



## Effect of partial substitution of dietary fish oil by vegetable oils on desaturation and $\beta$ -oxidation of [ $1\text{-}^{14}\text{C}$ ]18:3 $n-3$ (LNA) and [ $1\text{-}^{14}\text{C}$ ]20:5 $n-3$ (EPA) in hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.)

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### Abstract

The increasing worldwide aquaculture output and concomitant decrease in the stocks of feed-grade fish used for fish oil production has made fish oil replacement in feeds a priority for the aquaculture industry. The regulation of fatty acid metabolism in fish is important in order to determine strategies for the best use of plant oils in diets for commercially important cultured fish species. We have studied the desaturation/elongation and  $\beta$ -oxidation of  $^{14}\text{C}$ -linolenic (LNA) and  $^{14}\text{C}$ -eicosapentaenoic (EPA) acids in hepatocytes and pyloric caecal enterocytes in European sea bass fed diets with partial substitution (60%) of fish oil (FO) with vegetable oils (rapeseed, linseed and palm oil) blended in different proportions, for 64 weeks. The rate of desaturation of  $^{14}\text{C}$ -LNA was very low in hepatocytes from all treatments and no significant differences were observed among treatments. The rate of desaturation of  $^{14}\text{C}$ -LNA in enterocytes was higher than that in hepatocytes but still low (less than 5% of total radioactivity recovered). The desaturation of  $^{14}\text{C}$ -EPA in enterocytes was also higher than in hepatocytes, but again was low and no significant differences were found among treatments. The rates of  $\beta$ -oxidation of  $^{14}\text{C}$ -LNA and  $^{14}\text{C}$ -EPA were much higher than the rates of desaturation in both hepatocytes and enterocytes; however, no significant differences were observed in either hepatocytes or enterocytes among treatments. The rates of  $\beta$ -oxidation of  $^{14}\text{C}$ -LNA were considerably higher than those of  $^{14}\text{C}$ -EPA in both hepatocytes and enterocytes. In conclusion, European sea bass (a carnivorous marine fish) showed very low desaturation and elongation of LNA to EPA and DHA, and EPA to DHA, higher  $\beta$ -oxidation of LNA than EPA, and all desaturation and oxidation activities were significantly higher in enterocytes than in hepatocytes. A second major conclusion is that no clear quantitative nutritional

*Abbreviations:* AA, arachidonic acid (20:4 $n-6$ ); BHT, butylated hydroxytoluene; CPO, crude palm oil; DHA, docosahexaenoic acid (22:6 $n-3$ ); EFA, essential fatty acid; EPA, eicosapentaenoic acid (20:5 $n-3$ ); FAF-BSA, fatty acid-free bovine serum albumin; FO, fish oil; HBSS, Hanks balanced salt solution; HPTLC, high performance thin layer chromatography; HUFA, highly unsaturated fatty acids (carbon chain length  $\geq C_{20}$  with  $\geq 3$  double bonds); LA, linoleic acid (18:2 $n-6$ ); LNA,  $\alpha$ -linolenic acid (18:3 $n-3$ ); LO, linseed oil; PUFA, polyunsaturated fatty acid; RO, rapeseed oil; TLC, thin layer chromatography.

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effects on the desaturation/elongation and  $\beta$ -oxidation activities in either hepatocytes or enterocytes of sea bass were observed upon the inclusion of vegetable oils in the diet.

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*Keywords:* European sea bass; Enterocytes; Hepatocytes; PUFA; Desaturation;  $\beta$ -Oxidation; Rapeseed oil; Linseed oil; Palm oil; Fish oil

## 1. Introduction

Aquaculture has been successful in converting low value fish meal and oil, derived from industrial fisheries, into high value food for the human consumer. An increasing proportion of fish for human consumption is provided by aquaculture, which is expanding at 10% per year (Tidwell and Allan, 2002). As a consequence, demand for fish oil is increasing rapidly and current estimates suggest aquaculture feeds will consume around 90% of the world fish oil supplies by 2010 (Barlow, 2000). Marine fish are traditionally fed relatively high lipid diets using ingredients of marine origin containing high levels of  $n-3$  fatty acids, particularly  $n-3$  highly unsaturated fatty acids (HUFA) such as eicosapentaenoic (20:5 $n-3$ , EPA) and docosahexaenoic (22:6 $n-3$ , DHA) acids. However, stagnating worldwide supplies of marine oils and fish meal (Barlow, 2000) has forced the industry to investigate alternative lipid sources for use in marine fish diets. The only sustainable alternative to fish oils are plant (vegetable) seed oils, which are rich in  $C_{18}$  polyunsaturated fatty acids (PUFA) but lack the  $n-3$  HUFA abundant in fish oils. Vegetable oils such as rapeseed, linseed and olive are potential candidates to partially replace fish oil, and blends of these oils could also be an alternative in marine aquaculture diets. It is also of interest to investigate palm oil as a dietary oil source for marine fish as palm oil production is predicted to exceed soybean oil production within the next 10 years making it the most abundant vegetable oil in the world (Gunstone, 2001), and it has been used successfully in diets for salmonid species (Torstensen et al., 2000; Bell et al., 2002). Therefore, there is currently great interest in the regulation of HUFA biosynthesis in fish to determine the effectiveness with which vegetable oils can be utilized to replace FO in the diets of commercially important cultured fish species (Sargent et al., 2002).

PUFAs, linoleate (18:2 $n-6$ , LA) and linolenate (18:3 $n-3$ , LNA) cannot be synthesized *de novo* by animals, including fish, and therefore are termed es-

sential fatty acids (EFA) (Burr and Burr, 1929; Holman, 1986). The EFA requirement of fish differs between species. In freshwater fish, LA and/or LNA can satisfy the EFA requirement, whereas marine fish require the longer chain HUFA, EPA and DHA in the diet for optimal growth and health (Sargent et al., 1995, 1999). Supporting this, the conversion of LA to arachidonic acid (20:4 $n-6$ , AA) and LNA to EPA and DHA is well established for many freshwater species of fish, but the marine fish species studied so far cannot perform the conversions at a significant or appreciable rate (Sargent et al., 2002). The European sea bass, a strict carnivorous marine fish, is a highly prized species. Reared to a range of sizes, usually around 400–600 g, the fish is provided fresh, on ice to the market. With more than 50,000 tons coming from aquaculture, the supply of sea bass to Europe has increased and provides a high quality product. Although production of this species is a well-controlled process, knowledge of its nutritional requirements is still incomplete compared to other fish species, such as salmonids and carp (Oliva-Teles, 2000). In consequence, neither the EFA requirements or capacity of bioconversion of LNA to EPA and DHA, and EPA to DHA, nor the  $\beta$ -oxidation processes have been investigated in this species (Sargent et al., 2002). In addition, the replacement of dietary fish oil with vegetable oils resulted, in salmonids at least, in significantly increased activities of the fatty acyl desaturation/elongation pathway in hepatocytes and enterocytes, although flesh and liver  $n-3$  HUFA levels decreased significantly (Bell et al., 2001a, 2002; Tocher et al., 2002, 2003, 2004). Moreover, recent *in vivo* studies using stable isotopes (deuterium labelled LNA) had shown that dietary LNA was readily and substantially oxidized (Bell et al., 2001b) and that intestine was a tissue with high fatty acyl desaturation activity (Bell et al., 2003). In a previous study, we determined desaturation/elongation and  $\beta$ -oxidation of  $^{14}C$ -LNA in hepatocytes of European sea bass fed diets with 60% substitution of

fish oil (FO) with vegetable oils (rapeseed oil, RO; linseed oil, LO; and olive oil, OO), resulting in extremely low rates of fatty acid bioconversion and minimal nutritional regulation, with  $\beta$ -oxidation 10- to 30-fold higher than desaturation processes (Mourente and Dick, 2002).

In this frame of reference, an experiment was designed where European sea bass were fed for 64 weeks with diets in which 60% of FO was replaced with blends of rapeseed, linseed and palm oil to provide a similar balance of saturates, monoenes and PUFA to that found in fish oil, but without HUFA. The overall aims of this study were to investigate (i) the metabolism (desaturation/elongation and  $\beta$ -oxidation) of both  $[1-^{14}\text{C}]$ LNA and  $[1-^{14}\text{C}]$ EPA in isolated hepatocytes and pyloric caecal enterocytes of European sea bass, and (ii) the effects of partial substitution (60%) of dietary FO with vegetable oils on the rates of desaturation/elongation and  $\beta$ -oxidation of  $[1-^{14}\text{C}]$ LNA and  $[1-^{14}\text{C}]$ EPA, in order to increase the understanding of how dietary changes influence metabolic pathways determining tissue levels of fatty acids in this species.

