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Growth and Fatty Acid Composition of Juvenile Olive Flounder *Paralichthys olivaceus* Fed Diets Containing Different Levels and Ratios of Eicosapentaenoic Acid and Docosahexaenoic Acid

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Abstract

This study was carried out to investigate the influences of dietary levels, ratios and sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the growth and fatty acid compositions of juvenile olive flounder *Paralichthys olivaceus*. Sixteen diets containing five levels of EPA (0.5%, 1.0%, 1.5%, 2.0%, and 4.0%), five levels of DHA (0.5%, 1.0%, 1.5%, 2.0% and 4.0%), three ratios of EPA/DHA (75/25, 50/50 and 25/75), two levels of squid liver oil (5% and 10%) and a control diet containing 5% soybean oil were hand-fed to triplicate groups of fish (average weight, 9.7 ± 0.3 g) for 8 weeks. Survival, specific growth rate, feed efficiency and protein efficiency ratio of fish were not affected by dietary EPA and DHA levels or ratios. Also, the dietary treatment had no significant effect on the lipid and protein contents of muscle and whole body of fish. A corresponding increase in the EPA and DHA contents of fish occurred with increasing EPA and DHA levels in their diets. Our results suggest that juvenile olive flounder require a dietary EPA level of approximately 0.32% in the presence of 0.74% DHA for suitable survival and growth, and that EPA and DHA levels in fish muscle can increase to as much as 32% and 53%, respectively, of the total fatty acid content.

Key words: *Paralichthys olivaceus*, Dietary EPA and DHA level, EPA/DHA ratio

Introduction

The *n*-3 highly unsaturated fatty acids (*n*-3 HUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are essential fatty acids for normal growth, development and reproduction of marine fish (Furuita et al., 2002; Bell and Dick, 2004). HUFAs generally cannot be synthesized by marine fish and must be supplied in their diet (Izquierdo et al., 1989; Tocher, 2003; Turchini et al., 2007). The *n*-3 HUFA requirements of fish vary according to species and growth stage, and are also influenced by certain external factors such as seasonal changes and environmental conditions (Olsson et al., 2003; Sharma et al., 2010). Determining which HUFAs are essential for the normal growth and development of fish is an important step in the commercial viability of aquaculture for most fish. For larval flounder at the *Artemia* feeding stage, Izquierdo et al. (1992) stated that the *n*-3 HUFA requirement was 3.0-3.5%, while Furuita et al. (1999) reported that the DHA requirement was approximately 1.6% in the presence of 1.0% EPA. Meanwhile, Kim and Lee (2004) found that the *n*-3 HUFA requirement for juvenile flounder was approximately 0.8-1.0%. However, the influences of dietary EPA or DHA levels and ratios on juvenile development have not yet been studied.

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The relative proportions of EPA and DHA are equally important to the total *n*-3 HUFAs content when the different physiological roles of these two fatty acids are considered (Watanabe et al., 1989; Ibeas et al., 1997; Zuo et al., 2012). Bell et al. (1985) found that increasing the ratio of DHA to EPA from 0.1 to 0.5 in the diet of turbot juveniles remarkably increased the survival rate. An optimal 2:1 ratio of DHA to EPA was suggested for the diet of sea bass *Dicentrarchus labrax* larvae (Sargent et al., 1999). The optimal ratios of DHA and EPA vary for different stages, species and living environments (Estévez et al., 1999; Harel et al., 2001; Hamre and Harboe, 2008). The purpose of this study was to determine the impact of dietary levels, ratios and sources of EPA and DHA on growth performance, body composition and the fatty acid profile of juvenile olive flounder.

Materials and Methods

Experimental diets

Ingredients and proximate compositions of the experimental diets are presented in Table 1. Fish meal and casein were used as the main protein sources and wheat flour was applied as the primary carbohydrate source. Sixteen experimental diets were prepared, which contained five levels of pure EPA (0.5%, 1.0%, 1.5%, 2.0%, and 4.0% of dry matter) and DHA (0.5%, 1.0%, 1.5%, 2.0%, and 4.0% of dry matter), three ratios of EPA/DHA (75/25, 50/50, and 25/75), two levels of squid liver oil (5% and 10%) and a control diet containing 5% soybean oil. Crude protein and lipid levels of the experimental diets were maintained at 50% and 6.5%, respectively, based on the results of a previous study (Lee at al., 2000). The contents of *n*-3 HUFAs in the diets were maintained above 1.0%, according to the results of Kim and Lee (2004). The experimental ingredients (100 g) were mixed with water (40 g), pelletized with a laboratory pellet machine, and then dried overnight at room temperature. All diets were stored at –30°C until use. The fatty acid compositions of the experimental diets are presented in Table 2.

Experimental fish and feeding trial

Juvenile olive flounder were obtained from a local farm in Taean, Korea. Fish were transported to the experimental facilities, acclimated to the experimental conditions, and fed with a commercial diet for 2 weeks prior to the start of the feeding trial. Juveniles (average weight, 9.7 ± 0.3 g) were randomly distributed into 48 tanks (50 L water volume) at a density of 20 fish per tank. Three replicate groups of fish were hand-fed to apparent satiation twice a day (09:00 and 17:00 for 6 days per week) for 8 weeks. Water temperature was 16.7 ± 1.8°C and the photoperiod followed natural conditions during the feeding trial. Records were kept of the daily feed consumption, mortalities and feeding behavior of each tank.

