in black sea bass in which larvae fed ARA at levels from 0 to 6% TFA showed no differences in hypersaline (65 g/L) stress resistance on d24ph (Rezek et al., 2010). These authors noted, however, that abrupt exposure to high salinity overwhelmed the larva's ability to adapt to the salinity stress, causing rapid death (within 1 h) and preventing treatment effects on hypo-osmoregulatory ability of larvae to be observed (Rezek et al., 2010). Species-specific differences, fish age, temperature, and the magnitude of salinity challenge are important factors that should be considered when time to mortality after transfer (e.g. ST-50) is used as a measure of salinity tolerance. Intensity of salinity challenge should be lowered in earlier life stages. Alternatively, a median lethal salinity index may allow differences in resistance to hypersaline challenge to be discerned (Watanabe et al., 1985).

4.7. Na^+/K^+ ATPase mRNA expression

In this study, dietary arachidonic acid supplementation (6–12% TFA) increased ARA in larval tissues (>6.73–10.2% TFA) and the expression of NKA mRNA 24 h after exposure of larvae to a sub-lethal increase in salinity of 8 g/L (from 35 to 43 g/L). Gilthead sea bream fed high dietary ARA showed increased NKA activity accompanied by increased hypo-osmoregulatory ability after an acute hyposaline-transfer stress (from 42 to 25 g/L) (Van Anholt et al., 2004). Grouper *Epinephelus coioides* larvae (d20ph) raised at 30 °C and subjected to a salinity increase of 8 g/L (from 32 to 40 g/L) showed a decrease in NKA activity after 12 h, possibly due to failure of the NKA enzyme units under excessive hypersaline stress, causing complete mortality (Caberoy and Quinitio, 2000).

In the present study, expression of NKA mRNA in larval black sea bass was significantly elevated 24 h after a salinity increase of 8 g/L (from 35 to 43 g/L) in larvae fed 6–12% ARA, but not in larvae fed 0% ARA (control) or ALG. Increasing the salinity (from 0 to 31 g/L) during the smoltification process in Atlantic salmon *Salmo salar* increased expression of NKA mRNA after 24 h (D'cotta et al., 2000). Juvenile European sea bass *Dicentrarchus labrax* (Jensen et al., 1998) responded to hypersaline challenge (50–60 g/L) by increasing expression of gill NKA mRNA after 24 h, which was followed by an increase in NKA activity after 48 h. The results of this study indicate that measuring expression of NKA mRNA is a highly sensitive method of measuring hypo-osmoregulatory ability (and resistance to hypersalinity stress) in fish larvae. Faced with a potentially lethal salinity challenge, from 24 to 48 h is required for the larva's hypo-osmoregulatory system (NKA) to adapt to the salinity stress. Hence, to maximize the sensitivity of traditional salinity challenge tests (e.g. ST-50) that measure time to mortality as an index of stress, preliminary tests should be conducted to gauge the magnitude of challenge that does not induce complete mortality before 24–48 h of exposure.

The effects of dietary ARA supplementation on expression of NKA mRNA in black sea bass larvae were clearly demonstrated in this study. ARA, typically found in membrane phospholipids, can be converted to prostaglandins (PGs) (Bell et al., 1994, 2003; Sargent et al., 1999) in response to changes in extracellular ion concentrations, potentially increasing the sensitivity of the HPI axis in fish and the release of cortisol from the interrenals (Bell et al., 1983; Linares and Henderson, 1991; Van Anholt et al., 2004). Increased cortisol stimulates NKA activity and chloride cell density which are essential in the active transport of ions across cell membranes (Dang et al., 2000; Singer et al., 2003). Striped bass fed dietary ARA and subjected to a hypersaline challenge showed elevated tissue ARA, cortisol and increased hypersaline stress tolerance (Harel et al., 2001). Based on maximum expression of NKA mRNA in black sea bass larvae (Fig. 3), ARA supplementation at 6–12% TFA in combination with adequate levels of DHA and EPA ($\geq$2:1) is recommended. This combination would promote optimal growth, survival and hypersaline stress resistance in black sea bass larvae to increase the potential for successful rearing, especially when exposed to acute stressors, such as an unexpected change in rearing salinity. Whereas traditional salinity challenge tests that measure survival as an index of stress are relatively insensitive for marine fish larvae, measuring expression of NKA mRNA is a highly sensitive method of detecting subtle differences in hyper- or hypo-osmoregulatory ability.

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