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Aquaculture 210 (2002) 285–304

Aquaculture

www.elsevier.com/locate/aqua-online

Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment

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Received 26 June 2001; received in revised form 14 September 2001; accepted 14 September 2001

Abstract

The role of dietary ratios of docosahexaenoic acid (DHA, 22:6$n-3$), eicosapentaenoic acid (EPA, 20:5$n-3$) and arachidonic acid (AA, 20:4$n-6$) on early growth, survival, lipid composition, and pigmentation of yellowtail flounder was studied. Rotifers were enriched with lipid emulsions containing high DHA (43.3% of total fatty acids), DHA + EPA (37.4% and 14.2%, respectively), DHA + AA (36.0% and 8.9%), or a control emulsion containing only olive oil (no DHA, EPA, or AA). Larvae were fed differently enriched rotifers for 4 weeks post-hatch. At week 4, yellowtail larvae fed the high DHA diet were significantly larger (9.7 ± 0.2 mm, $P < 0.05$) and had higher survival ($22.1 \pm 0.4\%$), while larvae fed the control diet were significantly smaller (7.3 ± 0.2 mm, $P < 0.05$) and showed lower survival ($5.2 \pm 1.9\%$). Larval lipid class and fatty acid profiles differed significantly among treatments with larvae fed high polyunsaturated fatty acid (PUFA) diets having higher relative amounts of triacylglycerols (18–21% of total lipid) than larvae in the control diet (11%). Larval fatty acids reflected dietary levels of DHA, EPA and AA while larvae fed the control diet had reduced amounts of monounsaturated fatty acids (MUFA) and increased levels of PUFA relative to dietary levels. A strong relationship was observed between the DHA/EPA ratio in the diet and larval size ($r^2 = 0.75$, $P = 0.005$) and survival ($r^2 = 0.86$, $P = 0.001$). Following metamorphosis, the incidence of malpigmentation was higher in the DHA + AA diet (92%) than in all other treatments (~ 50%). Results suggest that yellowtail larvae require a high

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PII: S0044-8486(01)00849-3

level of dietary DHA for maximal growth and survival while diets containing elevated AA exert negative effects on larval pigmentation.

Keywords: Essential fatty acids; Fish larvae; Growth; Pigmentation; *Limanda ferruginea*

1. Introduction

The importance of PUFA in larval fish nutrition has been extensively investigated during the past 20 years (Wantanabe, 1993; Watanabe and Kiron, 1994; Sargent et al., 1999). Docosahexaenoic acid (DHA, 22:6$n-3$), eicosapentaenoic acid (EPA, 20:5$n-3$), and arachidonic acid (AA, 20:4$n-6$) are essential fatty acids (EFA) for many marine species. However, live-foods that are commonly used for first-feeding of marine larvae, such as rotifers and *Artemia* sp., are naturally low in these polyunsaturated fatty acids (PUFA). Therefore, enrichment of live foods with lipids rich in PUFA prior to feeding is necessary.

Recently, the importance of considering the relative amounts of DHA, EPA, and AA simultaneously has been demonstrated (McEvoy et al., 1998; Estevez et al., 1999; Sargent et al., 1999). This is due to competitive interactions between not only DHA and EPA but also between EPA and AA. The DHA and EPA competition results from both molecules using the same enzymes to esterify fatty acids into phospholipid structures (Mourente et al., 1991; Sargent et al., 1999). Given that DHA is naturally found at very high levels in neural tissue, it is thought to play a specialised role in neural membrane structure and function (Bell and Dick, 1991). Therefore, elevated dietary EPA relative to DHA is postulated to have a negative impact on larval neural function and thus growth and survival (Bell et al., 1995; Rodriguez et al., 1997).

The competitive interactions between EPA and AA are important in the formation of eicosanoids. Eicosanoids are a group of biologically active molecules, once known as local hormones, which include prostaglandins, thromboxanes, and leukotrienes. Eicosanoids play a wide variety of localised physiological roles in fish that can range from ionic regulation to the induction of egg shedding in ripe females (Sargent, 1995). EPA and AA are both substrates for the formation of eicosanoids, with AA being the preferred substrate and producing eicosanoids of higher biological activity (Bell et al., 1994). EPA produces eicosanoids of lower biological activity and therefore modulates the efficiency of AA. Atlantic halibut and turbot larvae developed high levels of malpigmentation when fed elevated AA relative to EPA (McEvoy et al., 1998; Estevez et al., 1999). These results were hypothesised to be a result of stress induced by increased eicosanoid activity (Estevez et al., 1999, Sargent et al., 1999).

The yellowtail flounder is a candidate species for cold-water aquaculture in Atlantic Canada. However, high mortality and malpigmentation are still substantial challenges to the successful early culture of yellowtail. Both these issues have been associated with the PUFA content of live food used in first-feeding in a number of marine flatfish species (Rainuzzo et al., 1997; Venizelos and Benetti, 1999). Currently, there are no studies that have addressed the optimal lipid requirements of yellowtail flounder larvae.

Therefore, this study was designed to investigate the role of dietary ratios of DHA/EPA and EPA/AA on growth, survival, and pigmentation success of yellowtail flounder.

2. Materials and methods

2.1. Emulsions

The four experimental emulsions used included three emulsions that were high in PUFA and one control emulsion that was high in monounsaturated fatty acids (MUFA) (Table 1). Two of the PUFA emulsions were formulated by blending different ratios of DHA (DHASCO)- and AA (ARASCO)-rich TAG oils and a marine oil (TG 22/33, Marine Lipids Leknes, Norway). The DHA-rich oil was extracted from the heterotrophically grown dinoflagellate, *Crypthecodinium* sp., and the AA-rich oil was extracted from

Table 1
Lipid composition of the four experimental emulsions (mean ± SEM, $n = 3$)

	DHA	DHA + EPA	DHA + AA	Control
Lipid mixture (%w/w)	100% DHASCO	50% DHASCO + 50% marine oil	75% DHASCO + 25% ARASCO	Olive oil
Lipid class (% of total lipid)[a]				
Triacylglycerols	86.3 ± 1.3	64.1 ± 0.5	83.6 ± 1.3	87.0 ± 1.5
Alcohols	2.1 ± 1.0	12.3 ± 0.5	2.8 ± 0.9	2.4 ± 0.3
Sterols	2.7 ± 0.8	7.9 ± 2.6	3.0 ± 0.4	1.4 ± 0.2
Acetone mobile polar lipids	2.5 ± 0.1	5.8 ± 1.3	2.3 ± 0.7	0.8 ± 0.1
Phospholipids	3.3 ± 0.2	6.1 ± 1.0	5.4 ± 1.5	6.0 ± 0.5
Fatty acids (% total)				
14:0	14.0 ± 0.1	8.4 ± 0.0	11.5 ± 0.3	0.6 ± 0.0
16:0	10.7 ± 0.0	6.9 ± 0.0	10.3 ± 0.1	12.6 ± 0.2
18:0	1.1 ± 0.1	1.9 ± 0.0	2.9 ± 0.1	2.2 ± 0.2
ΣSFA[b]	26.1 ± 0.2	17.8 ± 0.1	25.1 ± 0.6	16.8 ± 1.4
16:1$n-7$	1.1 ± 0.0	1.2 ± 0.0	1.0 ± 0.0	1.2 ± 0.9
18:1$n-9$	24.7 ± 0.1	18.6 ± 0.2	23.1 ± 0.6	69.5 ± 1.0
ΣMUFA[c]	26.2 ± 0.2	21.5 ± 0.6	24.6 ± 0.7	71.3 ± 2.0
18:2$n-6$	2.6 ± 0.0	2.5 ± 0.0	3.9 ± 0.1	10.1 ± 0.1
20:4$n-6$ (AA)	0.7 ± 0.0	1.6 ± 0.0	8.9 ± 0.2	0.0 ± 0.0
20:5$n-3$ (EPA)	0.1 ± 0.1	14.2 ± 0.2	0.1 ± 0.0	0.0 ± 0.0
22:5$n-3$	0.5 ± 0.0	2.6 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
22:6$n-3$ (DHA)	43.3 ± 0.4	37.4 ± 0.3	36.0 ± 0.4	0.5 ± 0.1
ΣPUFA[d]	47.7 ± 0.5	60.7 ± 0.6	50.3 ± 0.7	11.9 ± 0.3