Sample collection and chemical analysis

At the end of the feeding trial, all of the fish in each tank were collectively weighed after anesthetizing with tricaine methane sulfonate (MS222; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 ppm after starvation for 24 hours. Total length, body weight, liver weight and intestine weight of five fish from each tank were measured. Five fish from each tank were taken and pooled to determine whole body composition. The dorsal muscle and liver of 10 fish from each tank were removed and stored at –75°C for subsequent proximate analysis. The crude protein content was determined using the Kjeldahl method with the AutoKjeldahl System (Buchi, Flawil, Switzerland). The crude lipid content was determined by the ether-extraction method using a Soxhlet extractor (VELP Scientifica, Milano, Italy). The moisture content was determined with a dry oven (105°C for 6 h), and the ash content was determined using a muffler furnace (600°C for 4 h). Lipid for fatty acid analyses was extracted with a mixture of chloroform and methanol (2:l, v/v) according to the methods described by Folch et al. (1957), and fatty acid methyl esters were prepared by trans-esterification with 14% BF_3-MeOH (Sigma-Aldrich). Fatty acid methyl esters were analyzed using a gas chromatograph (Clarus 600; PerkinElmer, Shelton, CT, USA) with a flame ionization detector, equipped with an SP-2560 capillary column (L × I.D. 100 m × 0.25 mm; film thickness 0.20 μm; Supelco, Bellefonte, PA, USA). Injector and detector temperatures were both 240°C. The column temperature was programmed from 140°C to 240°C at a rate of 5°C/min. Helium was used as the carrier gas. Fatty acids were identified by comparison with retention times of the standard fatty acid methyl esters (PUFA 37 component FAME Mix; Supelco).

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Significant differences ($P < 0.05$) among the means were determined using a Duncan's multiple range test (Duncan, 1955).

Results

The growth performances of fish fed with the experimental diets for 8 weeks are shown in Table 3. Increasing the EPA and DHA levels in diets up to 4.0% had no significant effect on survival and growth performance of the juvenile olive flounder. Also, no significant differences were found in the specific growth rate, feed efficiency, protein efficiency ratio, or daily feed intake as dietary EPA/DHA was increased from 25% to

Table1. Ingredients and nutrient of the experimental diets

	Diets															
	CON	EPA5	EPA10	EPA15	EPA20	EPA40	DHA5	DHA10	DHA15	DHA20	DHA40	E75/D25	E50/D50	E25/D75	SLO5	SLO10
Ingredients (%)																
Casein	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Mackerel fish meal*	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Wheat flour	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9
Dehulled soybean meal	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Dextrin	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	10.0
Wheat gluten	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Squid liver oil															5	10
Soybean oil	5.0	4.47	3.93	3.39	2.85	0.7	4.39	3.79	3.19	2.59	0.18	3.91	3.9	3.89		
EPA (93%)		0.53	1.07	1.61	2.15	4.3						0.85	0.55	0.25		
DHA (83%)							0.61	1.21	1.81	2.41	4.82	0.24	0.55	0.86		
Vitamin premix†	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix‡	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Choline salt (50%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Proximate composition (%, dry matter basis)																
Crude protein	49.2	50.3	51.0	51.0	50.4	49.0	50.7	49.7	50.7	50.0	50.0	50.4	49.6	51.1	50.9	51.0
Crude lipid	6.9	6.6	5.8	6.6	6.4	5.9	7.4	7.0	7.3	6.5	5.9	6.3	6.4	6.6	6.8	12.5
Ash	6.9	6.8	4.9	7.0	6.7	7.1	7.0	6.9	6.9	7.0	6.8	6.9	6.8	7.0	6.7	6.9

CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

*Imported from Chile, †Vitamin premix contained the following amount which were diluted in cellulose (g/kg premix): L-ascorbic acid, 121.2; DL-α-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003, ‡Mineral premix contained the following ingredients (g/kg premix): MgSO4·7H_2O, 80.0; NaH_2PO_4·2H_2O, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4$·7H_2O, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$·6H_2O, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4$·H_2O, 2.0; $CoCl_2$·6H_2O, 1.0.

Table 2. Major fatty acids composition (% of total fatty acids) of the experimental diets

	Diets															
	CON	EPA5	EPA10	EPA15	EPA20	EPA40	DHA5	DHA10	DHA15	DHA20	DHA40	E75/D25	E50/D50	E25/D75	SLO5	SLO10
Fatty acids (% of total fatty acids)																
C14:0	0.9	0.7	0.6	0.5	0.5	0.5	0.7	0.6	0.6	0.5	0.4	0.6	0.6	0.6	2.2	1.8
C16:0	17.0	15.0	12.0	10.4	9.0	6.0	13.0	13.0	11.0	9.1	6.0	12.	12.	12.5	22.0	18.0
C16:1	0.7	0.5	0.4	0.4	0.4	0.4	0.5	0.5	0.5	0.4	0.3	0.4	0.5	0.5	2.2	2.1
C17:1	0.3	0.2	0.2	0.4	0.2	0.1	0.3	0.2	0.1	0.3	0.1	0.2	0.3	0.28	0.7	0.8
C18:0	0.2	0.1	0.1	0.5	0.14	0.10	0.2	0.1	0.1	0.14	0.07	0.1	0.2	0.1	0.3	0.6
C18:1*n*-9	16.5	12.0	10.0	8.3	7.5	5.0	11.3	10.3	9.0	7.0	4.2	9.8	10.1	10.0	17.0	14.6
C18:2*n*-6	40.8	32.3	27.4	22.4	20.0	12.1	33.7	27.0	23.7	17.7	10.0	26.5	27.2	27.3	14.0	12.0
C18:3*n*-3	4.2	3.0	2.4	2.0	1.8	1.0	3.0	2.3	2.1	1.5	0.8	2.2	2.4	2.4	2.8	2.0
C20:2	0.1	0.2	0.3	0.3	0.4	0.2	0.2	0.1	0.14	0.08	0.14	0.2	0.16	0.17	0.6	2.8
C22:2	1.0	1.7	2.1	2.3	2.5	0.4	1.4	1.2	1.2	1.2	1.2	1.8	1.6	1.3	1.4	1.1
C20:5*n*-3	5.2	22.2	34.4	44.0	50.2	69.2	5.6	4.3	5.0	4.6	5.0	28.0	19.3	12.0	10.0	11.0
C22:5*n*-3	1.0	0.9	0.2	0.2	0.2	0.17	3.3	2.2	2.8	3.5	4.4	1.2	1.4	1.8	1.8	1.4
C22:6*n*-3	12.0	11.5	9.3	7.9	7.0	4.6	26.7	38.2	43.9	53.8	67.4	16.4	23.7	31.1	25.1	32.0
Fatty acids (% in diet, dry matter basis)*																
EPA	0.32	1.31	1.78	2.59	2.87	3.64	0.37	0.27	0.32	0.27	0.26	1.57	1.10	0.70	0.59	1.22
DHA	0.74	0.67	0.48	0.46	0.40	0.24	1.76	2.39	2.86	3.12	3.55	0.92	1.35	1.83	1.52	3.57
EPA + DHA	1.06	1.98	2.26	3.05	3.27	3.88	2.13	2.66	3.18	3.39	3.81	2.49	2.45	2.53	2.11	4.79

CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

*Fatty acids (% in diet), calculated (dietary total lipid × area % × 0.892) (Yoshimatsu et al. 1997).

Table 3. Growth performance and feed utilization of juvenile olive flounder fed the experimental diets for 8 weeks

Diets	IAW (g)	SUR (%)	SGR (%)*	FE (%)†	DFI (%)‡	PER (%)§
CON	9.7 ± 0.1ns	85.0 ± 2.9ns	2.2 ± 0.1ns	107 ± 5.7ns	1.7 ± 0.1ns	2.2 ± 0.1ns
EPA5	9.9 ± 0.3	93.3 ± 4.4	2.2 ± 0.1	110 ± 4.4	1.7 ± 0.1	2.2 ± 0.1
EPA10	9.6 ± 0.1	93.3 ± 4.4	2.3 ± 0.1	109 ± 4.0	1.8 ± 0.1	2.1 ± 0.1
EPA15	10.0 ± 0.5	96.7 ± 3.3	2.2 ± 0.2	106 ± 0.4	1.8 ± 0.1	2.1 ± 0.1
EPA20	9.7 ± 0.3	98.3 ± 1.7	2.1 ± 0.1	111 ± 2.2	1.7 ± 0.1	2.2 ± 0.1
EPA40	9.8 ± 0.2	88.3 ± 4.4	2.3 ± 0.2	99 ± 9.4	1.8 ± 0.1	2.0 ± 0.2
DHA5	9.6 ± 0.2	96.7 ± 1.7	2.0 ± 0.2	106 ± 3.9	1.7 ± 0.1	2.1 ± 0.1
DHA10	9.7 ± 0.5	93.3 ± 3.3	2.2 ± 0.2	109 ± 3.4	1.7 ± 0.1	2.2 ± 0.1
DHA15	9.9 ± 0.4	91.7 ± 4.4	2.1 ± 0.1	108 ± 2.4	1.7 ± 0.1	2.1 ± 0.1
DHA20	9.8 ± 0.3	95.0 ± 2.9	2.1 ± 0.1	102 ± 7.3	1.8 ± 0.2	2.1 ± 0.1
DHA40	9.4 ± 0.2	93.3 ± 4.4	2.0 ± 0.3	105 ± 15.3	1.7 ± 0.1	2.1 ± 0.3
E75/D25	9.7 ± 0.4	78.3 ± 9.3	2.3 ± 0.4	96 ± 11.7	1.8 ± 0.1	1.9 ± 0.2
E50/D50	9.6 ± 0.2	91.7 ± 4.4	2.2 ± 0.2	113 ± 9.1	1.6 ± 0.1	2.3 ± 0.2
E25/D75	9.9 ± 0.3	96.7 ± 1.7	2.0 ± 0.1	110 ± 4.3	1.7 ± 0.1	2.1 ± 0.1
SLO5	9.7 ± 0.5	88.3 ± 4.4	2.2 ± 0.3	105 ± 4.3	1.7 ± 0.1	2.1 ± 0.1
SLO10	9.6 ± 0.1	95.0 ± 2.9	2.1 ± 0.1	112 ± 3.4	1.7 ± 0.1	2.2 ± 0.1

Values are presented as means ± SE of three replication.
CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, values are not significant ($P > 0.05$); IAW, initial weight; SUR, survival.
*Specific growth rate (SGR) = (ln (final weight) - ln (initial weight)) × 100/days of feeding, †Feed efficiency (FE) = wet weight gain × 100/feed intake, ‡Daily feed intake (DFI) = feed intake × 100/[(initial weight + final weight + dead fish weight) × days reared/2], §Protein efficiency ratio (PER) = (wet weight gain/protein intake) × 100.

