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Effects of dietary fish oil substitution by *Echium* oil on enterocyte and hepatocyte lipid metabolism of gilthead seabream (*Sparus aurata* L.)

[1&]Mercedes Díaz-López, [1] Mª José Pérez, [1]Nieves Guadalupe Acosta, [2]Salvador Jerez, [3]Roberto Dorta-Guerra, [4]Douglas R. Tocher, [1]Antonio Lorenzo, [1]Covadonga Rodríguez

[1] Departamento de Biología Animal (U.D.I. Fisiología Animal), Facultad de Biología, Universidad de La Laguna, 38206 La Laguna, Spain.

[2] Centro Oceanográfico de Canarias (I.E.O.), Crta. de San Andrés s/n, 38120 S/C de Tenerife, Spain.

[3] Departamento de Estadística, I.O. y Computación, Facultad de Matemáticas, Universidad de La Laguna, 38206 La Laguna, Spain.

[4]Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK.

[&] Corresponding author:
Mercedes Díaz López.
Departamento de Biología Animal (U.D.I. Fisiología Animal) Facultad de Biología, Universidad de La Laguna, C/ Astrofísico Francisco Sánchez s/n, 38206 La Laguna, Tenerife, Spain.
Tel: +34-922-318337
Fax: +34-922-318311
E-mail address: mercidl81@hotmail.com

Abstract

The fatty acid profile of vegetable oils (VOs), together with the poor ability of marine fish to convert polyunsaturated fatty acids (PUFA) to highly unsaturated fatty acids (HUFA), lead to important changes in the nutritional value of farmed fish fed VO, which include increased fat and 18:2n-6 and reduced n-3 HUFA. *Echium* oil (EO) has a good n-3/n-6 balance as well as an interesting profile with its high content of unusual fatty acids (SDA, 18:4n-3 and GLA, 18:3n-6) that are of increasing pharmacological interest. The effects of substituting 50 % of dietary fish oil (FO) by EO on gilthead seabream (*Sparus aurata* L.) enterocyte and hepatocyte lipid metabolism were studied. After 4 months of feeding, cell viability, total lipid contents and lipid class compositions were not affected by EO. The cells clearly reflected the fatty acid profile of the EO showing increased SDA, GLA and its elongation product 20:3n-6, and only minorly decreased n-3 HUFA compared to other VO. Metabolism of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 was also unaffected by EO in terms of total uptake, incorporation, β-oxidation and elongation-desaturation activities.

Keywords:

1. Introduction

Diets for the major carnivorous finfish species farmed in Europe have traditionally been based on fish meal and fish oil (FO) (Turchini et al., 2009). However, aquaculture will potentially utilize 80-100 % of the global fish oil supply by 2010 (Bell and Waagbo, 2008; Turchini et al., 2009). As a consequence, the sustainable development of aquaculture requires dietary FO to be substituted with vegetable oils (VO) that are available, sustainable and cost effective (Sargent et al., 2002; Bell and Waagbo, 2008; Turchini et al., 2009).

The poor ability of marine fish to convert linolenic (18:3n-3, LNA) and linoleic (18:2n-6, LA) acids to the highly unsaturated fatty acids (HUFA), 20:5n-3 (eicosapentaenoic, EPA), 22:6n-3 (docosahexaenoic, DHA) and 20:4n-6 (arachidonic, ARA) compared to fresh water fish may be associated with the latter having higher concentrations of LNA and LA and limited EPA and, especially, DHA in their diet compared to the former (Sargent et al., 2002; Bell and Tocher, 2009). Therefore, HUFA are essential fatty acids (EFA) for marine fish, and are important as structural components of cell membranes (Sargent et al., 2002; Marsh, 2008), eicosanoids precursors (Tocher, 2003; Bell et al., 2006; Villalta et al., 2007) and regulators of gene expression (Zheng et al., 2005; Miller et al., 2008; Leaver et al., 2008). The fatty acid profile of VO, together with this poor ability of marine fish to convert C_{18} polyunsaturated fatty acids

(PUFA) to HUFA, lead to important changes in the fatty acid composition of flesh of fish fed VOs, which include decreased n-3 HUFA and increased fat and LA contents (Sargent et al., 2002; Menoyo et al., 2004; Bell et al., 2006; Benedito-Palos et al., 2007; Díaz-López et al., 2009; Fountoulaki et al., 2009). Excessive consumption of VO and, particularly 18:2n-6, has been related to some detrimental effects on health of fish (McKenzie, 2001; Sargent et al., 2002; Montero et al., 2003, 2008). For these reasons, replacement of dietary FO must be approached with caution, and is only possible in marine fish when HUFA are present in feeds at sufficient quantities to meet EFA requirements, and to maintain nutritional value for human consumption. In this sense, up to 50-70% substitution of FO by VO has been reported to maintain the correct growth and health of some marine fish species including seabream and seabass (Sargent et al., 2002; Montero et al., 2003; Menoyo et al., 2004; Mourente et al., 2005; Benedito-Palos et al., 2007; Díaz-López et al., 2009; Fountoulaki et al., 2009). However, research is now focused at identifying VOs or blends that avoid excessive deposition of fat and LA, provide a good n-3/n-6 HUFA ratio, and maximize potential for conversion of C_{18} n-3PUFA to EPA and DHA. In this sense, there are several studies demonstrating that dietary VOs induce the conversion of LA to ARA, and LNA to EPA and DHA in both hepatocytes and enterocytes in salmonids, although total n-3 HUFA levels are still significantly decreased in flesh and liver (Bell et al., 2001, 2002; Tocher et al., 2002, 2004, 2006; Fonseca-Madrigal et al., 2006). However, HUFA synthesis is very low and generally not increased by dietary VOs in marine fish (Mourente et al., 2005; Almaida-Pagán et al., 2007).