[a] Also contained < 1% hydrocarbons, ketones, diacylglycerols, and free fatty acids.
[b] Includes *ai*—15:0, 15:0, *i*—17:0, *ai*—17:0, 17:0, 20:0.
[c] Includes 18:1$n-11$, 18:1$n-7$, 18:1$n-5$, 20:1$n-9$, 20:1$n-7$, 22:1$n-11$, 22:1$n-9$, and 24:1.
[d] Includes 16:2$n-6$, 16:2$n-4$, 16:3$n-4$, 16:4$n-3$, 18:3$n-3$, 18:4$n-3$, 20:2$n-6$, 20:3$n-6$, 20:4$n-3$, 22:4$n-6$ and 22:5$n-6$.

the heterotrophically grown fungus, *Mortierella alpina* (DHASCO and ARASCO, respectively, Martek BioSci., Columbia, MD). The fatty acid composition of DHASCO and ARASCO was 49% DHA and 54% AA, respectively, and both had less than 0.5% EPA. The marine oil contained approximately 20% DHA and 30% EPA. The control emulsion was prepared using only olive oil, which was low in PUFA. A mixture of 2% alginic acid, 2% polyoxyethylene sorbitan monooleate (Tween 80), 1% ascorbic acid, 1% vitamin E, 1% silicon based anti-foaming agent (Sigma, St. Louis, MO, USA) and 5% soy lecithin (80% PC: Archer Daniels Midland, Decatur, IL, USA) was added to the oils. Oil mixtures were emulsified with equal amounts of distilled water by first homogenising at low speed (Ultra-turrax T8, IKA Labortechnik, Staufen, Germany) for 15 s and then sonicating for an additional 15 s at one third of the maximum sonication energy level (Sonifier 450: Branson Sonic Power: Danbury, CT.). Emulsions were stored under nitrogen at 4 °C for daily use.

2.2. Live food

Rotifers were cultured on baker's yeast, *Saccharomyces cerevisiae*, and culture Selco (INVE, Dendermond, Belgium) for 5 days prior to enrichment. Rotifer enrichments were carried out in small 10-l vessels at a density of 5×10^5 rotifers l^{-1}. Rotifer batches were enriched for approximately 12 h at 0.1 g of experimental oil l^{-1} of rotifer culture. Emulsions were added at time zero and after 6 h of enrichment. Rotifers were sampled from each enrichment vessel in triplicate for lipid analysis once per week.

All larval groups were fed identically enriched *Artemia* (Great Salt Lake, UT). Enrichment alternated daily between 24 h of DHA Selco (INVE) or Algamac (Bio-Marine, Hawthorne, CA, USA). Second instar stage *Artemia* were stocked in 300-l tanks at a density of 2×10^5 animals l^{-1}. Temperature was maintained at 26 °C and vigorous bottom aeration was applied. Enrichment was added at a concentration of 2 g/10^6 animals. After 12 h of enrichment, *Artemia* were transferred to a new enrichment vessel to receive a second 12-h enrichment. *Artemia* were sampled in triplicate four times during the experimental period.

2.3. Larviculture

Yellowtail flounder (*Limanda ferruginea)* broodstock were collected from Witless Bay (47°16.5′ N 52°48.8′ W), Newfoundland and brought to the Ocean Sciences Centre (Logy Bay, Newfoundland) in late June of 1998. Eggs for this experiment were obtained between July 6 and 7, 1998 and were pooled to obtain the required quantity. They were then incubated in a 300-l cylindro-conical upwelling tank and hatched at approximately 60°-days.

Hatched larvae were transferred to eight 230-l round, flat-bottomed tanks at a density of 60 larvae $l^{-1.}$ Water flow was 2 l min^{-1} and aeration was provided by one airstone placed in the centre of each tank. Larvae were reared at ambient temperature ~ 13 °C (10.5–16.5 °C) under continuous light (~ 1000 lx at the water surface).

Differentially enriched rotifers were added to tanks twice per day at a density of 7000 prey l^{-1}, from day 2 to 28 post-hatch (Puvanendran and Brown, 1995; Rabe and Brown,

2000). Tanks were 'greened' daily with a 50:50 mix of 10 l of *Isochrysis galbana* and *Nannochloropsis* spp. This combined algae mixture had a concentration of 6.1×10^{10} cells l^{-1}. After day 30, identically enriched *Artemia* were added to all tanks twice per day at a density of 2000 prey l^{-1}.

2.4. Growth and survival

Standard length, body depth, and dry weight were measured at hatching and at weeks 2, 3, and 4. Standard length was defined as the length in millimeters from the tip of the snout to the end of the notochord. Body depth was defined as the width of the larvae just posterior to the anus not including the fin fold. Larvae were sampled and placed in beakers that were kept on ice and were then sacrificed using an overdose of 3-amino-benzoate methane sulphonate (MS 222). Larval measurements were completed within 30 min of death to minimise shrinkage due to osmotic loss. These measurements were completed on 15 larvae $tank^{-1}$ $week^{-1}$ using a dissecting microscope and a depression slide.

After morphometric measurements, larvae were washed with 3% ammonium formate to remove salt, and five larvae were placed on a 1.5-cm^2 preweighed aluminium foil. This method resulted in three samples of five larvae being analysed for dry weight $tank^{-1}$ $week^{-1}$. The foils were dried at 60 °C for 24 h. Foils were then stored in a desiccator and reweighed.

Survival was determined after the rotifer-feeding period, at week 4, by counting larvae in subsamples of water. Aeration was increased prior to sampling to provide enhanced mixing of the tank water and then each tank was thoroughly stirred with a glass rod. Five subsamples of 4 l of water were counted per tank to calculate an average number of larvae per tank.

2.5. Lipid samples and lipid analysis

Triplicate samples ranging between 9 and 30 mg dry weight of larvae were taken from each tank at hatching and after weeks 3 and 4. Lipid samples were placed directly in chloroform and stored under nitrogen at −20 °C until extraction. Lipids were extracted in chloroform/methanol according to Parrish (1998) using a modified Folch procedure (Folch et al., 1957).

Lipid classes were determined using thin layer chromatography with flame ionisation detection (TLC/FID) with a MARK V Iatroscan (Iatron Laboratories, Tokyo, Japan) as described by Parrish (1987). Extracts were spotted on silica gel coated Chromarods and a three-stage development system was used to separate lipid classes.