Table 4. Morphological parameters of juvenile olive flounder fed the experimental diets for 8 weeks

Diets	Condition factor*	Hepatosomatic index†	Viscerasomatic index‡
CON	1.0 ± 0.03ns	2.8 ± 0.4ns	3.7 ± 0.3ns
EPA5	1.0 ± 0.01	2.8 ± 0.2	3.5 ± 0.2
EPA10	1.0 ± 0.02	2.8 ± 0.1	3.3 ± 0.1
EPA15	1.1 ± 0.05	2.7 ± 0.3	2.7 ± 0.5
EPA20	0.9 ± 0.07	2.5 ± 0.3	2.8 ± 0.4
EPA40	1.0 ± 0.05	2.5 ± 0.4	3.3 ± 0.2
DHA5	1.0 ± 0.04	2.7 ± 0.4	3.3 ± 0.2
DHA10	1.0 ± 0.01	2.6 ± 0.2	3.4 ± 0.2
DHA15	1.0 ± 0.03	2.4 ± 0.3	3.1 ± 0.3
DHA20	1.1 ± 0.07	2.5 ± 0.2	3.4 ± 0.1
DHA40	0.9 ± 0.02	2.1 ± 0.1	4.0 ± 0.3
E75/D25	1.0 ± 0.04	2.4 ± 0.1	3.2 ± 0.3
E50/D50	1.0 ± 0.03	2.8 ± 0.3	3.3 ± 0.3
E25/D75	1.0 ± 0.02	2.5 ± 0.3	3.5 ± 0.1
SLO5	0.9 ± 0.03	3.2 ± 0.2	3.7 ± 0.2
SLO10	1.0 ± 0.03	3.6 ± 0.4	3.8 ± 0.2

Values are presented as means ± SE of three replication.
CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, values are not significant ($P > 0.05$).
*[fish weight (g)/fish length (cm)^3] × 100, †(liver weight/body weight) × 100, ‡(viscera weight/body weight) × 100.

75% ($P > 0.05$). Morphological parameters such as the condition factor, hepatosomatic index and viscerasomatic index of experimental fish did not change significantly in response to changes in dietary EPA or DHA levels or ratios (Table 4). The moisture, crude lipid and ash contents of whole body and dorsal muscle of fish were not significantly affected by dietary EPA and DHA levels and ratios (Table 5). A remarkable increase was detected in the EPA and DHA contents of fish with increasing EPA and DHA levels in the diet, and the highest content of EPA was observed in fish fed 4.0% EPA diet. Similarly, the highest content of DHA was observed in fish fed the 4.0% DHA diet. In our experiment, no significant effect of additive ratios of EPA and DHA was observed on the survival or growth of juvenile olive flounder. The ratios of EPA/DHA in fish tissues clearly showed a similar trend to the dietary composition. Fatty acid compositions of dorsal muscle, liver and whole body in juvenile olive flounder are presented in Tables 6, 7, and 8, respectively. The fatty acid compositions of liver, dorsal muscle and whole body of the fish well reflected the fatty acid compositions of the dietary lipids. However, DHA concentration in muscle was higher than its respective concentration in the diet. Saturated fatty acids, such as the 14:0 and 16:0 contents in dorsal muscle, liver and whole body of fish fed the diet containing squid liver oil were significantly higher than those of the fish fed the other diets. Fish fed the control diet showed the highest content of 18:2*n*-6 in muscle, liver and whole body and the content of 18:2*n*-6 in fish tended to decrease with increasing EPA and DHA levels in the diets.

Discussion

The results of the present study indicate that different levels of neither EPA nor DHA had significant effects on the survival, growth performance, morphological parameters, or body composition of juvenile olive flounder. These results indicate that juvenile olive flounder can tolerate a wide range (1.06-4.79%) of EPA and DHA variation in their diet. Similar results have been obtained from previous studies; olive flounder larvae were able to grow and survive past metamorphosis on *Artemia metanauplii* with very low DHA content (0.1% of the total fatty acids) (Izquierdo et al., 1992). Also, a low EPA + DHA content (1.06%) in the control diet supplemented by soybean oil seems to meet the *n*-3 HUFA requirements of juvenile olive flounder. In a previous study by Kim and Lee (2004), the *n*-3 HUFA requirement for juvenile olive flounder was reported at 0.8-1.0%. For juveniles of several species, including the red seabream *Pagrus major*, European sea bass *Dicentrarchus labrax* L., red drum *Sciaenops ocellatus* L., and rockfish *Sebastes schlegeli*, the *n*-3 HUFA requirements can be met by contents ≤1% of the dry weight of the diet (Tocher, 2010). However, Furuita et al. (1999) noted that growth of olive flounder larvae was improved linearly by increasing *n*-3 HUFA in *Artemia* up to 3.8%. A lower value for juveniles compared to the larval stage was also reported for turbot (Gatesoupe et al., 1977). A possible explanation for these differences is that fish larvae grow much faster than the juveniles and therefore need more DHA and EPA for their vision and nervous development (Watanabe and Kiron, 1994). The growth performance of juvenile flounder investigated in this study was not affected when dietary *n*-3 HUFA levels became excessive. A similar phenomenon was also observed in other fish studies, such as rockfish (Lee, 2001) and starry flounder (Lee et al., 2003). However, negative effects of excessive *n*-3 HUFAs were observed in channel catfish (Stickney and Andrews, 1972), rainbow trout (Takeuchi and Watanabe, 1979), and flounder (Kim and Lee, 2004).

The different responses of fishes to excessive *n*-3 HUFAs in their diets may be due to differences in fish species, interactions with other dietary nutrients, or culture conditions (Lee, 2001; Østbye et al., 2011). The exact reason for the different responses in this study and the previous study by Kim and Lee (2004) is not clear, but it may be due to the differences in dietary lipid sources (defatted fishmeal and ethyl laurate vs. fishmeal and soybean oil as lipid sources), fatty acid compositions (*n*-3 HUFAs vs. EPA or DHA), or fish culture environments, *e.g.*, water temperatures (21.1°C vs. 16.7°C). Some evidence exists to indicate that natural lipid sources containing glycerol are better utilized by fish, compared to purified fatty acid in the form of ethyl esters (Ibeas et al., 2000). The results obtained by Rodríguez et al. (1994) showed that gilthead seabream larvae fed rotifers enriched on methyl esters of *n*-3 fatty acids displayed a much lower weight gain compared with larvae fed rotifers enriched with triacylglycerols of *n*-3 fatty acids. Poor growth was also observed in red seabream larvae when fed with rotifers (Izquierdo et al., 1989) or *Artemia* (Takeuchi et al., 1992) enriched on methyl esters of fatty acids. The poor bioavailability of ethyl esters may be related to the less effective increases in activity and gene expression of enzymes involved in fatty acid oxidation (Hong et al., 2003), or it may be caused by toxic methanol produced during the digestion of methyl esters (Ibeas et al., 2000). On the other hand, the optimum temperature for growth of olive flounder has been reported to be 20-25°C (Iwata et al., 1994). Low temperatures may lead to the solidification of saturated fatty acids, thus reducing membrane fluidity and disrupting membrane function.