## 2. Materials and methods

### 2.1. Animals, diets and experimental design

European sea bass (*Dicentrarchus labrax* L.), average total length  $7.9 \pm 0.5$  cm and average total body mass  $5.2 \pm 1.0$  g were purchased from MARESA, Huelva, SW Spain and transported to the aquaculture laboratory facilities at the University of Cádiz in the Faculty of Marine and Environmental Sciences, Puerto Real (Cádiz). Fish were transported at a density of approximately  $1.8 \text{ kg/m}^3$ , salinity 39‰, temperature 20 °C, with seawater in transport tank saturated with oxygen. On arrival at the wet laboratory, fish were stocked in six 5000 l rectangular tanks at 600 fish/tank (approximately  $0.6 \text{ kg/m}^3$ ), salinity 39‰, temperature 20 °C and saturated with oxygen. After 2 weeks acclimation (July 2002), fish were fed to satiation with mechanic belt automatic feeders with three iso-energetic and iso-nitrogenous experimental diets formulated with a constant lipid content of about 22% and produced by Nutreco ARC Stavanger (Norway). The diets contained approximately 47% protein,

primarily fish meal, and 21.4%, 24.1% and 21.5% lipids for diets of pellet sizes, 2, 3 and 5 mm, respectively. Two experimental diets contained 60% of three vegetable oils, rapeseed oil, palm oil and linseed oil, blended to provide a similar balance of saturates, monoenes and PUFA to that found in fish oil, but without HUFA. The control diet contained anchovy oil and the added oil combinations for the experimental diets were as follows: diet A: 100% anchovy oil (control); diet B: 40% anchovy oil, 35% linseed oil, 15% palm oil and 10% rapeseed oil; diet C: 40% anchovy oil, 24% linseed oil, 12% palm oil and 24% rapeseed oil. Table 1 shows the formulation and proximate compositions of the experimental diets. Total lipid and fatty acid compositions of the diets are shown in Table 2.

### 2.2. Sample collection and biometric determinations

Fish were grown during 64 weeks and were sampled at the beginning and the end of the experiment for biometry (total length, live mass, liver live and dry mass, hepatosomatic index and specific growth rates) plus sampling of liver and pyloric caeca for fatty acid analyses and lipid metabolism (desaturation/elongation and  $\beta$ -oxidation) studies. Live masses were determined by blotting on filter paper before weighing, and dry mass was determined after heating in an oven at 60 °C for 24 h and cooling in vacuum before weighing. Hepatosomatic index (HSI) was calculated and growth assessed by measuring the specific growth rate (SGR) as % weight gain  $\text{day}^{-1}$  (Wootten, 1990). Mortality was measured at the end of the experiment and expressed as percentage of fish surviving. For analytical studies, intact livers and pyloric caeca were dissected from 3 fish per dietary treatment, immediately frozen in liquid nitrogen and kept at  $-80$  °C until analysis. For metabolic studies, intact livers and pyloric caeca were dissected from 4 fish per dietary treatment and hepatocytes and enterocytes isolated as described below.

### 2.3. Lipid analysis

Total lipid was extracted after homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957) and essentially as

Table 1  
Formulation and proximate composition of experimental diets (g/kg feed)

Diets	A	B	C
<i>Components</i>			
Fish meal <sup>a</sup>	400.0	400.0	400.0
Maize gluten <sup>b</sup>	262.7	262.7	262.7
Wheat <sup>c</sup>	152.3	152.3	152.3
Oil	160.0	160.0	160.0
Premixes <sup>d</sup>	25.0	25.0	25.0
<i>Composition (%) of added oil</i>			
Anchovy oil (FO) <sup>e</sup>	100	40	40
Rapeseed oil (RO) <sup>f</sup>	0	10	24
Linseed oil (LO) <sup>g</sup>	0	35	24
Palm oil (PO) <sup>h</sup>	0	15	12
<i>Total lipids (dry mass %)</i>			
Diet 1 (2 mm)	20.8	25.2	20.2
Diet 2 (3 mm)	25.5	26.4	22.4
Diet 3 (5 mm)	20.2	21.2	21.1
<i>Gross composition (%)</i>			
Crude protein	47.8	46.2	47.8
Crude lipid	19.9	18.5	23.4
Carbohydrate	15.4	18.1	13.2
Ash	6.7	6.3	6.2
Moisture	10.2	10.9	9.4

<sup>a</sup> Scandinavian LT-fish meal (Nordsildmel, Norway).

<sup>b</sup> Cargill, Staley, USA.

<sup>c</sup> Statkom, Oslo, Norway.

<sup>d</sup> Vitamin and mineral premix added min. to NRC recommendations.

<sup>e</sup> Anchovy oil (Denofa, Fredrikstad, Norway) supplemented with 200 ppm BHT.

<sup>f</sup> Crude rapeseed oil (Oelmühle Hamburg, Germany) no antioxidant added.

<sup>g</sup> Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500 ppm Ronoxan A (Roche, Basel, Switzerland).

<sup>h</sup> Crude palm oil.

described by Christie (1982). The lipid extract was desiccated overnight under vacuum following the removal of solvent by evaporation under a stream of nitrogen. After being weighed, the lipid extract was redissolved in chloroform/methanol (2:1, v/v).

Fatty acid methyl esters (FAME) from total lipids were prepared by acid-catalyzed transmethylation for 16 h at 50 °C, using tricosanoic acid (23:0) as internal standard (Christie, 1989). FAME were extracted and purified as described previously (Tocher and Harvie, 1988) and were separated in

a Hewlett-Packard 5890A Series II gas chromatograph equipped with a chemically bonded (PEG) Supelcowax-10 fused silica wall coated capillary column (30 m × 0.32 mm i.d., Supelco Inc., Bellefonte, USA), “on column” injection system and flame ionization detection. Hydrogen was used as the carrier gas with an oven thermal gradient from an initial 50 °C to 180 °C at 25 °C/min, and then to a final temperature of 235 °C at 3 °C/min with the final temperature maintained for 10 min. Individual FAME were identified by comparison with known

Table 2

Total lipid content (% of dry mass) and fatty acid composition (mass percentage of total fatty acids) of the experimental diets