The first separation consisted of 20-min developments in 99:1:0.05 hexane/diethyl ether/formic acid. The second separation consisted of a 40-min development in 80:20:1 hexane/diethyl ether/formic acid. The last separation consisted of 15-min developments in 100% acetone followed by 10-min developments in 5:4:1 chloroform/methanol/water. After each separation, the rods were scanned and the three chromatograms were combined using T-data scan software (RSS, Bemis, TN, USA). The signal detected in millivolts was quantified using lipid standards (Sigma).

Total lipid as well as neutral and polar lipids were analysed for fatty acid composition. Prior to separation of total lipids, tricosanoic acid methyl ester (23:0) was added to larval fish extracts at an amount that was approximately 10% of the total fatty acids. Preliminary analysis of samples without added 23:0 revealed that it would be a suitable internal standard. Total lipids were separated into neutral and polar lipids using column chromatography (Yang, 1995; Budge, 1999). Lipid extracts were applied to the top of the column and the neutral lipids were eluted with 3 ml of 99:1:0.5 (v/v/v) chloroform/methanol/formic acid. The remaining polar lipids were removed using 6 ml of methanol.

Fatty acid methyl esters (FAME) were prepared by transesterification with 10% BF_3 in methanol at 85 °C for 1 h (Morrison and Smith, 1964; Budge, 1999). A Varian model 3400 GC equipped with a Varian 8100 autosampler was used for fatty acid analysis (Varian, CA, USA). The column was an Omegawax 320 column, 30 m, 0.32-mm i.d., 0.25-μm film thickness (Supelco, Bellefonte, PA, USA). Hydrogen was used as the carrier gas and the flow rate was set at 2 ml min^{-1}. The column temperature profile was: 65 °C for 0.5 min, hold at 195 °C for 15 min after ramping at 40 °C min^{-1}, and hold at 220 °C for 0.75 min after ramping at 2 °C min^{-1}. The injector temperature increased from 150 to 250 °C at 200 °C min^{-1}. Peaks were detected by flame ionisation with the detector held at 260 °C. Fatty acid peaks were integrated using Varian Star Chromatography Software (version 4.02) and identification was made with reference to known standards (PUFA 1 and 37 Component FAME Mix, Supelco Canada, ON).

2.6. Pigmentation and eye migration

Pigmentation, eye migration, and orientation of the fish were classified at week 13. Scales based on categories previously defined for Atlantic halibut (Gara et al., 1998; Table 2) were used to summarise the range in pigmentation (1–6) and eye migration (0–3). These two classifications were made on 50 fish per tank while viewing each fish

Table 2
Categories of pigmentation and eye migration used in evaluation of yellowtail flounder (after Gara et al., 1998)

Categories of pigmentation	Definition
1	No pigmentation visible
2	Pigmentation only visible on the head
3	Pigmentation visible on the head and tail
4	Pigmentation visible on the head and abdomen
5	Completely pigmented on the ocular side
6	Completely pigmented on both the ocular and blind side
Categories of eye migration	**Definition**
0	Blind side eye not yet visible
1	Blind side eye only partially visible but not the full diameter
2	Blind side eye diameter fully visible but not past the dorsal margin
3	Blind side eye visible and fully past the dorsal margin

on a petri dish from directly above. Also, the side on which the fish were lying was recorded.

2.7. Statistical analysis

Differences in growth and lipid profiles between treatments were analysed using an ANOVA with tanks nested into treatments (General Linear Model, Minitab Version 10.5). In all cases, the effect of tanks was not found to be significant and was dropped from the model. The interaction between treatments and time was significant. Therefore, one-way ANOVAs with Tukey's multiple comparison test were used to compare weekly differences in growth and lipid composition between treatments. Significance was set at $\alpha = 0.05$. Residuals versus fitted values were examined to check for normality and heteroscedasticity. Body depth and dry weight data were log transformed and certain percentage data was arcsine-square root transformed in order to meet the assumption of the model.

Pigmentation and eye migration and side of settlement data were analysed for difference between treatments using a non-parametric G-test based on the chi-square distribution. When differences between treatments were detected, a priori multiple comparison tests were performed at $\alpha = 0.05$.

3. Results

3.1. Lipid composition of rotifers

Following enrichment, all rotifer groups contained approximately 16% lipid (Table 3). TAG was the major lipid class in all rotifer groups reflecting high levels of TAG in enrichment emulsions (Table 1). The PUFA-enriched rotifers had significantly higher

Table 3
Lipid class composition of rotifers enriched for 12 h using four different oil emulsions (mean ± SEM, $n = 4$)

	DHA	DHA + EPA	DHA + AA	Control
Total lipid (μg mg^{-1} dry weight)*	169.7 ± 3.4	168.2 ± 15.3	156.5 ± 22.4	165.2 ± 9.6
Lipid class (% total lipid)				
Steryl/wax esters	4.1 ± 1.4	4.1 ± 1.1	4.4 ± 0.9	3.9 ± 1.4
Methyl ketones	2.1 ± 0.6	1.9 ± 0.7	2.7 ± 0.5	2.0 ± 0.5
Triacylglycerols	55.5 ± 1.5[a]	52.3 ± 2.3[a]	53.9 ± 3.1[a]	38.0 ± 4.3[b]
Free fatty acids	4.3 ± 0.9[a]	4.5 ± 0.9[a]	2.8 ± 0.8[a]	20.5 ± 3.0[b]
Sterols	2.7 ± 0.6	2.3 ± 0.8	2.8 ± 0.3	2.7 ± 0.2
Acetone mobile polar lipids	3.1 ± 0.8	5.3 ± 0.8	3.9 ± 1.1	2.6 ± 0.9
Phospholipids	24.9 ± 0.5	26.1 ± 1.8	24.8 ± 0.9	26.7 ± 2.0

[a,b] Different letters represent a significant difference among groups; $P < 0.05$, $F_{3,12}$, one-way ANOVA with Tukey's multiple comparison test.

* Also contained <2.5% ethyl ketones, glyceryl esters, alcohols, and diacylglycerols.

Table 4
Fatty acid composition of rotifers enriched for 12 h using four different oil emulsions (mean ± SEM, $n=4$)