Table 5. Chemical composition (%, wet weight basis) of the whole body and the dorsal muscle of juvenile olive founder fed the experimental diets for 8 weeks

Diets	Moisture	Crude protein	Crude lipid	Ash
Whole body				
CON	74.3 ± 0.60^{ns}	15.9 ± 0.20^{ns}	2.6 ± 0.08^{ns}	3.7 ± 0.27^{ns}
EPA5	74.3 ± 0.68	15.3 ± 0.36	2.9 ± 0.33	3.2 ± 0.13
EPA10	74.5 ± 0.33	16.1 ± 0.37	3.0 ± 0.43	3.4 ± 0.09
EPA15	75.9 ± 0.47	15.4 ± 0.04	2.5 ± 0.32	3.3 ± 0.14
EPA20	74.7 ± 0.71	15.6 ± 0.38	2.7 ± 0.33	3.3 ± 0.22
EPA40	74.5 ± 0.38	16.0 ± 0.11	3.4 ± 0.19	3.2 ± 0.27
DHA5	75.6 ± 0.36	15.5 ± 0.53	3.1 ± 0.14	3.3 ± 0.14
DHA10	75.5 ± 0.66	15.5 ± 0.16	2.5 ± 0.28	3.1 ± 0.03
DHA15	75.1 ± 0.80	15.4 ± 0.39	2.6 ± 0.06	3.3 ± 0.23
DHA20	75.2 ± 0.64	15.8 ± 0.25	3.3 ± 0.27	3.4 ± 0.19
DHA40	76.4 ± 0.50	15.2 ± 0.40	3.1 ± 0.27	3.4 ± 0.13
E75/D25	74.3 ± 0.47	15.5 ± 0.16	3.6 ± 0.39	3.3 ± 0.28
E50/D50	75.1 ± 0.32	15.4 ± 0.32	2.4 ± 0.07	3.3 ± 0.14
E25/D75	74.9 ± 0.41	15.5 ± 0.43	3.1 ± 0.15	3.3 ± 0.18
SLO5	74.8 ± 0.38	15.9 ± 0.18	3.0 ± 0.31	3.5 ± 0.10
SLO10	74.2 ± 0.61	15.8 ± 0.31	3.0 ± 0.38	3.1 ± 0.28
Dorsal muscle				
CON	77.2 ± 0.37^{ns}	18.8 ± 0.22^{ns}	0.7 ± 0.15^{ns}	1.3 ± 0.07^{ns}
EPA5	77.3 ± 0.32	18.5 ± 0.11	1.1 ± 0.41	1.3 ± 0.02
EPA10	76.3 ± 0.28	18.7 ± 0.23	0.9 ± 0.34	1.4 ± 0.02
EPA15	76.6 ± 0.53	20.3 ± 1.21	0.8 ± 0.07	1.5 ± 0.03
EPA20	77.0 ± 0.48	20.4 ± 1.04	0.6 ± 0.09	1.3 ± 0.04
EPA40	75.7 ± 0.26	19.6 ± 0.67	1.1 ± 0.49	1.3 ± 0.04
DHA5	76.3 ± 0.42	21.0 ± 0.68	0.5 ± 0.04	1.4 ± 0.07
DHA10	76.4 ± 0.62	20.2 ± 0.46	0.7 ± 0.11	1.4 ± 0.01
DHA15	78.4 ± 0.95	20.2 ± 0.45	1.0 ± 0.21	1.4 ± 0.13
DHA20	76.6 ± 0.34	19.5 ± 0.50	0.7 ± 0.14	1.4 ± 0.07
DHA40	77.2 ± 0.19	18.8 ± 0.35	0.7 ± 0.30	1.3 ± 0.02
E75/D25	77.1 ± 0.38	19.1 ± 0.84	1.0 ± 0.09	1.3 ± 0.02
E50/D50	76.7 ± 0.27	20.0 ± 1.62	1.1 ± 0.20	1.4 ± 0.02
E25/D75	77.0 ± 0.59	20.0 ± 0.17	0.7 ± 0.20	1.4 ± 0.01
SLO5	76.9 ± 0.66	18.4 ± 0.47	0.5 ± 0.09	1.4 ± 0.05
SLO10	77.2 ± 0.44	18.6 ± 0.11	0.7 ± 0.13	1.4 ± 0.01

Values are presented as means ± SE of three replication.
CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, values are not significant ($P > 0.05$).