Dietary treatments	A	B	C
Total lipid (%)	20.2 ± 3.1	21.2 ± 4.3	21.1 ± 2.8
<i>Fatty acid</i>			
14:0	4.3 ± 0.1 <sup>a</sup>	1.9 ± 0.2 <sup>b</sup>	1.9 ± 0.2 <sup>b</sup>
15:0	0.5 ± 0.0	0.3 ± 0.1	0.6 ± 0.0
16:0	14.0 ± 0.2 <sup>a</sup>	13.3 ± 0.1 <sup>b</sup>	12.5 ± 0.1 <sup>c</sup>
18:0	3.0 ± 0.1 <sup>a</sup>	3.1 ± 0.1 <sup>a</sup>	2.7 ± 0.0 <sup>b</sup>
Total saturated	22.9 ± 0.1 <sup>a</sup>	19.5 ± 0.2 <sup>b</sup>	18.6 ± 0.3 <sup>c</sup>
16:1 <i>n</i> -9	4.9 ± 0.0 <sup>a</sup>	2.8 ± 0.0 <sup>b</sup>	2.7 ± 0.0 <sup>b</sup>
16:1 <i>n</i> -7	8.7 ± 0.6 <sup>a</sup>	8.6 ± 1.9 <sup>a</sup>	7.5 ± 0.3 <sup>b</sup>
18:1 <i>n</i> -9	9.4 ± 0.2 <sup>c</sup>	16.9 ± 0.4 <sup>b</sup>	21.6 ± 0.4 <sup>a</sup>
18:1 <i>n</i> -7	2.2 ± 0.1 <sup>a</sup>	1.8 ± 0.0 <sup>c</sup>	2.0 ± 0.0 <sup>b</sup>
20:1 <i>n</i> -9	2.3 ± 0.0 <sup>a</sup>	1.9 ± 0.0 <sup>c</sup>	2.0 ± 0.0 <sup>b</sup>
22:1 <i>n</i> -11	2.0 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>b</sup>	1.7 ± 0.0 <sup>b</sup>
Total monoenes	30.7 ± 0.7 <sup>c</sup>	34.6 ± 1.5 <sup>b</sup>	38.1 ± 0.4 <sup>a</sup>
16:2	1.3 ± 0.1 <sup>a</sup>	0.9 ± 0.1 <sup>b</sup>	0.9 ± 0.0 <sup>b</sup>
16:3	0.7 ± 0.0 <sup>a</sup>	0.4 ± 0.0 <sup>b</sup>	0.3 ± 0.0 <sup>b</sup>
16:4	0.9 ± 0.1 <sup>a</sup>	0.4 ± 0.0 <sup>b</sup>	0.4 ± 0.0 <sup>b</sup>
18:2 <i>n</i> -6	4.9 ± 0.0 <sup>c</sup>	9.0 ± 0.7 <sup>b</sup>	10.4 ± 0.2 <sup>a</sup>
18:3 <i>n</i> -3	1.5 ± 0.0 <sup>c</sup>	12.3 ± 0.7 <sup>a</sup>	10.3 ± 0.3 <sup>b</sup>
18:4 <i>n</i> -3	2.0 ± 0.0 <sup>a</sup>	1.1 ± 0.1 <sup>b</sup>	1.0 ± 0.0 <sup>b</sup>
20:4 <i>n</i> -6	0.6 ± 0.0 <sup>a</sup>	0.3 ± 0.0 <sup>b</sup>	0.3 ± 0.0 <sup>b</sup>
20:4 <i>n</i> -3	0.5 ± 0.0 <sup>a</sup>	0.3 ± 0.0 <sup>b</sup>	0.3 ± 0.0 <sup>b</sup>
20:5 <i>n</i> -3	9.8 ± 0.1 <sup>a</sup>	5.3 ± 0.3 <sup>b</sup>	5.2 ± 0.1 <sup>b</sup>
22:5 <i>n</i> -3	1.2 ± 0.0 <sup>a</sup>	0.7 ± 0.0 <sup>b</sup>	0.6 ± 0.0 <sup>b</sup>
22:6 <i>n</i> -3	11.0 ± 0.3 <sup>a</sup>	6.3 ± 0.5 <sup>b</sup>	6.2 ± 0.1 <sup>b</sup>
Total polyenes	35.4 ± 0.6	37.8 ± 2.1	36.3 ± 0.9
Total <i>n</i> -6	7.4 ± 0.1 <sup>c</sup>	10.7 ± 0.5 <sup>b</sup>	11.8 ± 0.2 <sup>a</sup>
Total <i>n</i> -3	27.9 ± 0.5 <sup>a</sup>	27.0 ± 1.7 <sup>ab</sup>	24.5 ± 0.6 <sup>b</sup>
HUFA <i>n</i> -6	1.0 ± 0.1 <sup>a</sup>	0.6 ± 0.0 <sup>b</sup>	0.4 ± 0.1 <sup>bc</sup>
HUFA <i>n</i> -3	22.8 ± 0.5 <sup>a</sup>	12.7 ± 0.9 <sup>b</sup>	12.4 ± 0.3 <sup>b</sup>

Results are means ± S.D. (*n* = 3). An S.D. of 0.0 implies an S.D. of <0.05. Significant effects due to dietary oil supplementation were determined by one-way analysis of variance and Tukey multiple range test. Values bearing different superscript letter in the same row are significantly different (*P* < 0.05). HUFA, highly unsaturated fatty acid; nd, not detected.

standards and quantified by means of a direct-linked PC and Hewlett-Packard ChemStation software.

#### 2.4. Preparation of isolated hepatocytes and caecal enterocytes

Fish were killed by immersion in ice-cold seawater and the livers and intestinal tracts removed immediately. The gall bladder and main blood vessels were dissected from the liver, and the liver perfused via the hepatic vein with solution A (calcium- and magnesium-free Hank's balanced salt solution (HBSS)+10 mmol/l HEPES+1 mmol/l EDTA). The liver was chopped finely and about 1 g incubated with 20 ml of solution A containing 0.1% (w/v) collagenase at 20 °C for 45 min. The digested liver was filtered through 100 µm nylon gauze and the cells collected by centrifuging at 300×g for 5 min. The cell pellet was washed with 20 ml of solution A containing fatty acid-free bovine serum albumin (10 g/l, FAF-BSA) and recentrifuged. The hepatocytes were resuspended in 10 ml of Medium 199 containing 10 mmol/l HEPES, 2 mmol/l glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

The intestinal tract was removed and pyloric caeca dissected, cleaned of adhering adipose tissue, slit open and luminal contents rinsed away with solution A. The caeca samples were chopped finely and incubated with 20 ml of solution A containing 0.1% (w/v) collagenase at 20 °C for 45 min. The digested caecal tissues were filtered through 100 µm nylon gauze and the cells collected and washed exactly as described for hepatocytes. The enterocytes were resuspended in 10 ml of Medium 199 containing 10 mmol/l HEPES, 2 mmol/l glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. One hundred microliters of the hepatocyte and enterocyte cell suspensions were retained for protein determination according to the method of Lowry et al. (1951) after incubation with 0.4 ml of 2.5 g/l SDS/1 M NaOH for 45 min at 60 °C.

#### 2.5. Incubation of hepatocyte and enterocyte preparations with [1-<sup>14</sup>C]18:3n-3 and [1-<sup>14</sup>C]20:5n-3

Six milliliters of hepatocyte/enterocyte suspension were dispensed into 25 cm<sup>2</sup> tissue culture flasks and incubated with 0.3 µCi (~1 µM) [1-<sup>14</sup>C]18:3n-3 or

[1-<sup>14</sup>C]20:5n-3, added as a complex with FAF-BSA in phosphate buffered saline prepared as described in Ghioni et al (1997). After addition of isotope, the flasks were incubated at 20 °C for 2 h. After incubation, the cell layer was dislodged by gentle rocking and the cell suspension transferred to glass conical test tubes and 1 ml of each suspension withdrawn into a 2 ml microcentrifuge tube for β-oxidation assay as described below. The cell suspensions remaining in the glass conical centrifuge tubes were used for the desaturation/elongation assay as described below.

#### 2.6. Assay of hepatocyte and enterocyte fatty acyl desaturation/elongation activities

The cell suspensions were centrifuged at 500×g for 2 min, the supernatants discarded and the cell pellets washed with 5 ml of ice-cold 1% HBSS/FAF-BSA and recentrifuged. The supernatants were again discarded before extraction of total lipid using ice-cold chloroform/methanol (2:1, v/v) containing 0.01% (w/v) BHT essentially according to Folch et al. (1957) and as described in detail previously (Tocher and Ghioni, 1999). Total lipid was transmethylated and FAME prepared as described above. The methyl esters were redissolved in 100 µl hexane containing 0.01% BHT and applied as 2.5 cm streaks to TLC plates impregnated by spraying with 2 g silver nitrate in 20 ml acetonitrile and pre-activated at 110 °C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, v/v) (Wilson and Sargent, 1992). Autoradiography was performed with Kodak MR2 film (Sigma, Poole, UK) for 6 days at room temperature. Areas of silica corresponding to individual PUFA were scraped into scintillation mini-vials containing scintillation fluid (Beckman Ready Safe™) and radioactivity determined in a TRI-CARB 2300TR liquid scintillation analyzer (United Technologies Packard, Pangbourne, UK). Results were corrected for counting efficiency and quenching of <sup>14</sup>C under exactly these conditions.

#### 2.7. Assay of hepatocyte and enterocyte fatty acyl oxidation activities

The assay of fatty acid oxidation in intact hepatocytes requires the determination of acid-soluble radioactivity as described in detail previously (Frøy-

land et al. 2000; Torstensen et al. 2000). Briefly, the 1 ml of cell suspension was homogenized and centrifuged at  $2000\times g$  for 10 min in a microcentrifuge. Five hundred microliters of the supernatant were taken into a clean 2 ml microcentrifuge tube and 100  $\mu$ l of 6% HBSS/FAF-BSA solution was added. After mixing thoroughly, the protein was precipitated by the addition of 1 ml of ice-cold 4 M perchloric acid. After vortexing, the tubes were centrifuged at  $5000\times g$  for 10 min. Five hundred microliters of aliquots were carefully transferred to a scintillation vial, 4 ml of scintillant added and radioactivity in the acid soluble fraction determined as described above.