Fatty acid (% total fatty acids)	DHA	DHA + EPA	DHA + AA	Control
14:0	6.9 ± 0.3[a]	3.7 ± 0.4[b]	5.4 ± 0.2[a,b]	1.8 ± 0.5[c]
16:0	10.3 ± 0.9	9.3 ± 0.9	9.7 ± 0.8	10.1 ± 1.4
18:0	2.8 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
ΣSFA[1]	21.0 ± 1.2	17.6 ± 1.3	19.6 ± 1.2	16.7 ± 1.6
16:1*n* − 7	4.9 ± 0.4	4.9 ± 0.4	4.7 ± 0.5	5.6 ± 0.5
18:1*n* − 9	26.7 ± 1.0[a]	20.8 ± 0.4[b]	26.4 ± 0.8[a]	52.1 ± 1.0[c]
18:1*n* − 7	0.7 ± 0.4	2.0 ± 0.4	1.1 ± 0.4	1.8 ± 0.9
20:1*n* − 9	2.0 ± 0.1	2.3 ± 0.3	2.0 ± 0.1	2.0 ± 0.2
ΣMUFA[2]	36.8 ± 1.3[a]	33.4 ± 0.4[a]	36.3 ± 0.8[a]	63.5 ± 1.3[b]
18:2*n* − 6	4.5 ± 0.3[a]	4.4 ± 0.4[a]	5.5 ± 0.4[a]	10.2 ± 0.5[b]
20:3*n* − 6	0.2 ± 0.0	1.2 ± 0.8	0.7 ± 0.0	0.3 ± 0.0
20:4*n* − 6 (AA)	1.2 ± 0.0[a]	2.2 ± 0.4[b]	7.1 ± 0.2[c]	0.7 ± 0.0[a]
20:4*n* − 3	0.7 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.0
20:5*n* − 3 (EPA)	3.5 ± 0.3[a]	11.0 ± 0.2[c]	3.2 ± 0.2[a,b]	2.5 ± 0.2[b]
22:5*n* − 3	2.0 ± 0.3[a]	4.1 ± 0.3[b]	2.0 ± 0.1[a]	1.5 ± 0.1[a]
22:6*n* − 3 (DHA)	28.2 ± 0.5[a]	21.5 ± 0.7[b]	23.4 ± 0.8[b]	1.7 ± 0.1[c]
ΣPUFA[3]	42.1 ± 0.4[a]	49.0 ± 1.0[b]	44.2 ± 1.2[a]	19.8 ± 0.5[c]
DHA/EPA	8.2 ± 0.7[a]	1.9 ± 0.1[b]	7.5 ± 0.6[a]	0.7 ± 0.0[c]
DHA/AA	24.5 ± 1.1[a]	10.5 ± 1.4[b]	3.3 ± 0.0[c]	2.5 ± 0.2[c]
EPA/AA	3.0 ± 0.3[a]	5.4 ± 0.7[a]	0.5 ± 0.0[b]	3.7 ± 0.4[a]

[a,b] Different letters represent a significant difference among groups; $P<0.05$, $F_{3,12}$, one-way ANOVA with Tukey's multiple comparison test.

[1] Includes *ai*—15:0, 15:0, *i*—17:0, *ai*—17:0, 17:0, and 20:0.

[2] Includes 18:1*n* − 11, 18:1*n* − 5, 20:1*n* − 7, 22:1*n* − 11, 22:1*n* − 9, and 24:1.

[3] Includes 16:2*n* − 4, 16:3*n* − 4, 16:4*n* − 3, 18:3*n* − 3, 18:4*n* − 3, 20:2*n* − 6, 22:4*n* − 6, and 22:5*n* − 6.

levels of TAG (~ 54%) than the control group (38%) and significantly lower levels of free fatty acids (~ 3.9%) than in the control treatment (21%). Phospholipid (PL) levels were similar ($P>0.05$) in all treatments and accounted for ~ 26% of total lipid.

The fatty acid composition of the enriched rotifers is given in Table 4. All rotifer groups contained ~ 19% saturated fatty acids (SFA). The control diet had higher levels of MUFA (64%, $P<0.05$) and lower levels of PUFA (~ 20%, $P<0.05$) than seen in the three PUFA treatments. However, the DHA + EPA treatment had the highest concentration of PUFA (49%, $P<0.05$). Levels of DHA, EPA, and AA in the rotifers reflected that of enrichment emulsions. The three PUFA treatments resulted in high levels of DHA (21.5–28.2%) with various levels of EPA (3.2–11.0%) and AA (1.2–7.1%). The control treatment had low concentrations of all these PUFA (<2.5%). The ratio of DHA/EPA in the rotifers varied significantly between groups from a high of 8:1 in the DHA treatment to a low of 0.7:1 in the control diet. The EPA/AA ratio was highest in the DHA + EPA treatment (5:1) and significantly lower in the DHA + AA enriched group (0.5:1).

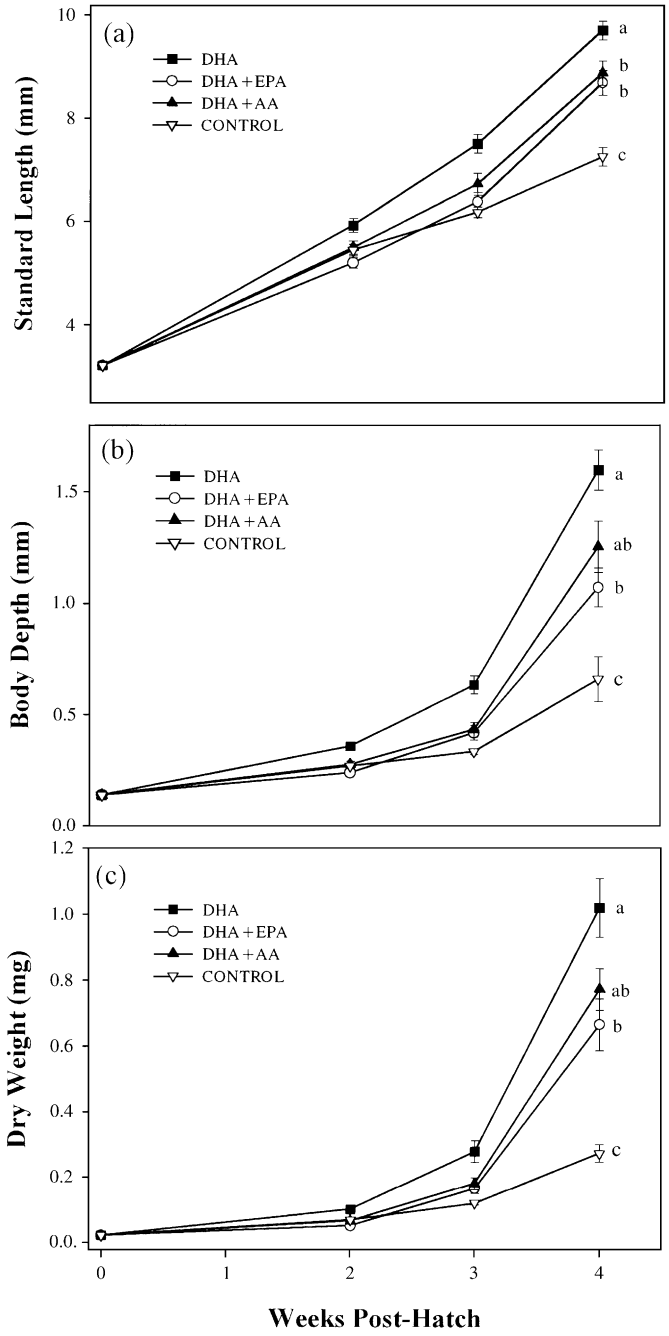

Fig. 1. Morphometric measurements and mass of yellowtail flounder larvae fed four types of differently enriched rotifers for the first 4 weeks post-hatch. Data are mean ± SEM. (a) Standard length, $n = 30$ ($P < 0.05$, $F_{3,116}$). (b) Body depth, $n = 30$ ($P < 0.05$, $F_{3,116}$). (c) Dry weight, $n = 6$ ($P < 0.05$, $F_{3,20}$). [a,b,c]Different letters represent significant differences among dietary groups (ANOVA, Tukey's multiple comparison).