Table 6. Major fatty acids composition (% of total fatty acids) of dorsal muscle in juvenile olive flounder fed experimental diet for 8 weeks

Fatty acids	Diets																
	CON	EPA5	EPA10	EPA15	EPA20	EPA40	DHA5	DHA10	DHA15	DHA20	DHA40	E75/D25	E50/D50	E25/D75	SLO5	SLO10	SEM*
C14:0	1.3^{ns}	1.2	1.2	1.3	1.3	1.2	1.2	1.2	1.3	1.2	1.3	1.3	1.2	1.1	1.3	1.3	0.02
C16:0	16.2^{a}	16.8^{abc}	17.6^{bcd}	17.0^{abc}	17.6^{bcd}	18.3^{d}	17.2^{abcd}	16.5^{ab}	16.2^{a}	16.4^{ab}	17.0^{abc}	17.2^{abcd}	17.3^{abcd}	17.9^{cd}	21.3^{e}	20.5^{e}	0.22
C16:1	1.0^{ns}	0.7	0.3	1.4	1.4	0.5	0.3	1.1	0.5	0.5	0.9	0.7	0.5	1.1	0.5	0.4	0.08
C17:1	0.1^{ns}	0.2	0.3	0.3	0.5	0.4	0.1	0.2	0.4	0.4	0.5	0.5	0.4	0.4	0.6	0.4	0.03
C18:0	0.5^{ns}	0.4	0.5	0.5	0.4	0.4	0.4	0.5	0.4	0.5	0.5	0.4	0.4	0.4	0.5	0.5	0.01
C18:1*n*-9	19.6^{h}	16.0^{cde}	16.8^{defg}	17.0	15.5^{cd}	13.6^{b}	17.1^{defg}	16.7^{defg}	16.1^{cdef}	15.0	11.0	17.2^{efg}	17.6^{fg}	15.6^{cd}	17.8^{g}	15.7^{cde}	0.29
C18:2*n*-6	38.7^{h}	35.3^{g}	30.5^{f}	26.8^{e}	23.6^{d}	14.4^{b}	30.8^{f}	28.8^{ef}	26.4^{e}	20.8^{c}	8.8^{a}	28.6^{ef}	27.6^{e}	27.6^{e}	9.5^{a}	8.0^{a}	1.33
C18:3*n*-3	3.4^{e}	3.2^{e}	2.6^{d}	2.6^{d}	2.2^{d}	1.3^{b}	2.7^{d}	2.5^{d}	2.2^{cd}	1.8^{bc}	0.9^{a}	2.6^{d}	2.2^{cd}	2.4^{d}	0.8^{a}	0.8^{a}	0.12
C20:2	0.6^{ns}	0.3	0.2	0.5	0.8	0.2	0.5	0.7	0.7	0.6	0.5	0.8	0.2	0.9	0.4	0.4	0.05
C20:3*n*-6	1.5^{ns}	1.5	1.4	1.3	1.4	1.4	1.6	1.5	1.4	1.4	1.3	1.5	1.4	1.5	1.4	1.4	0.02
C20:5*n*-3	3.3^{a}	9.8^{f}	12.0	15.6^{h}	19.5^{i}	31.5^{j}	5.3^{b}	3.1^{a}	3.3^{a}	3.3^{a}	3.7^{a}	9.1^{e}	7.0^{c}	5.3^{b}	8.4^{d}	8.2^{d}	1.07
C22:5	0.4^{ns}	0.5	0.5	0.4	0.4	0.5	0.6	0.4	0.5	0.4	0.6	0.6	0.5	0.5	0.8	0.7	0.03
C22:6*n*-3	13.0	13.4^{ab}	16.0^{b}	14.8^{ab}	15.0	15.8^{b}	21.8^{d}	26.3^{f}	30.4^{g}	38.0	53.0	19.1^{c}	23.7^{de}	25.8^{ef}	35.2^{h}	40.7^{j}	1.65
EPA+DHA	16.4^{a}	23.2^{b}	28.0^{cd}	30.5^{de}	34.5^{f}	47.5^{h}	27.2^{c}	29.6^{cde}	33.8^{f}	41.3^{g}	56.6^{i}	28.4^{cde}	30.8^{de}	31.2^{e}	43.8^{g}	49.1^{h}	1.50

Values (means ± SE of three replication) in the same row not sharing a common superscript are significantly different ($P < 0.05$).
CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, values are not significant ($P > 0.05$).
*Standard error of the treatment mean calculated from the residual mean square in the analysis of variance.

Table 7. Major fatty acids composition (% of total fatty acids) of liver in juvenile olive flounder fed experimental diet for 8 weeks