### 2.8. Materials

[ $1\text{-}^{14}\text{C}$ ]18:3 $n$ -3 and [ $1\text{-}^{14}\text{C}$ ]20:5 $n$ -3 (40–60 mCi/mmol) were obtained from Perkin Elmer Life Sciences. HBSS, Medium 199, HEPES buffer, glutamine, penicillin, streptomycin, collagenase (type IV), FAF-BSA, BHT and silver nitrate were obtained from Sigma-Aldrich, Química S.A. (Madrid, Spain). Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) plates, precoated with silica gel 60 (without fluorescent indicator), were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Romil Ltd., England, UK.

### 2.9. Statistical analysis

All the data are presented as means  $\pm$  S.D. ( $n=3, 4$  or 30 as stated). The significance of diet on growth, liver fatty acid composition, hepatocyte fatty acid desaturation and  $\beta$ -oxidation were determined by one-way ANOVA. The differences between means were determined by Tukey's multiple comparison test. Percentage data and data that were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. Differences were regarded as significant when  $P<0.05$  (Zar, 1984).

## 3. Results

### 3.1. Dietary fatty acid compositions

Diet A or control diet (containing 100% FO) contained approximately 22% total saturates, primarily 16:0, about 30% total monounsaturated fatty acids, mainly 18:1 $n$ -9 and 16:1 $n$ -7, 7.4% total  $n$ -6 fatty acids, predominantly 18:2 $n$ -6, and 28%  $n$ -3 fatty acids, of which 23% were  $n$ -3 HUFA, 9.8% EPA, 11% DHA and 1.5% 18:3 $n$ -3 (Table 2). Diet B, 60% of dietary FO substituted with a blend of 10% RO, 35% LO and 15% PO, showed increased percentages of 18:1 $n$ -9 (16.9%), 18:2 $n$ -6 (9.0%) and 18:3 $n$ -3 (12.3%) with concomitant decreased proportions of

Table 3

Effect of partial replacement (60%) of dietary fish oil (diet A) with vegetable oils (diets B and C) on growth and performance of European sea bass fed experimental diets during 64 weeks

Dietary treatment	Initial	A	B	C
Fish length (cm)	7.9 $\pm$ 0.5	24.9 $\pm$ 1.3	23.2 $\pm$ 1.4	24.1 $\pm$ 1.4
Fish live mass (g)	5.2 $\pm$ 1.0	176.2 $\pm$ 32.9	143.2 $\pm$ 29.4	159.8 $\pm$ 34.2
Liver live mass (g)	0.08 $\pm$ 0.02	3.2 $\pm$ 0.7	2.7 $\pm$ 0.8	3.5 $\pm$ 0.6
Liver dry mass (%)	31.8 $\pm$ 1.2	56.0 $\pm$ 3.9	50.9 $\pm$ 3.4	57.8 $\pm$ 2.2
Liver total lipid content (% dry mass)		54.8 $\pm$ 7.6	52.9 $\pm$ 0.9	54.7 $\pm$ 5.8
HSI <sup>a</sup>	1.4 $\pm$ 0.2	2.01 $\pm$ 0.27	1.9 $\pm$ 0.2	2.1 $\pm$ 0.3
Pyloric caeca dry mass (%)		22.0 $\pm$ 0.9	23.1 $\pm$ 3.2	23.6 $\pm$ 1.0
Pyloric caeca total lipid content (% dry mass)		29.8 $\pm$ 8.8	27.4 $\pm$ 4.5	15.3 $\pm$ 4.7
Condition factor (K)		1.14	1.15	1.14
Daily growth rate ( $G_w$ ) <sup>b</sup>		0.00786	0.00740	0.00764
%W/day <sup>c</sup>		0.8	0.7	0.8

Data are mean  $\pm$  S.D. ( $n=30$  for all data other than lipid content of pyloric caeca where  $n=4$ ). Values corresponding to different dietary treatments bearing different superscript letter in the same row are significantly different ( $P<0.05$ ).

<sup>a</sup> Hepato Somatic Index.

<sup>b</sup> Daily growth rate =  $(\ln W_1 - \ln W_0)/t$ .

<sup>c</sup> %W/day =  $(e^{G_w} - 1)100$ .  $K = (W/L^3) * 100$ .

$n-3$  HUFA (12.7%), EPA (5.3%), DHA (6.3%), 16:0, total saturates and 16:1 $n-9$ . Diet C, 60% dietary FO substituted with a blend of 24% RO, 24% LO and 12% PO, showed increased the percentages of 18:1 $n-9$  (21.6%), 18:2 $n-6$  (10.4%) and 18:3 $n-3$  (10.3%) with concomitant decreases in total saturates, total ( $n-3$ ), total  $n-3$  HUFA (12.7%), EPA (5.2%) and DHA (6.2%).

### 3.2. Effect of dietary treatments on growth performance

Neither fish length nor fish live mass or specific growth rate (SGR) were affected by partial inclusion of vegetable oils in the diets (Table 3). There was 100% survival and good fish health status in all treatments. Specifically, fish were examined for health condition profile and health assessment according to Morgan and Iwama (1997). No differences were encountered among fish from different dietary treatments and all fish presented normal fins, opercules, eyes and gills, abundant mesenteric fat, normal spleen, hindgut and kidney, and liver presented a fatty or light brown color with dark green to bluegreen bile and full gall bladder.

### 3.3. Effect of diet on liver and pyloric caeca total lipid fatty acid compositions

Liver dry mass was above 50% and total lipid content was approximately 54% on a dry mass basis and no statistical differences were found among treatments (Table 3). Liver lipid was predominantly neutral (storage) lipids in all treatments, with approximately 75% of total lipids being triacylglycerols (data not shown). The principal fatty acids of liver total lipid from fish fed control diet (FO) were, in rank order, 18:1 $n-9$  (28.8%), 16:0 (16.6%), 22:6 $n-3$  (10.2%), 20:5 $n-3$  (6.2%) and 16:1 $n-9$  (6.1%) (Table 4). In the livers of fish fed diet B, the levels of 18:1 $n-9$ , 18:2 $n-6$  and 18:3 $n-3$  increased to 34.1%, 6.4% and 7.5%, respectively, whereas DHA and EPA were reduced to 5.4% and 3.2%, respectively. In livers of fish fed diet C, 18:1 $n-9$ , 18:2 $n-6$  and 18:3 $n-3$  increased to 39.2%, 6.2% and 5.1%, respectively, whereas DHA and EPA decreased to 4.2% and 2.5%, respectively.

Pyloric caeca dry mass was approximately 22% and total lipid content were close to 30% in treatments A

Table 4

Total lipid fatty acid composition (mass percentage of total fatty acids) of livers from European sea bass fed the experimental diets during 64 weeks