3.2. Rearing success

Feeding differently enriched rotifers over the first 4 weeks resulted in differences in standard length ($P<0.05$, $F_{3,344,}$ Fig. 1a), body depth ($P<0.05$, $F_{3,344,}$ Fig. 1b), and dry weight ($P<0.05$, $F_{3,56,}$ Fig. 1c). By week 4, larvae in the DHA treatment were significantly larger than those in all other groups while the control treatment was significantly smaller. There was no significant difference in growth between larvae from the DHA + EPA and DHA + AA treatments.

Trends in survival reflected those seen in growth, with larvae in the DHA enriched treatment showing the highest survival (22%) and those in the control group the lowest (5%). Larvae from the DHA + AA group had a survival of 19% while the DHA + EPA group had 12% survival (Table 5).

In Table 5, pigmentation data are categorised as either normal (stages 5–6) or malpigmented on the ocular side (stages 1–4). High proportions of malpigmented fish were observed in all dietary groups at the end of the experiment. However, at least 39% of fish were normally pigmented in the DHA, DHA + EPA, and control groups. In the DHA + AA treatment, there was a significantly higher proportion of albinos ($P<0.05$) with only 8% of the fish normally pigmented on their ocular side.

Eye migration is presented as either complete (stage 3) or incomplete (0–2). In all treatments, the percentage of fish with complete eye migration averaged ~ 52%. The DHA + EPA treatment had the highest levels of complete eye migration at 75% ($P<0.05$). The percentage of fish that were settled on their right side was highest in the control larvae (Table 5).

3.3. Lipid class composition of larvae

Total lipids from larvae fed DHA and DHA + EPA diets increased from ~ 60 μg mg^{-1} dry weight at hatch to ~ 126 μg mg^{-1} at week 4, while larvae from the control and DHA + AA diets had only ~ 87 μg mg^{-1} at week 4 (Fig. 2a). TAG increased significantly in larvae fed the PUFA enriched diets, from hatch until the end of the rotifer stage (Fig. 2b). At weeks 3 and 4, larvae fed the high PUFA diets had significantly higher percentages of TAG (~ 18–22%, $P<0.05$) than larvae in the control group (11%). PL also increased in all treatments over the first 4 weeks post-

Table 5
Rearing success of yellowtail flounder fed differently enriched rotifers for the first 4 weeks post-hatch

Diet	Survival	Percentage normal pigmentation	Percentage complete eye migration	Percentage right-sided orientation
DHA	22.2 ± 0.8	47	47	61
DHA + EPA	12.3 ± 4.0	39	75[a]	73
DHA + AA	19.0 ± 3.4	8[a]	46	57
Control	5.2 ± 3.6	46	42	84

Data are the average of duplicate tanks ± SD.

[a] Represents a significant difference in the odds of having normal pigmentation or complete eye migration, G-test, Chi-square $df=3$.

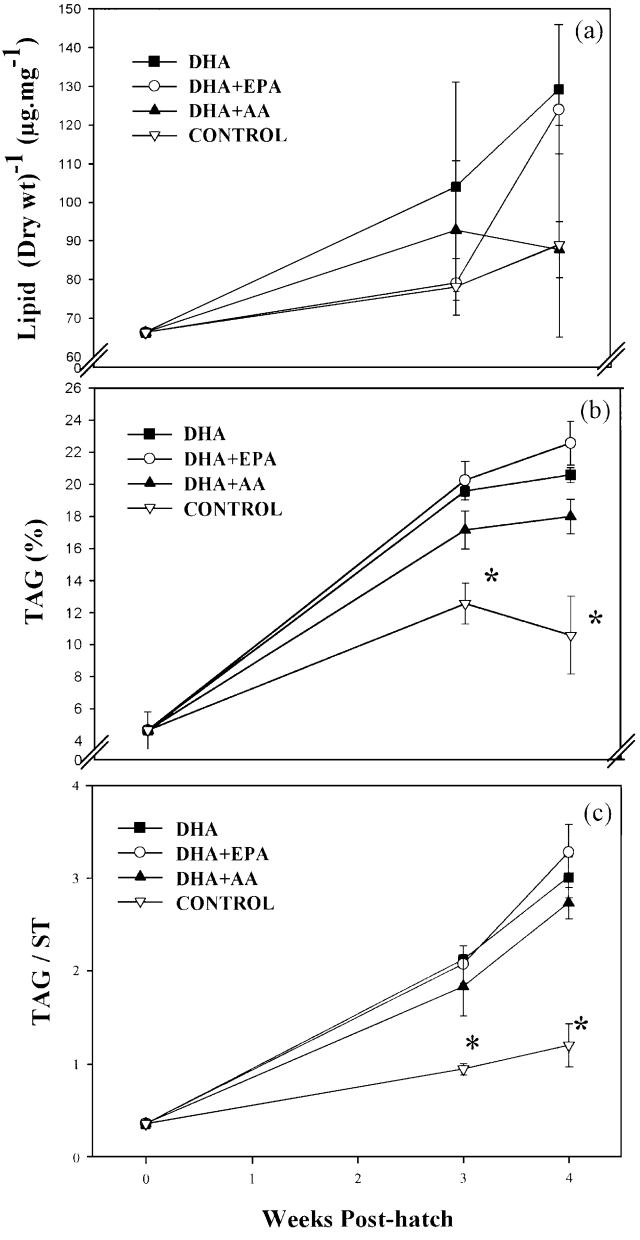

Fig. 2. Lipids in yellowtail flounder fed four types of differently enriched rotifers for the first 4 weeks post-hatch. Data are mean ± SEM ($n=6$). (a) Lipid (dry weight^{-1}); (b) TAG (%); (c) TAG/ST. * Represents a significant difference among dietary groups, ANOVA, Tukey's multiple comparison test ($P<0.05$, $F_{3,20}$).

hatch; however, there was no significant difference between dietary groups (~ 65% of total lipid).

The larval condition index TAG/ST increased in all treatments during the first 4 weeks from a low of 0.4 at hatching (Fig. 2c). Larvae in the high PUFA treatments showed higher condition index at weeks 3 and 4 than larvae in the control group (Fig. 2c, $P<0.05$).

3.4. Larval fatty acid composition

Larvae fed the DHA diet contained higher levels of DHA ($P<0.05$) than all other groups while larvae in the control group had significantly lower levels (Table 6). The DHA + EPA treatment had the highest proportions of EPA and larvae in the DHA + AA group had the highest level of AA ($P<0.05$). Larvae in the control treatment had the

Table 6
Fatty acid composition of larvae at hatch and after 4 weeks of feeding on differently enriched rotifers (mean ± SEM, $n=6$)