Fatty acids	Diets																
	CON	EPA5	EPA10	EPA15	EPA20	EPA40	DHA5	DHA10	DHA15	DHA20	DHA40	E75/D25	E50/D50	E25/D75	SLO5	SLO10	SEM*
C14:0	1.8^{a}	2.2^{abcd}	2.5^{cd}	2.7^{de}	2.6^{cd}	3.1^{ef}	2.3^{abcd}	2.3^{abcd}	2.2^{abcd}	2.1^{abc}	4.0^{g}	1.9^{ab}	2.4^{bcd}	2.4^{bcd}	4.1^{g}	3.5^{f}	0.10
C16:0	12.8^{a}	13.5^{ab}	14.6^{abc}	14.5^{abc}	17.0	19.5^{e}	14.7^{abc}	16.0^{cd}	14.7^{abc}	14.4^{abc}	23.3^{g}	15.4^{bcd}	15.6^{cd}	15.8^{cd}	21.4^{f}	17.4^{d}	0.42
C16:1	1.1^{d}	0.2^{ab}	0.2^{ab}	0.3^{abc}	0.2^{ab}	0.2^{ab}	0.2^{abc}	0.3^{abc}	0.3^{abc}	0.3^{abc}	0.3^{abc}	0.2^{a}	0.3^{abc}	0.3^{abc}	0.6^{c}	0.6^{bc}	0.04
C17:1	0.3^{bc}	0.3^{bc}	0.4^{bc}	0.3^{b}	0.3^{b}	0.4^{bc}	0.4^{bc}	0.4^{c}	0.3^{bc}	0.3^{bc}	0.1^{a}	0.4^{bc}	0.3^{b}	0.4^{bc}	0.1^{a}	0.4^{bc}	0.01
C18:0	0.8^{c}	0.3^{a}	0.3^{a}	0.3^{ab}	0.4^{ab}	0.5^{ab}	0.3^{a}	0.3^{a}	0.3^{ab}	0.4^{ab}	0.6^{b}	0.4^{ab}	0.3^{a}	0.4^{ab}	1.0^{d}	0.9^{cd}	0.03
C18:1*n*-9	27.0	33.0^{bc}	32.3^{bc}	32.4^{bc}	30.9^{abc}	32.2^{bc}	31.6^{bc}	31.4^{bc}	31.3^{abc}	28.4^{ab}	30.4^{abc}	30.3^{abc}	34.5^{c}	33.5^{c}	33.5^{c}	33.8^{c}	0.40
C18:2*n*-6	40.9^{e}	35.8^{d}	33.2^{cd}	30.2^{c}	26.5^{b}	14.5^{a}	35.3^{d}	32.3^{cd}	30.5^{c}	29.8^{bc}	14.0^{a}	33.3^{cd}	32.8^{cd}	33.6^{cd}	12.1^{a}	15.6^{a}	1.3
C18:3*n*-3	2.8^{e}	2.5^{de}	2.2^{cde}	1.8^{bc}	1.6^{bc}	1.0^{a}	2.7^{e}	2.2^{cde}	1.9^{bcd}	1.8^{bc}	0.7^{a}	2.5^{de}	2.1^{cde}	2.2^{cde}	0.9^{a}	1.3^{ab}	0.10
C20:2	1.9^{a}	1.9^{a}	1.5^{a}	2.0^{a}	2.2^{a}	2.1^{a}	2.3^{a}	2.0^{a}	2.1^{a}	1.8^{a}	1.9^{a}	1.9^{a}	2.1^{a}	2.1^{a}	7.2^{c}	3.9^{b}	0.22
C20:3*n*-6	3.9^{f}	3.2^{cde}	3.0^{bcd}	2.9^{bc}	2.8^{b}	1.8^{a}	3.3^{de}	3.1^{bcd}	3.2^{cde}	3.0^{bcd}	1.7^{a}	3.5^{e}	3.0^{bcd}	3.2^{cde}	1.7^{a}	1.9^{a}	0.10
C20:5*n*-3	3.1^{bcd}	3.5^{cd}	5.6^{e}	9.1^{f}	11.6^{g}	19.9^{h}	1.1^{a}	1.0^{a}	1.2^{a}	1.4^{ab}	1.7^{abc}	4.9^{de}	2.5^{abc}	1.7^{abc}	4.6^{de}	6.3^{e}	0.72
C22:5	0.2^{cde}	0.2^{cde}	0.2^{bc}	0.3^{def}	0.3^{efg}	0.3^{g}	0.3^{def}	0.3^{efg}	0.3^{gh}	0.4^{h}	0.2^{cd}	0.3^{g}	0.3^{def}	0.3^{efg}	0.2^{ab}	0.1^{a}	0.01
C22:6*n*-3	3.1^{ab}	2.9^{a}	3.5^{ab}	3.0^{a}	3.5^{ab}	3.5^{ab}	5.5^{c}	8.1^{d}	11.2^{e}	15.5^{g}	20.7^{h}	4.6^{bc}	3.4^{ab}	3.9^{ab}	12.1^{e}	13.9^{f}	0.79
EPA + DHA	6.4^{a}	6.7^{a}	9.4^{b}	12.3^{c}	15.4^{d}	23.8^{f}	7.4^{ab}	9.3^{b}	12.7^{c}	17.2^{d}	22.6^{e}	9.9^{b}	6.2^{a}	5.9^{a}	17.0^{d}	20.4^{e}	0.87

Values (means±SE of three replication) in the same row not sharing a common superscript are significantly different ($P < 0.05$).
CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, values are not significant ($P > 0.05$).
*Standard error of the treatment mean calculated from the residual mean square in the analysis of variance.

Table 8. Major fatty acids composition (% of total fatty acids) of whole body in juvenile olive flounder fed experimental diet for 8 weeks