Dietary treatments	A*	B	C
14:0	1.8 ± 0.3 <sup>a</sup>	1.3 ± 0.2 <sup>ab</sup>	1.0 ± 0.1 <sup>b</sup>
15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:0	16.6 ± 2.4	16.1 ± 2.1	17.3 ± 2.1
18:0	3.1 ± 0.7	3.7 ± 0.2	3.4 ± 0.7
Total saturated	22.4 ± 3.1	21.8 ± 2.5	22.5 ± 2.9
16:1 $n-9$	6.1 ± 0.8 <sup>a</sup>	3.6 ± 1.0 <sup>b</sup>	3.9 ± 0.2 <sup>b</sup>
16:1 $n-7$	2.7 ± 1.0	2.4 ± 1.0	2.2 ± 0.2
18:1 $n-9$	28.8 ± 2.0 <sup>b</sup>	34.1 ± 2.2 <sup>ab</sup>	39.2 ± 2.3 <sup>a</sup>
18:1 $n-7$	3.4 ± 0.4 <sup>a</sup>	2.5 ± 0.2 <sup>b</sup>	2.7 ± 0.2 <sup>ab</sup>
20:1 $n-9$	2.1 ± 0.1	2.0 ± 0.2	2.1 ± 0.3
22:1 $n-11$	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
Total monoenes	44.7 ± 1.2 <sup>b</sup>	46.2 ± 1.5 <sup>b</sup>	51.3 ± 1.6 <sup>a</sup>
16:2	0.4 ± 0.1 <sup>a</sup>	0.3 ± 0.0 <sup>ab</sup>	0.2 ± 0.0 <sup>b</sup>
16:3	0.3 ± 0.0 <sup>a</sup>	0.2 ± 0.0 <sup>b</sup>	0.2 ± 0.0 <sup>b</sup>
16:4	0.5 ± 0.0 <sup>a</sup>	0.3 ± 0.1 <sup>b</sup>	0.3 ± 0.0 <sup>b</sup>
18:2 $n-6$	3.3 ± 0.3 <sup>b</sup>	6.4 ± 1.5 <sup>a</sup>	6.2 ± 1.3 <sup>a</sup>
18:3 $n-3$	0.8 ± 0.2 <sup>b</sup>	7.5 ± 2.8 <sup>a</sup>	5.1 ± 1.2 <sup>a</sup>
18:4 $n-3$	1.1 ± 0.0	0.9 ± 0.1	0.7 ± 0.1
20:4 $n-6$	0.5 ± 0.1 <sup>a</sup>	0.3 ± 0.1 <sup>b</sup>	0.2 ± 0.0 <sup>b</sup>
20:4 $n-3$	0.5 ± 0.0 <sup>a</sup>	0.3 ± 0.1 <sup>b</sup>	0.2 ± 0.0 <sup>b</sup>
20:5 $n-3$	6.2 ± 0.7 <sup>a</sup>	3.2 ± 1.0 <sup>b</sup>	2.5 ± 0.6 <sup>b</sup>
22:5 $n-3$	1.2 ± 0.1 <sup>a</sup>	0.6 ± 0.2 <sup>b</sup>	0.4 ± 0.1 <sup>b</sup>
22:6 $n-3$	10.2 ± 1.6 <sup>a</sup>	5.4 ± 1.8 <sup>b</sup>	4.2 ± 0.9 <sup>b</sup>
Total polyenes	25.9 ± 2.9	27.0 ± 1.0	21.1 ± 4.4
<i>Unknown</i>			
Total $n-9$	37.7 ± 1.5 <sup>b</sup>	40.4 ± 1.5 <sup>b</sup>	45.7 ± 2.0 <sup>a</sup>
Total $n-7$	6.2 ± 1.3	5.1 ± 0.9	5.0 ± 0.4
Total $n-6$	4.9 ± 0.5	7.6 ± 1.5	7.2 ± 1.5
Total $n-3$	21.1 ± 2.5 <sup>a</sup>	19.3 ± 0.5 <sup>ab</sup>	13.8 ± 2.9 <sup>b</sup>
HUFA $n-6$	0.9 ± 0.1 <sup>a</sup>	0.5 ± 0.1 <sup>b</sup>	0.4 ± 0.0 <sup>b</sup>
HUFA $n-3$	18.4 ± 2.4 <sup>a</sup>	9.6 ± 3.1 <sup>b</sup>	7.5 ± 1.7 <sup>b</sup>

Results are means ± S.D. ( $n=3$ ). An S.D. of 0.0 implies an S.D. of <0.05. Significant effects due to dietary oil supplementation were determined by one-way analysis of variance and Tukey multiple range test. Values bearing different superscript letter in the same row are significantly different ( $P<0.05$ ). HUFA, highly unsaturated fatty acid.

\* Control diet.

(control) and B and about half that value for treatment C, although no significant differences were found among treatments (Table 3). The principal fatty acids of pyloric caeca total lipid from fish fed control diet (FO) were, in rank order, 18:1 $n-9$  (15.0%), 16:0 (14.3%), 22:6 $n-3$  (11.9%), 16:1( $n-7$ ) (9.7%) and 20:5 $n-3$  (8.3%) (Table 5). In the pyloric caeca of fish fed diet B, the levels of 18:1 $n-9$ , 18:2 $n-6$  and 18:3 $n-3$  increased to 18.0%, 9.1% and 14.1%, respec-

Table 5

Total lipid fatty acid composition (mass percentage of total fatty acids) of pyloric caeca from European sea bass fed the experimental diets during 64 weeks

Dietary treatments	A	B	C
14:0	2.7 ± 0.6 <sup>a</sup>	1.2 ± 0.2 <sup>b</sup>	1.8 ± 0.0 <sup>a</sup>
15:0	0.7 ± 0.0	0.7 ± 0.0	0.9 ± 0.2
16:0	14.3 ± 0.3 <sup>a</sup>	10.5 ± 0.1 <sup>c</sup>	12.2 ± 0.8 <sup>b</sup>
18:0	4.2 ± 0.6 <sup>b</sup>	4.4 ± 0.5 <sup>b</sup>	7.8 ± 0.8 <sup>a</sup>
Total saturated	22.8 ± 0.3 <sup>a</sup>	17.7 ± 0.2 <sup>b</sup>	23.2 ± 1.6 <sup>a</sup>
16:1 $n$ -9	5.0 ± 0.6 <sup>a</sup>	2.2 ± 0.1 <sup>b</sup>	1.3 ± 0.1 <sup>c</sup>
16:1 $n$ -7	9.7 ± 0.7 <sup>b</sup>	8.1 ± 0.1 <sup>b</sup>	13.9 ± 2.1 <sup>a</sup>
18:1 $n$ -9	15.0 ± 1.1 <sup>b</sup>	18.0 ± 0.6 <sup>a</sup>	9.7 ± 1.2 <sup>c</sup>
20:1 $n$ -9	2.1 ± 0.2 <sup>a</sup>	2.1 ± 0.2 <sup>a</sup>	1.3 ± 0.1 <sup>b</sup>
22:1 $n$ -11	1.1 ± 0.3 <sup>a</sup>	1.2 ± 0.0 <sup>a</sup>	0.4 ± 0.0 <sup>b</sup>
Total monoenes	36.8 ± 0.7 <sup>a</sup>	34.4 ± 0.8 <sup>a</sup>	29.2 ± 1.4 <sup>b</sup>
16:2	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2
16:3	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
16:4	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.1
18:2 $n$ -6	3.5 ± 0.6 <sup>b</sup>	9.1 ± 0.3 <sup>a</sup>	3.7 ± 0.2 <sup>b</sup>
18:3 $n$ -3	1.0 ± 0.2 <sup>c</sup>	14.1 ± 0.4 <sup>a</sup>	2.7 ± 0.0 <sup>b</sup>
18:4 $n$ -3	1.1 ± 0.3 <sup>a</sup>	0.6 ± 0.1 <sup>b</sup>	0.2 ± 0.0 <sup>c</sup>
20:4 $n$ -6	1.3 ± 0.3 <sup>b</sup>	0.9 ± 0.3 <sup>b</sup>	2.4 ± 0.1 <sup>a</sup>
20:4 $n$ -3	0.4 ± 0.0 <sup>a</sup>	0.2 ± 0.0 <sup>b</sup>	0.2 ± 0.0 <sup>b</sup>
20:5 $n$ -3	8.3 ± 0.5 <sup>a</sup>	4.8 ± 0.5 <sup>b</sup>	7.5 ± 0.7 <sup>a</sup>
22:5 $n$ -3	1.1 ± 0.1 <sup>a</sup>	0.6 ± 0.0 <sup>b</sup>	0.7 ± 0.1 <sup>b</sup>
22:6 $n$ -3	11.9 ± 1.4 <sup>b</sup>	7.5 ± 1.1 <sup>c</sup>	17.8 ± 1.9 <sup>a</sup>
Total polyenes	31.6 ± 0.9 <sup>b</sup>	40.1 ± 1.9 <sup>a</sup>	38.2 ± 1.8 <sup>a</sup>
Total $n$ -6	6.8 ± 0.5 <sup>c</sup>	11.4 ± 0.3 <sup>a</sup>	8.0 ± 0.1 <sup>b</sup>
Total $n$ -3	24.8 ± 0.7 <sup>b</sup>	28.7 ± 1.7 <sup>a</sup>	30.1 ± 1.9 <sup>a</sup>
HUFA $n$ -6	1.8 ± 0.3 <sup>b</sup>	1.0 ± 0.3 <sup>c</sup>	2.7 ± 0.1 <sup>a</sup>
HUFA $n$ -3	21.8 ± 1.1 <sup>b</sup>	13.3 ± 1.7 <sup>c</sup>	26.4 ± 1.9 <sup>a</sup>

Results are means ± S.D. ( $n=3$ ). An S.D. of 0.0 implies an S.D. of <0.05. Significant effects due to diet were determined by one-way analysis of variance and Tukey's multiple range test. Values within a row bearing different superscript letters in the same row are significantly different ( $P<0.05$ ). HUFA, highly unsaturated fatty acid.

tively, whereas DHA and EPA were reduced to 7.5% and 4.8%, respectively. In pyloric caeca of fish fed diet C, 18:1 $n$ -9 decreased to 9.7%, 18:2 $n$ -6 remained at the same level and 18:3 $n$ -3 increased to 2.7%, whereas DHA increased to 17.8% and EPA remained at the same level.