	100% hatch	DHA	DHA + EPA	DHA + AA	Control
Total fatty acids (μg mg^{-1} dry weight)		64.01 ± 13.00	60.6 ± 3.6	39.1 ± 0.7	48.1 ± 13.4
Percentage total fatty acids					
14:0	2.1 ± 0.3	4.1 ± 0.2^{a}	3.2 ± 0.3^{b}	3.4 ± 0.0^{b}	1.6 ± 0.4^{c}
16:0	21.3 ± 0.6	11.0 ± 0.2^{a}	11.3 ± 0.2^{a}	11.9 ± 0.2^{a,b}	12.7 ± 0.6^{b}
18:0	5.3 ± 2.1	4.9 ± 0.1^{a}	5.6 ± 0.1^{b}	5.4 ± 0.0^{a,b}	6.0 ± 0.3^{b}
ΣSFA[1]	30.8 ± 1.9	21.5 ± 0.6	21.9 ± 0.6	22.1 ± 0.3	22.1 ± 1.5
16:1$n-7$	3.9 ± 0.8	5.0 ± 0.1^{a}	5.8 ± 0.1^{b}	5.0 ± 0.1^{a}	6.2 ± 0.1^{b}
18:1$n-11$	9.9 ± 0.9	1.0 ± 0.2	1.4 ± 0.0	1.2 ± 0.0	1.1 ± 0.1
18:1$n-9$	4.7 ± 0.0	21.4 ± 0.5^{a}	18.9 ± 0.9^{b}	18.5 ± 0.6^{b}	26.5 ± 0.7^{c}
18:1$n-7$	0.9 ± 0.4	2.5 ± 0.1^{a}	2.9 ± 0.1^{b}	2.3 ± 0.1^{c}	3.3 ± 0.1^{d}
20:1$n-9$	0.3 ± 0.3	2.0 ± 0.1^{a}	2.2 ± 0.1^{a,b}	2.1 ± 0.0^{a}	2.5 ± 0.0^{b}
ΣMUFA[2]	20.6 ± 0.5	34.1 ± 1.2^{a}	33.8 ± 1.5^{a}	31.2 ± 1.0^{a}	42.1 ± 1.1^{b}
18:2$n-6$	1.2 ± 0.3	5.0 ± 0.2^{a}	4.8 ± 0.1^{a}	5.3 ± 0.2^{b}	10.8 ± 0.0^{c}
20:4$n-6$ (AA)	2.7 ± 0.1	2.2 ± 0.0^{a}	2.6 ± 0.2^{b}	8.0 ± 0.2^{c}	2.8 ± 0.1^{d}
20:5$n-3$ (EPA)	15.6 ± 1.9	4.8 ± 0.0^{a}	10.1 ± 0.4^{b}	4.6 ± 0.4^{a}	6.7 ± 0.0^{c}
22:5$n-3$	3.5 ± 0.4	2.0 ± 0.0^{a}	3.6 ± 0.1^{b}	2.1 ± 0.1^{a}	3.5 ± 0.1^{b}
22:6$n-3$ (DHA)	23.4 ± 0.3	27.1 ± 1.1^{a}	18.7 ± 1.0^{b}	22.3 ± 1.0^{c}	7.0 ± 0.2^{d}
ΣPUFA[3]	48.6 ± 2.4	44.4 ± 1.6^{a}	44.3 ± 1.9^{a}	46.7 ± 2.4^{a}	35.8 ± 0.9^{a}
DHA/EPA	1.5 ± 0.2	5.6 ± 0.2^{a}	1.9 ± 0.0^{b}	4.9 ± 0.6^{c}	1.1 ± 0.0^{d}
DHA/AA	8.8 ± 0.1	12.5 ± 0.5^{a}	7.2 ± 0.2^{b}	2.8 ± 0.1^{c}	2.5 ± 0.2^{d}
EPA/AA	5.9 ± 0.6	2.2 ± 0.0^{a}	3.9 ± 0.2^{b}	0.6 ± 0.1^{c}	2.4 ± 0.1^{d}

[a,b,c] Different letters represent a significant difference among dietary groups; $P<0.05$, $F_{3,20}$, one-way ANOVA with Tukey's multiple comparison test.

[1] Includes *ai*—15:0, 15:0, *i*—17:0, *ai*—17:0, 17:0, and 20:0.

[2] Includes 18:1$n-5$, 20:1$n-7$, 22:1$n-11$, 22:1$n-9$, and 24:1.

[3] Includes 16:2$n-4$, 16:3$n-4$, 16:4$n-3$, 18:3$n-3$, 18:4$n-3$, 20:2$n-6$, 20:3$n-6$, 20:4$n-3$, 22:4$n-6$, and 22:5$n-6$.

lowest proportions of DHA, EPA, and AA with a higher level of 18:2$n-6$ and 18:1$n-9$ ($P<0.05$, Table 6). The DHA/EPA ratio in larval total lipids was different between all dietary groups, with the DHA treatment having the highest and the control treatment the lowest (Table 6, $P<0.05$). Larval growth (body depth = 0.7–1.6 mm, $r^2=75.4\%$, $P=0.005$) and survival (survival = 5–22%, $r^2=86.5\%$, $P=0.001$) at week 4 was positively correlated with the dietary DHA/EPA ratio (Table 5).

Levels of dietary fatty acids affected PUFA retention in larval tissue (Fig. 3). Larvae in the high PUFA diets had fatty acid profiles that closely reflected dietary levels while in the control treatment, larvae showed preferential retention of PUFA and a decrease in 18:1$n-9$ compared to dietary levels (Fig. 3). All larvae had lower relative levels of 18:1$n-9$ in their bodies than in their diet. In all cases, larvae had higher levels of AA than were present in the diet and this was especially true of the control treatment which had 0.7% AA in the diet and 2.8% AA in the larvae. EPA was present in larval tissue in higher amounts than found in rotifers except for in the DHA + EPA treatment, which had approximately the same amount. The control larvae also showed higher levels of 22:5$n-3$ and DHA in their polar lipid (5.1% and 10.5% respectively) than was seen in control enriched rotifers (1.5% and 1.7%).

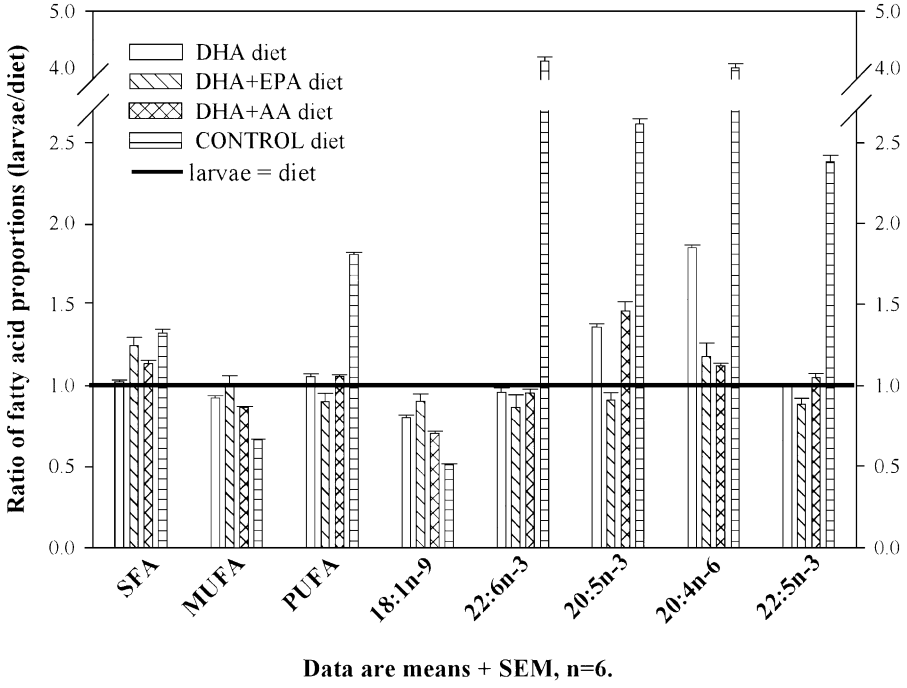

Fig. 3. Relative proportions of specific fatty acids in larvae compared to dietary levels after 4 weeks of feeding on differently enriched rotifers. Data are mean ± SEM, $n=6$. The solid bar indicates that the proportion of fatty acids in the larvae equals that of those in the diet.