Fatty acids	Diets														
	CON	EPA5	EPA10	EPA15	EPA20	EPA40	DHA5	DHA10	DHA15	DHA20	DHA40	E75/D25	E50/D50	E25/D75	SLO5	SLO10	SEM*
C14:0	2.0^{ab}	1.9^{ab}	2.0^{ab}	1.8^{ab}	2.2^{ab}	2.0^{ab}	1.7^{a}	1.9^{ab}	2.3^{b}	2.1^{ab}	2.3^{b}	1.9^{ab}	1.9^{ab}	1.9^{ab}	3.9^{c}	4.1^{c}	0.11
C16:0	15.2^{a}	15.1^{a}	16.6^{ab}	15.4^{a}	16.3^{ab}	21.4^{c}	15.9^{ab}	15.6^{ab}	17.1^{b}	16.0^{ab}	15.6^{ab}	15.4^{a}	15.4^{ab}	16.2^{a}	22.2^{c}	21.9^{c}	0.36
C16:1	0.3^{a}	0.4^{a}	0.5^{ab}	0.4^{a}	0.5^{ab}	0.5^{ab}	0.4^{a}	0.4^{a}	0.4^{a}	0.4^{a}	0.4^{ab}	0.4^{ab}	0.4^{ab}	0.5^{ab}	0.9^{c}	0.7^{bc}	0.03
C17:1	0.4^{a}	0.4^{a}	0.5^{a}	0.4^{a}	0.4^{a}	0.5^{a}	0.5^{a}	0.5^{a}	0.4^{a}	0.3^{a}	0.5^{a}	0.3^{a}	0.4^{a}	0.5^{a}	0.5^{a}	1.2^{b}	0.05
C18:0	0.3^{ab}	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.1^{a}	0.4^{b}	0.1^{a}	0.2^{ab}	0.2^{ab}	0.6^{c}	0.4^{b}	0.02
C18:1*n*-9	19.4^{ab}	18.8^{ab}	19.5^{ab}	18.5^{ab}	16.6^{a}	19.7^{ab}	21.7^{bc}	18.3^{ab}	18.5^{ab}	16.7^{a}	15.2^{a}	18.6^{ab}	17.4^{ab}	17.8^{ab}	25.1^{c}	22.0^{bc}	0.44
C18:2*n*-6	42.5^{g}	41.4^{g}	37.8^{f}	35.5^{de}	31.6^{c}	17.7^{b}	37.9^{f}	37.9^{f}	35.2^{d}	31.7^{c}	14.9^{a}	35.5^{de}	37.9^{f}	37.6^{ef}	14.7^{a}	13.3^{a}	1.44
C18:3*n*-3	4.0^{fg}	4.2^{g}	3.5^{cdef}	3.4^{cde}	2.9^{c}	1.9^{b}	3.5^{cde}	3.7^{defg}	3.2^{cd}	3.0^{c}	1.4^{ab}	3.6^{def}	3.8^{efg}	3.7^{defg}	1.7^{ab}	1.3^{a}	0.14
C20:2	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.2^{a}	0.3^{ab}	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.9^{d}	0.5^{c}	0.02
C20:3*n*-6	2.0^{e}	1.7^{cde}	2.0^{e}	1.7^{cde}	1.6^{cd}	1.0^{ab}	1.8^{de}	1.9^{de}	1.5^{c}	1.5^{c}	0.9^{a}	1.7^{cde}	1.9^{e}	1.9^{de}	1.2^{b}	1.1^{ab}	0.05
C20:4*n*-6	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.01
C20:5*n*-3	4.4^{ab}	7.1^{cd}	9.8^{f}	14.1^{g}	19.1^{h}	26.8^{i}	4.7^{ab}	3.5^{a}	3.7^{ab}	3.9^{ab}	4.6^{ab}	8.9^{ef}	7.1^{cd}	5.4^{bc}	7.9^{de}	7.7^{de}	0.91
C22:6*n*-3	8.3^{ab}	7.8^{ab}	6.6^{a}	7.4^{a}	7.3^{a}	7.2^{a}	10.5^{bc}	14.9^{de}	15.9^{e}	22.5^{g}	42.7^{h}	12.1^{c}	11.9^{c}	12.6^{cd}	19.2^{f}	25.1^{g}	1.34
EPA + DHA	12.8^{a}	14.9^{ab}	16.4^{abcd}	21.4^{e}	26.4^{f}	34.0^{g}	15.3^{abc}	18.4^{bcde}	19.7^{de}	26.5^{f}	47.3^{h}	21.0^{e}	19.0^{cde}	18.1^{bcde}	27.2^{f}	32.8^{g}	1.29

Values (means ± SE of three replication) in the same row not sharing a common superscript are significantly different ($P < 0.05$).
CON, control diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, values are not significant ($P > 0.05$).
*Standard error of the treatment mean calculated from the residual mean square in the analysis of variance.

The increase in the level of unsaturated fatty acids in cells during cold acclimation appears to be a mechanism for conserving membrane fluidity (Farkas et al., 1980).

The essential fatty acid values of EPA and DHA were compared in juvenile fish by feeding the fish diets that contained various ratios of EPA to DHA. No correlation between dietary EPA/DHA ratios and fish growth performance was observed in this study. These results were similar to those from previous studies on turbot and flounder (Dickey-Collas and Geffen, 1992; Furuita et al., 1998, 1999). However, other studies on larval yellowtail (Furuita et al., 1996), larval and juvenile striped jack (Takeuchi et al., 1996) and larval flounder fed a microdiet (Watanabe and Kiron, 1994) have shown that DHA is superior to EPA as an essential fatty acid. These observations indicated that the requirement for DHA in the larval stage is higher than that of juveniles. The results of the present experiment indicate that adding either EPA, DHA, or a combination thereof has no significant effect on fish growth. However, in an earlier study by Kim and Lee (2004), a combination of EPA and DHA in the diet was more effective on the growth of juvenile flounder than the use of EPA only. The difference in results may have arisen when using diets that contained soybean oil masked the effects of EPA and DHA (Kim et al., 2012).

In this experiment, the liver, muscle and whole body fatty acid compositions were reflective of the respective dietary fatty acids. However, the DHA content in muscle was greater than its respective level in the diet, regardless of the dietary treatment. Similar results were also described for flounder in multiple studies (Kim and Lee, 2004; Kim et al., 2012). The selective deposition of DHA may be related to the high specificity of a synthesis enzyme, such as 1-lysophosphatidylacyl CoA transferase for DHA. Also, the increased DHA/EPA ratio in muscle indicated a selective catabolism of EPA relative to DHA in fatty acid oxidative processes. The relative resistance of DHA to β-oxidation stems from the complex catabolic pathway of this fatty acid (Caballero, 2002; Mourente and Bell, 2006).

In conclusion, the results of this study suggest that juvenile olive flounder require a low level of dietary EPA for suitable survival and growth, *e.g.*, approximately 0.32% EPA in the presence of 0.74% DHA. Additionally, the EPA and DHA levels in the fish muscle may increase to up to 32% and 53%, respectively, of the total fatty acid content. Nevertheless, excessive levels of EPA or DHA supplements in the diet had no negative effects on the growth of juvenile olive flounder.

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