### 3.4. Effect of diet on hepatocyte and pyloric caecal fatty acid desaturation/elongation activities

The desaturation and elongation of <sup>14</sup>C-18:3 $n$ -3 and <sup>14</sup>C-20:5 $n$ -3 in hepatocytes and pyloric caeca enterocytes from sea bass after 64 weeks of feeding with experimental diets is shown in Table 6. The rate of desaturation of <sup>14</sup>C-18:3 $n$ -3 to all products was, in

general, very low in hepatocytes from all treatments, being only between 0.21 and 0.23 pmol/mg protein/h. This presented only between 7.7% and 13.4% of the total radioactivity recovered from the assay, with the majority of that representing  $\Delta 6$  desaturase products and only very low levels of  $\Delta 5$  desaturated products. No significant differences were observed among different dietary treatments (Table 6). The rate of desaturation of <sup>14</sup>C-18:3 $n$ -3 to all products in pyloric caecal enterocytes was, in general, higher than that in hepatocytes (up to three-fold), varying between 0.66 and 1.09 pmol/mg protein/h, but this represented only 2.1% of total radioactivity recovered, with 97.9% of total radioactivity recovered as the substrate [1-<sup>14</sup>C]18:3 $n$ -3. The rates of synthesis of the pentaene+hexaene fraction were significantly higher (two-

Table 6

Desaturation and elongation (pmol/mg protein/h) of <sup>14</sup>C-18:3 $n$ -3 and <sup>14</sup>C-20:5 $n$ -3 by hepatocytes and enterocytes of European sea bass fed the experimental diets, containing partial substitution of dietary fish oil with vegetable oils

Dietary treatments	A*	B	C
<i>Hepatocytes</i>			
<sup>14</sup> C-18:3 $n$ -3			
18:4 $n$ -3	0.09 ± 0.08	0.06 ± 0.02	0.05 ± 0.01
20:3 $n$ -3	0.11 ± 0.07	0.06 ± 0.00	0.13 ± 0.06
20:4 $n$ -3	0.03 ± 0.01	0.03 ± 0.00	0.05 ± 0.01
22:4 $n$ -3	0.07 ± 0.05	0.08 ± 0.02	0.07 ± 0.03
Pentaenes+hexaenes	0.04 ± 0.02	0.04 ± 0.01	0.06 ± 0.01
Total desaturated	0.23 ± 0.03	0.21 ± 0.02	0.23 ± 0.01
<sup>14</sup> C-20:5 $n$ -3			
22:5 $n$ -3	0.48 ± 0.42	0.20 ± 0.03	0.36 ± 0.31
22:6 $n$ -3	0.20 ± 0.09	0.22 ± 0.05	0.47 ± 0.35
<i>Enterocytes</i>			
<sup>14</sup> C-18:3 $n$ -3			
18:4 $n$ -3	0.13 ± 0.02	0.17 ± 0.03	0.17 ± 0.02
20:3 $n$ -3	0.40 ± 0.05	0.42 ± 0.05	0.44 ± 0.14
20:4 $n$ -3	0.20 ± 0.08 <sup>a</sup>	0.07 ± 0.01 <sup>b</sup>	0.11 ± 0.02 <sup>ab</sup>
22:4 $n$ -3	0.21 ± 0.04 <sup>b</sup>	0.41 ± 0.12 <sup>ab</sup>	0.59 ± 0.16 <sup>a</sup>
Pentaenes+hexaenes	0.12 ± 0.02 <sup>b</sup>	0.21 ± 0.03 <sup>a</sup>	0.22 ± 0.04 <sup>a</sup>
Total desaturated	0.66 ± 0.04 <sup>c</sup>	0.86 ± 0.03 <sup>b</sup>	1.09 ± 0.03 <sup>a</sup>
<sup>14</sup> C-20:5 $n$ -3			
22:5 $n$ -3	0.32 ± 0.11 <sup>b</sup>	0.78 ± 0.22 <sup>a</sup>	0.86 ± 0.19 <sup>a</sup>
22:6 $n$ -3	0.32 ± 0.10 <sup>c</sup>	0.56 ± 0.07 <sup>b</sup>	1.05 ± 0.63 <sup>a</sup>

Results are means ± S.D. ( $n=3$ ). An S.D. of 0.0 implies an S.D. of <0.05. Significant effects due to diet were determined by one-way analysis of variance and Tukey's multiple range test. Values within a row bearing different superscript letters in the same row are significantly different ( $P<0.05$ ).



fold) in enterocytes of fish fed on experimental diets than in control fish although still represented only a very low level (Table 6). However, no significant dietary effect was observed on total desaturation of  $[1-^{14}\text{C}]18:3n-3$  in either hepatocytes or enterocytes fed experimental diets containing blends of vegetable oils (Table 6). The desaturation of  $[1-^{14}\text{C}]20:5n-3$  to DHA was also higher in enterocytes than in hepatocytes, but, whereas no significant differences were observed in desaturation rates among treatments in hepatocytes, a significant increasing effect was observed in enterocytes of fish fed diets containing vegetable oils.

The total desaturation activity of  $^{14}\text{C}$ -EPA in hepatocytes varied between 0.20 and 0.47 pmol/mg protein/h detected as hexaenes, which represented only between 1.0% and 1.9% of total radioactivity recovered (Table 6). The vast majority of precursor  $[1-^{14}\text{C}]20:5n-3$  remained unmetabolized at between 96.4% and 96.6% of total activity recovered. No significant differences were observed among different treatments. The total desaturation activity of  $^{14}\text{C}$ -EPA in pyloric caeca enterocytes varied between 0.32 and 1.05 pmol/mg protein/h detected as hexaenes, representing only between 0.8% and 1.3% of total radioactivity recovered. Almost 98% of radioactivity was recovered unmetabolized as  $[1-^{14}\text{C}]20:5n-3$ . No significant differences were observed among dietary treatments (Table 6). Recovery of radioactivity as hexaenes for treatments B and C in pyloric caeca were higher than the equivalent values in hepatocytes.