4. Discussion

This is the first study to examine the effect of dietary lipid on the early growth and development of yellowtail flounder larvae. Diets were chosen to provide maximal information on not only the importance of DHA (22:6$n-3$) but also EPA (20:5$n-3$) and AA (20:4$n-6$).

4.1. Rotifer lipid composition

Rotifers enriched with the control emulsion (~ 70% 18:1$n-9$) had the same amount of PL but significantly higher levels of free fatty acid (FFA) than all other rotifer groups. This indicates that rotifers were able to hydrolyse the TAG from the emulsions but were unable to reassimilate such high levels of 18:1$n-9$ into their own membrane or storage lipids. Rainuzzo et al. (1994) demonstrated that rotifers fed wax ester emulsions were able to assimilate these fatty acids into their TAG lipid fraction. Oleic acid (18:1$n-9$) is commonly used as a control enrichment (Wantanabe, 1993; Watanabe and Kiron, 1994; Furuita et al., 1998); however, lipid class data for other rotifers enriched with high levels of this fatty acid are not available.

A challenge to providing live-food with proper enrichment is the loss of lipid that can occur in first-feeding tanks prior to larval consumption (McEvoy et al., 1998). Olsen et al. (1993) demonstrated that at temperatures <8 °C rotifers show essentially no loss of lipid for up to 4 days; however, this rate increased exponentially with temperatures above 8 °C. Further, Rainuzzo et al. (1989) found that at 10 °C rotifers lost only 10% of their $n-3$ fatty acids per day. In our study, temperatures during the rotifer-feeding period were <12 °C. Also, high water exchange in larval tanks (2-l min^{-1}) ensured that live-food did not remain for prolonged periods. Approximately 10 tank volumes were exchanged every 24 h and observations of prey levels showed that only 1.5% of the initial feed remained after just 2 h.

4.2. Larval growth and survival

After 4 weeks, larvae in the DHA diet were significantly larger while larvae in the control diet were significantly smaller than all other treatments. Rabe and Brown (2000) examined a pulse feeding strategy for rearing yellowtail flounder. They found that after 4 weeks of feeding on rotifers that were maintained on culture Selco and enriched with microalgae (*Isochrysis galbana*), larvae reached a standard length of ~ 9 mm. We found that larvae ranged between 7.3 and 9.7 mm in standard length after week 4, which demonstrates that our experimental emulsions produced growth comparable to trials using more typical enrichment methods.

Trends in survival reflected those seen in growth, with larval survival in the DHA treatment the highest (~ 22%) and those in the control treatment the lowest (~ 5%). Survival is often low and variable for new cold water aquaculture species. Rabe and Brown (2000) reported survival for yellowtail flounder during the first 2 weeks post-hatch to range between 1.4% and 17.3% and survival from weeks 2 to 7 ranged from 1.5% to 13.8%. These studies represent findings for larvae that were reared in

small containers (30–300 l), and it is probable that higher survival would easily be achieved using larger first-feeding tanks.

A positive correlation between dietary DHA/EPA and larval growth and survival was found in this study. Rodriguez et al. (1997) investigated the effect of dietary ratios of DHA/EPA on early development of larval gilthead seabream. They used DHA/EPA ratios in the range 1.4:1–0.3:1 and found a significant positive relationship between the DHA/EPA ratio in the larval polar lipids and larval total length after 2 weeks. Furuita et al. (1999) examined the effect of EPA and DHA on the growth, survival and salinity tolerance of Japanese flounder larvae. They found no difference in growth in relation to EPA and DHA. However, larvae receiving the high DHA diet demonstrated better 'vitality' after exposure to stress tests (high salinity water: 65‰). Estevez et al. (1999) also tested a range of DHA/EPA ratios (0.1–3.1) on the early development of turbot larvae and found that there was no significant difference between dietary groups in either growth or survival.

The biological significance of dietary DHA/EPA can be viewed in terms of competitive interactions between fatty acids for incorporation into phospholipids, specifically competition for the enzymes that esterify fatty acids onto the glycerophospho-base structure (Sargent et al., 1999). Previous studies have shown that high levels of di-22:6$n-3$ species are present in the eyes and brains of Atlantic cod, European sea bass, turbot and herring (Bell and Dick, 1991; Mourente et al., 1991; Bell et al., 1995, 1996). Diets deficient in DHA have been shown to change the fatty acid composition of neural tissue and decrease foraging efficiency at low light intensities in juvenile herring (Bell et al., 1995). Yellowtail flounder larvae are also visual predators and it is therefore probable that a dietary DHA deficiency could affect their early foraging behaviour and thus growth and survival.

Lipid composition of eggs/yolk has been suggested as an indicator for determining the nutritional requirements of newly hatched larvae. Typically a dietary DHA/EPA ratio of 2:1 is found in marine species and has thus been suggested as adequate for larval feeding (Tocher and Sargent, 1984; Sargent, 1995; Sargent et al., 1999). However, analysis of newly hatched larvae here revealed a DHA/EPA ratio of 1.5:1. These eggs were collected from fish that were captured only a week prior to spawning and, therefore, the egg composition should reflect a wild broodstock diet. There is no previously published data on the lipid composition of yellowtail flounder eggs and larvae. Still, for other marine species, evidence points to a connection between lipid composition and egg and early larval survival. Navas et al. (1997) reported that decreased TAG and increased $n-3$ fatty acids were correlated with higher egg quality in European sea bass. Similarly, Zhu (1998) found that decreased TAG and increased EPA were associated with better fertilisation success in Atlantic halibut.

This is the first study to demonstrate a relationship between dietary DHA/EPA and increased growth and survival using a ratio as high as 8:1. The elevated DHA used here was achieved by utilising a speciality TAG oil. This oil was produced by a heterotrophic dinoflagellate, *Crypthecodinium cohnii*, which contains DHA as the sole HUFA (Martek BioSci.). The majority of studies in larval nutrition use emulsions based on fish oils that typically have a DHA/EPA ratio of $<2:1$; with tuna orbital oil providing the highest ratio at $\sim 3.6:1$ (McEvoy et al., 1996; Estevez et al., 1999). This relationship between

unusually high dietary DHA/EPA and increased rearing success is new and calls into question the theory of 'nature knows best'. Further studies using novel DHA enrichment products, similar to that used here, may show this relationship in other marine species.

4.3. Pigmentation

There were high levels of malpigmention in all dietary treatments. However, larvae that received high levels of dietary AA during the first 4 weeks post-hatch showed a significantly lower proportion of normally pigmented fish (8%) than all other dietary groups (>39%). Although pigmentation can only be categorised after metamorphosis, the factors that determine pigmentation are influential during the larval phase. These results indicate that the sensitive period for determination of pigmentation in yellowtail flounder is within the first 4 weeks post-hatch. Flexion of the notochord (45° upward) and an increase in body depth relative to standard length both occurred during week 4. Both of these observations indicate that week 4 represented the onset of metamorphosis. At week 6, the beginning of eye migration and the first incidence of settling behaviour were observed.

Seikai et al. (1987) estimated the larval stage at which nutrition had an impact on later pigmentation in Japanese flounder. Live-feed known to induce albinism (*Artemia* nauplii and rotifers) or normal pigmentation (copepods and rotifers) were fed in different sequences in order to deduce the 'pigmentation window'. This critical period was found to occur at about 8-mm standard length during the premetamorphic period when larvae began to increase in body depth. Naess and Lie (1998) conducted a similar experiment with Atlantic halibut and also found that the sensitive period was the initial stage of metamorphosis: standard length 16 mm and dry weight 2.8 mg. In agreement with this, the 'pigmentation window' for yellowtail flounder also occurs during the premetamorphic stage, standard length <9 mm and body depth <1.6 mm.

Relatively little is known about the significance of AA on early growth and development of larval fish and the existing data point to species-specific and age-specific requirements. Here, AA had no positive effect on growth or survival and had a negative effect on pigmentation, when included at 0.8% dietary dry weight (7% of total fatty acids). Castell et al. (1994) found that AA had growth promoting effects in juvenile turbot when included at between 0.3% and 1.0% of dietary dry weight. Bessonart et al. (1999) also found that gilthead sea bream showed increased growth and survival when AA was included at between 1.0% and 1.8% of their dietary dry weight. However, this effect was masked when the DHA/EPA ratio was altered.

McEvoy et al. (1998) and Estevez et al. (1999) investigated the effects of EPA and AA on pigmentation in Atlantic halibut and turbot. In both these studies, high levels of DHA (6–12%) were present in all diets and the EPA/AA ratio was varied. It was concluded that given adequate DHA, pigmentation was dependent on AA and not EPA. A high incidence of malpigmentation was found in turbot larvae that received diets containing EPA/AA ratios of 1.4:1 and 0.3:1. In the present study, the DHA + AA diet also produced high levels of malpigmentation and the EPA/AA ratio was 0.5:1. Estevez et al. (1999) noted that malpigmentation occurred when levels of AA and EPA increased and decreased, respectively, in the phosphatidylinositol (PI) fraction of the brain. Given

that AA is the preferred dietary precursor for the production of eicosanoids (Bell et al., 1994), elevated albinism was hypothesised to result from the effects of increased brain eicosanoid production on the nervous and endocrine functions during metamorphosis.

Sargent et al. (1999) hypothesised that increased levels of dietary AA may cause a generalized state of stress due to elevated eicosanoid production. In the present study, larval survival was estimated during week 4, and this process involved vigorous aeration and stirring of tank water. Therefore, it is possible that this stressful event may have influenced pigmentation in all treatments. It is likely that stressful activities such as enumeration or transferring of larvae should be avoided until well after the 'pigmentation window'. If high levels of malpigmentation can be attributed to stress, then improvements in pigmentation of yellowtail flounder may be achieved using lower stocking densities, lower water exchange (turbulence), larger tanks, lower light intensity, increased tank algae concentrations and antibiotic baths.

4.4. Larval lipid composition

Lipid class analysis showed that larvae fed the control treatment had significantly lower levels of TAG and TAG/ST ratios than larvae in all other treatments. Relative improvements in larval condition in other species, such as herring and Atlantic cod have been attributed to elevated total lipid, TAG per dry weight and TAG/ST ratios (Fraser, 1989; Lochman et al., 1995).

Yellowtail larvae fed the high PUFA diets had fatty acid compositions that generally reflected dietary proportions while larvae fed the control diet had elevated levels of PUFA and reduced amounts of 18:1$n-9$ compared to dietary levels. The high proportions of AA ($\sim 4\times$), DHA ($\sim 4\times$), and EPA ($\sim 2.5\times$) show evidence for preferential retention of these fatty acids and point to their dietary essentiality while high levels of 22:5$n-3$ ($\sim 2.5\times$) may represent evidence for chain elongation. Larvae in the PUFA-enriched treatments (DHA present as 22–28% of dietary fatty acids) did not show any preferential retention of DHA compared to dietary amounts, indicating that the requirement for DHA may be lower than 22%. Interestingly, all treatments except for the DHA+EPA had higher amounts of EPA in the larvae than in the diet. This could indicate that EPA is not needed at the high concentrations found in the DHA+EPA enrichment (11%). Similarly, AA was also conserved in all groups but to a lesser extent in larvae receiving a diet containing higher levels of AA. Curiously, larvae fed the DHA-enriched diet had higher incorporation for AA ($\sim 1.8\times$) than larvae fed the DHA+EPA ($\sim 1.2\times$)-enriched diet, which could indicate a competitive interaction between EPA and AA for incorporation into phospholipids.

Elevated levels of specific fatty acids in larval tissue compared to dietary amounts have been referred to as incorporation efficiency (Castell et al., 1994). However, it is difficult to differentiate whether this elevation is due to preferential retention/utilisation or chain elongation/desaturation. The ability of larval yellowtail flounder to desaturate and elongate 18:3$n-3$ or EPA to DHA has not yet been investigated. However, conversion of 18:3$n-3$ to DHA has been shown to be very limited in studies on brain cell cultures from juvenile turbot (Tocher et al., 1992; Tocher, 1993). Further, when juvenile herring were fed a pelleted diet devoid of 22:5$n-3$, DHA and low in EPA,

there was incorporation of 22:5$n-3$ in the eye phospholipid with no increase in DHA after 4 weeks (Bell et al., 1995). This indicates that herring possess a limited ability to chain elongate 18:3$n-3$ and EPA to 22:5$n-3$ but cannot complete the final desaturation step to form DHA. Based on these results, it is probable that yellowtail flounder have very low levels of desaturase activity and are dependent on preformed dietary DHA.

5. Conclusions

Dietary PUFA have a pronounced effect on early growth, survival, and pigmentation in yellowtail flounder larvae. High dietary levels of DHA relative to EPA had growth-promoting effects and this relationship was observed over a wide range of DHA/EPA ratios (1:1 to 8:1). Elevated dietary AA resulted in an increase in malpigmentation and, therefore, the 'pigmentation window' was concluded to be within the first 4 weeks post hatch. Further investigations into the role of husbandry-induced stress on pigmentation are recommended as high levels of malpigmentation were observed in all treatments. Enrichment of live-food with DHA is essential for this species; however, further studies are needed to confirm whether enrichment with EPA and AA is necessary. Based on the incorporation efficiency of PUFA into larval tissue and on the relative amounts of PUFA in the neutral and polar lipid fractions, a ratio of DHA/EPA/AA of 10:1.5:1 could be used as a starting point for further investigations into the dietary requirements of yellowtail flounder larvae.

Acknowledgements

We are grateful to Olav Lingstad and Danny Boyce for help with the design of both larval fish and live-food rearing systems. Dr. Suzanne Budge and Jeanette Wells provided valuable technical assistance with lipid analysis. We thank the live-food technicians Kim Fifield, Dena Wisemen and Tracy Granter for culturing unenriched rotifers and microalgae. Both NSERC and a Special Resource Scholarship from the provincial government of Newfoundland awarded to L.A. Copeman provided financial support for this study.

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