### 3.5. Effect of diet on hepatocyte and enterocyte fatty acid oxidation activities

The rates of  $\beta$ -oxidation of  $[1-^{14}\text{C}]18:3n-3$  were up to 700-fold higher than the rate of desaturation of  $[1-^{14}\text{C}]18:3n-3$  in hepatocytes and up to –200-fold higher in isolated enterocytes from pyloric caeca. The rates of  $\beta$ -oxidation of  $[1-^{14}\text{C}]20:5n-3$  in hepatocytes were 136-fold, 106-fold and 42-fold higher than the rates of desaturation of  $[1-^{14}\text{C}]20:5n-3$  to hexaenes, for treatments A, B and C, respectively. Similarly, the rates of  $\beta$ -oxidation of  $[1-^{14}\text{C}]20:5n-3$  in enterocytes were 161-fold, 72-fold and 42-fold higher than the rates of desaturation of  $[1-^{14}\text{C}]20:5n-3$  to hexaenes, for treatments A, B and C, respectively. However, no significant differences were observed among rates of

Table 7

$\beta$ -Oxidation activities (pmol/mg protein/h) of  $^{14}\text{C}$ -18:3n-3 and  $^{14}\text{C}$ -20:5n-3 by hepatocytes and enterocytes of European sea bass fed the experimental diets, containing partial substitution of dietary fish oil with vegetable oils

Dietary treatments	A	B	C
<i>Hepatocytes</i>			
$^{14}\text{C}$ -18:3n-3	138.1 $\pm$ 13.1	139.5 $\pm$ 8.7	159.8 $\pm$ 7.7
$^{14}\text{C}$ -20:5n-3	27.3 $\pm$ 1.5	23.3 $\pm$ 1.5	19.9 $\pm$ 2.3
<i>Enterocytes</i>			
$^{14}\text{C}$ -18:3n-3	182.4 $\pm$ 11.2 <sup>ab</sup>	164.1 $\pm$ 15.9 <sup>b</sup>	212.7 $\pm$ 15.7 <sup>a</sup>
$^{14}\text{C}$ -20:5n-3	51.4 $\pm$ 10.5	39.9 $\pm$ 14.9	44.4 $\pm$ 4.1

Results are means  $\pm$  S.D. ( $n=3$ ). An S.D. of 0.0 implies an S.D. of  $<0.05$ . Significant effects due to diet were determined by one-way analysis of variance and Tukey's multiple range test. Values within a row bearing different superscript letters in the same row are significantly different ( $P<0.05$ ).

$\beta$ -oxidation in either hepatocytes or enterocytes, with the exception of  $[1-^{14}\text{C}]18:3n-3$   $\beta$ -oxidized in isolated enterocytes, with higher values in treatments A (control) and C compared to treatment B (Table 7).

## 4. Discussion

The pathway from LA to AA and from LNA to EPA and DHA involves desaturations at the  $\Delta 6$  and  $\Delta 5$  positions of the carbon chain, and an intermediate chain elongation step. Synthesis of DHA from EPA requires an additional desaturation and chain elongations, although the mechanism is more complicated. EPA is elongated to 22:5n-3 and 24:5n-3, which is then converted by  $\Delta 6$  desaturation to 24:6n-3 and, finally, chain shortened to DHA in the peroxisomes (Buzzi et al., 1996, 1997, Sprecher and Chen, 1999; Sargent et al., 2002). However, there is wide variation among fish species in their ability to synthesize HUFA (Sargent et al., 1995, 1999, 2002). Freshwater species such as carp, tilapia and trout are capable of converting dietary  $\text{C}_{18}$  precursor fatty acids to AA, EPA and DHA. However, marine fish such as turbot and sea bream, which are inherently carnivorous, have very limited capacities to perform these conversions and, in consequence, have an absolute dietary requirement for  $\text{C}_{20}$  and  $\text{C}_{22}$  HUFA (Owen et al., 1975; Mourente and Tocher, 1994; Buzzi et al., 1997; Sargent et al., 1999, 2002). Thus, the physiologically active HUFAs (AA, EPA and DHA) are required for optimal health and

development of marine fish. The paradigm represented by a metabolic “freshwater” pattern versus a metabolic “marine” pattern in the bioconversion of  $C_{18}$  PUFAs to  $C_{20}$  and  $C_{22}$  HUFAs may be associated with adaptation of fish to a carnivorous lifestyle (Mourente and Tocher, 1993a,b, 1994, 1998; Henderson et al., 1995; Sargent et al., 1995; Tocher and Ghioni, 1999). The hypothesis being that consumption of a carnivorous (piscivorous) diet, naturally rich in HUFA, results in an evolutionary down-regulation of the desaturase and/or elongase enzyme activities required for the conversion of  $C_{18}$  PUFA to HUFA (Sargent et al., 2002).

In a previous study, we demonstrated that the European sea bass, as a carnivorous marine fish, presented a “marine” fish pattern in the metabolism of  $[1-^{14}C]18:3n-3$  to EPA and DHA (Mourente and Dick, 2002). Although the sea bass appeared to contain all the enzymic activities necessary to produce DHA, the amount of radioactivity recovered in DHA was very low and the overall rate of fatty acid bioconversion was also extremely low. In addition, and in contrast to other species studied, nutritional regulation of hepatocyte fatty acid desaturation was minimal. However, sea bass store a substantial amount of lipid in their livers, which may have influenced the results obtained. Therefore, in the present study, enterocytes were also investigated as they have been shown to have significant fatty acid desaturation and elongation activity in salmonids (Tocher et al., 2002, 2004; Bell et al., 2003). Furthermore, the deficiencies in the HUFA biosynthetic pathway have been linked to low  $C_{18-20}$  elongase in turbot cells (Ghioni et al., 1999) and very low  $\Delta 5$  desaturase activity in sea bream cells (Tocher and Ghioni, 1999). Therefore, the conversion of  $[1-^{14}C]20:5n-3$  to DHA, which bypasses these steps, was also investigated in both cell types. However, in the present study, investigating the effects of partial replacement of dietary fish oil (60%) with a blend vegetable oils (rapeseed, palm and linseed), the desaturation/elongation of  $[1-^{14}C]18:3n-3$  and  $[1-^{14}C]20:5n-3$  in both hepatocytes and enterocytes was confirmed as being very low. In addition, the majority of the radioactivity was recovered in  $\Delta 6$  products, whereas recovery of radioactivity in  $\Delta 5$  desaturated products was minimal. This is consistent with several earlier studies with hepatocytes from salmonids and freshwater fish, in which the recovery

of radioactivity from  $[1-^{14}C]18:3n-3$  in products of  $\Delta 6$  desaturation and  $C_{18-20}$  elongation ( $18:4n-3$  and  $20:4n-3$ , respectively) was generally greater than the recovery of radioactivity in  $\Delta 5$  desaturated products, irrespective of dietary oil (Bell et al., 2001a,b, 2002; Tocher et al., 2001a,b, 2002, 2003). In contrast, in our previous study on sea bass hepatocytes, a slightly different pattern to that observed in other species was observed in that DHA appeared to be the most abundant product of desaturation and elongation of  $[1-^{14}C]18:3n-3$ , albeit that overall recovery of radioactivity in desaturated products was very low. However, the results from the present study are entirely consistent with studies in salmonids and freshwater fish, with  $\Delta 6$  products being the predominant products recovered from the desaturation and elongation of  $[1-^{14}C]18:3n-3$  in hepatocytes from sea bass.

A further aim in the present study was to determine if enterocytes from pyloric caeca were a site of significant fatty acid desaturation/elongation in sea bass. The present study has shown for the first time and this was also the case in a carnivorous marine fish. However, the rates of desaturation/elongation, although higher in enterocytes than in hepatocytes, were still very low. The highest values for total desaturation/elongation activities found in either hepatocytes or enterocytes in sea bass in the present study are lower than those reported in salmon fed a diet containing fish oil (Tocher et al., 2002), and much lower than those in rainbow trout fed diets containing either fish oil or fish oil partially replaced by palm oil (Tocher et al., 2004).

The biochemical mechanisms underpinning the nutritional regulation of the fatty acyl desaturation/elongation pathway are unclear. In general terms, feeding vegetable oils could increase the activity of the PUFA desaturation/elongation through two mechanisms: (i) the pathway could be stimulated by increased substrate  $C_{18}$  PUFA concentrations and/or (ii) the lack of  $C_{20}$  and  $C_{22}$  HUFA could increase activity of the pathway through decreased product inhibition. Certainly, HUFA appear to suppress fatty acid desaturation in mammals (Nakamura and Nara, 2002, 2003). Moreover, a study concluded that both decreased product (HUFA) inhibition and increased substrate provision were factors determining fatty acid desaturation activity in salmon fed vegetable oil (Tocher et al., 2003). Therefore, in

addition to possible evolutionary adaptations, diet is also known to affect fatty acyl desaturase enzyme activities in fish (Tocher et al., 1996; Sargent et al., 2002), possibly in a very similar manner to the nutritional regulation of desaturase activities in mammals (Brenner, 1981). Specifically, the activity of the HUFA synthesis pathway is depressed when animals, including fish, are fed fish oil rich in  $n-3$  HUFA compared to animals fed vegetable oils rich in  $C_{18}$  EFA. In the present study, no nutritional regulation of the desaturation and elongation of  $[1-^{14}C]18:3n-3$  or  $[1-^{14}C]20:5n-3$  in hepatocytes was observed by partially substituting dietary FO with blends of rapeseed, linseed and palm oil. In contrast, a small but significant nutritional influence was detected in enterocytes (diet C > diet B > control diet) although the effect is not quantitatively comparable to that found in salmonids. Numerous studies have shown that inclusion of vegetable oils in diets for freshwater and diadromous fish species increases hepatic fatty acid desaturase activities (Buzzi et al., 1996; Bell et al., 1997; Tocher et al., 1997, 2000, 2001a,b). In Arctic charr, the conversion of intraperitoneally injected  $^{14}C$ -labelled LA and LNA was increased in fish fed diets containing only  $C_{18}$  PUFA compared to fish fed commercial diets containing fish oil (Olsen and Ringo, 1992). In rainbow trout, hepatic fatty acid desaturase activities were also induced when fed a diet containing olive oil compared to a diet containing fish oil (Buzzi et al., 1996). Hepatic desaturation of LA and LNA was significantly greater in Atlantic salmon parr in freshwater fed diets containing vegetable oils (rapeseed and linseed oils) compared to post-smolts in seawater fed diets containing fish oil (Bell et al., 1997; Tocher et al., 1997, 2000). Fatty acid desaturation/elongation in hepatocytes was also nutritional regulated in two warm freshwater species, the zebrafish and Nile tilapia (Tocher et al., 2001a). Moreover, several further studies have confirmed that PUFA desaturation and elongation in hepatocytes from salmonid fish were increased in fish fed diets rich in  $C_{18}$  EFA compared to fish fed standard diets containing FO rich in  $C_{20}$  and  $C_{22}$  HUFA (Bell et al., 2001a, 2002; Tocher et al., 2001b). Thus, fish fed dietary vegetable oil showed higher activities compared to those fish fed fish oil, although the desaturation of LNA was still insufficient to main-

tain tissue proportions of EPA and DHA in fish fed vegetable oil at the same level as in fish fed fish oil (Tocher et al., 2002).

A possible explanation for the lack of nutritional regulation of HUFA synthesis in sea bass hepatocytes could simply be related to the fact that the activity of the pathway is so low. However, there may be other explanations. For instance, that fatty acyl desaturation and elongation was both higher in enterocytes and also showed some minimal nutritional regulation compared to hepatocytes may suggest another factor is involved. Perhaps fatty acid metabolism in terms of HUFA synthesis is different in fish like sea bass that store substantial amounts of lipid in their liver, and/or high lipid levels in the hepatocytes may more directly interfere or compete in the assay. However, a full explanation of the apparent difference in fatty acyl desaturation and elongation activities in hepatocytes and enterocytes and their nutritional regulation in sea bass will require more research, including actual measurement of desaturase and elongase gene expression. A very recent study, reporting both gene expression and enzyme activities in an investigation of the nutritional regulation of the HUFA biosynthetic pathway in hepatocytes of Atlantic salmon fed with diets containing vegetable oils, presented evidence that transcriptional control of fatty acid desaturase and elongase gene expression is one mechanism, whereby dietary fatty acids could influence the activity of the HUFA biosynthetic pathway (Zheng et al., 2004).

A parallel aim in the present study was to determine the extent to which  $[1-^{14}C]18:3n-3$  and  $[1-^{14}C]20:5n-3$  were oxidized in comparison with the amount desaturated/elongated simultaneously, in a combined assay, in both hepatocytes and enterocytes. The results showed that, in both hepatocytes and enterocytes, more radioactivity was recovered in acid-soluble products than that was recovered in desaturated products, indicating that a greater amount of the exogenously added  $[1-^{14}C]18:3n-3$  and  $[1-^{14}C]20:5n-3$  were being oxidized. Thus, since sea bass showed very low desaturation/elongation activities, total  $\beta$ -oxidation activities for  $[1-^{14}C]18:3n-3$  and  $[1-^{14}C]20:5n-3$  in hepatocytes were over 600-fold and over 100-fold higher, respectively, than the values for total desaturation activities. In enterocytes, total  $\beta$ -oxidation

activities for  $[1-^{14}\text{C}]18:3n-3$  and  $[1-^{14}\text{C}]20:5n-3$  were over 200-fold and 100-fold higher than desaturation activities, respectively. Values for  $\beta$ -oxidation activities in sea bass were higher than those found for Atlantic salmon (Tocher et al., 2002) and rainbow trout (Tocher et al. 2004) fed with diets containing FO and FO partially replaced with linseed or palm oils. The fatty acid oxidation activities were generally higher in enterocytes compared to hepatocytes, as also observed in previous studies with salmonids fed with diets containing linseed oil and palm oil (Tocher et al., 2002, 2004). In addition, the present study showed that  $[1-^{14}\text{C}]18:3n-3$  was quantitatively oxidized to a significantly greater extent than  $[1-^{14}\text{C}]20:5n-3$  in both hepatocytes and enterocytes. Therefore, overall, sea bass as a marine fish showed that a greater proportion of fatty acid was  $\beta$ -oxidized compared to desaturated, independent of the cell type, in comparison to values obtained with salmonid fish. There are few data in the literature on  $\beta$ -oxidation of fatty acid in fish tissues such as hepatocytes or intestinal tissue, particularly in marine fish, with which to compare the data in the present study. Relatively high levels of fatty acid oxidation have been reported for red muscle and heart in trout, whereas liver, kidney and white muscle have only limited capacity to oxidize fatty acids (Henderson and Tocher, 1987). In recent studies, white muscle was shown to be a quantitatively important site of fatty acid catabolism in salmon, particularly juveniles (Frøyland et al., 1998, 2000), but liver was not a major site of mitochondrial or peroxisomal  $\beta$ -oxidation (Frøyland et al., 2000). However, in the present study, hepatocytes and enterocytes showed similar values for  $\beta$ -oxidation activities, although enterocyte fatty acid oxidation activities were generally slightly higher than in hepatocytes. Moreover, no nutritional effect on  $\beta$ -oxidation of  $[1-^{14}\text{C}]18:3n-3$  and  $[1-^{14}\text{C}]20:5n-3$  was observed in sea bass hepatocytes by the inclusion of vegetable oils blends in the diets. Similarly, no nutritional effect was observed in relation to the oxidation of  $[1-^{14}\text{C}]20:5n-3$  in enterocytes from sea bass. Although there were minor statistical differences in the  $\beta$ -oxidation of  $[1-^{14}\text{C}]18:3n-3$  in enterocytes, these did not fit any logical pattern with one vegetable oil diet showing the highest level of oxidation with the other vegetable oil diet the lowest,

with fish oil intermediate. The differences are small and likely to have no physiological significance.

## 5. Conclusions

In conclusion, the results obtained in the present study support the following hypothesis: (i) Partial replacement (60%) of fish oil with blends of rapeseed, linseed and palm oils in diets for European sea bass did not significantly compromise growth performance and survival of the fish during a 64 week trial. (ii) The rates of desaturation/elongation activities of  $^{14}\text{C}$ -LNA to EPA and DHA were very low in hepatocytes from all dietary treatments and no significant differences were observed among different treatments. The rates of desaturation of  $^{14}\text{C}$ -LNA in pyloric caeca enterocytes were higher than in hepatocytes but still very low. (iii) The total desaturation/elongation activities of  $^{14}\text{C}$ -EPA in enterocytes were higher than in hepatocytes but no significant differences were found among treatments. (iv) The rates of  $\beta$ -oxidation of  $^{14}\text{C}$ -LNA and  $^{14}\text{C}$ -EPA were much higher than the rates of desaturation and elongation in both hepatocytes and enterocytes, and no significant statistical differences were observed in either cell type among treatments. (v) European sea bass (a carnivorous marine fish) showed no clear quantitative nutritional effects by the inclusion of vegetable oils in the diet upon the desaturation/elongation and  $\beta$ -oxidation activities of  $^{14}\text{C}$ -LNA and  $^{14}\text{C}$ -EPA in isolated hepatocytes and pyloric caeca enterocytes.

## Acknowledgements

This work was supported by contract no. QLRT-2000-30058 of the project “Researching Alternatives to Fish Oils in Aquaculture, RAFOA” of the European Commission, Directorate General Fisheries as part of the Fifth Framework Programme “Quality of Life and Management of Living Resources”.

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