The *Echium* genus (*Boraginaceae*) seed oils are relatively rich in LNA and 18:4n-3 (stearidonic acid, SDA) as well as 18:3n-6 (γ-linolenic acid, GLA), and only contains moderate levels of LA compared to other VOs (Guil-Guerrero et al., 2000a,b). Thus, *Echium* oil (EO) has a good n-3/n-6 PUFA balance as well as an interesting profile with its high content of unusual fatty acids (SDA and GLA) that are of increasing pharmacological interest based on their competitive and inhibitory effects in the production of proinflammatory eicosanoids derived from ARA (Tocher, 2003; Villalta et al., 2007; Chilton et al., 2008; Whelan, 2009). The high levels of SDA, compared to LNA, may also facilitate its conversion into n-3 HUFA, since it does not require the first, rate-limiting, Δ6 desaturation step.

Gilthead seabream, *Sparus aurata* L., is the most important marine fish species in Mediterranean and Canarian aquaculture, and several studies have shown that it can generally be grown well on diets with FO partially replaced by VO (Montero et al., 2003; Caballero et al., 2003, 2004; Menoyo et al., 2004; Benedito-Palos et al., 2007; Díaz-López et al., 2009; Fountoulaki et al., 2009). However knowledge of seabream lipid metabolism is still incomplete compared to other fish species, such as salmonids, turbot, and seabass (Sargent et al., 2002;

Rodríguez et al., 2002; Mourente et al., 2005; Bell et al., 2001, 2002, 2006; Tocher et al., 2002, 2004, 2006; Fonseca-Madrigal et al., 2006). Thus, neither HUFA synthesis, nor β-oxidation processes have been investigated in gilthead seabream.

Hepatocytes and enterocytes play critical roles in lipid metabolism including uptake, oxidation, and conversion of fatty acids and the supply of HUFA to the other tissues. Much information of the biosynthetic pathways of HUFA synthesis and other aspects of fatty acid metabolism has been obtained by incubating isolated fish cells with radiolabeled fatty acids (Bell et al., 2001, 2002, 2006; Rodríguez et al., 2002; Tocher et al., 2002, 2004, 2006; Mourente et al., 2005; Fonseca-Madrigal et al., 2006; Almaida-Pagán et al., 2007). Therefore, the aim of the present study was to evaluate the effects of 50% substitution of dietary FO by EO, in gilthead seabream lipid profile and fatty acid metabolism in isolated hepatocytes and enterocytes.

2. Material and methods

2.1. Fish, diets and sampling

Gilthead seabream juveniles obtained from a local fish farm (CEDRA S.L.L.) were distributed into six 500L circular tanks (14 fish per tank) and reared under constantly flowing seawater, and natural photoperiod at 19-19.5 ºC, at Centro Oceanográfico de Canarias (I.E.O.) (Tenerife, Spain). After a 4-week acclimatization period, where fish were fed an extruded commercial diet (Aqualife 17, Biomar S.A., France), fish from three of the tanks were changed to a pelletized experimental diet containing 50 % FO and 50 % EO, manufactured by the Institute of Aquaculture at Stirling University (Scotland, U.K.). EO was produced by cold pressing of seeds from both *Echium plantagineum* and *Echium vulgare* (Goerlich Pharma, Spain). Proximate composition, lipid class and fatty acid profiles of the diets are shown in Table 1.

The fish were fed *ad libitum* twice a day, from Monday to Friday. The fish, initial mass 265.05 ± 49.75 g, were sampled at the beginning of the experimental trial and then monthly, to obtain final growth and specific growth rates (SGR). Mortality was registered daily. After 4 months of feeding, 6 fish per dietary treatment were randomly collected, anesthetized with 1 mL of chlorobutanol in ethanol per litre seawater and killed by a blow to the head and liver and gut rapidly removed for cell isolation and subsequent metabolic assays. The entire experiment was conducted in accordance with Spanish law 223/1988 (B.O.E. 18th March) for protection of experimental animals, in agreement with European law 89/609/CE.

2.2. Preparation of isolated enterocytes and hepatocytes.

After 48 h of starvation, the foregut, including pyloric caeca, was rapidly removed from the

carcass, cleaned of adhering adipose tissue, and contents removed with cold (4 ºC) physiological saline solution III (Dópido et al., 2004). The intestine was then filled, immersed and incubated with a hyperosmolar solution. The process was repeated twice with the same solution containing 0.2 mM EDTA and 0.5 mM dithiothreitol (DTT) as disaggregants and then the luminal solution, mainly enterocytes loosened from the epithelia, was filtered. The resultant cell suspension was centrifuged and the pellet resuspended and incubated in a similar solution containing collagenase (0.5 mg/mL). Hepatocytes were prepared basically as described by Rodríguez et al. (2002). Liver was cannulated through the hepatic portal vein or another suitable vessel to clear the liver of blood with a physiological solution. The liver was then finely chopped in a beaker with HBSS solution containing collagenase (1 mg/mL). Both gut and liver preparations were incubated with collagenase for 15 min at 25 ºC with shaking. The resultant cell suspensions were filtered through a 60-100 µm nylon mesh and centrifuged. The cell pellet was washed with 20 mL of HBSS containing 1% w/v fatty acid-free bovine serum albumin (FAF-BSA) and re-centrifuged at 700 *g* for 15 min. The isolated cells were then suspended in 50 mL of cold M199 medium (Rodríguez et al., 2002). Fifty to 100 µl samples of cell suspensions were collected and stored at -80 ºC for protein determination (Lowry et al., 1951).

2.3. Enterocyte and hepatocyte viability.

Cell viability was assessed by Trypan-blue dye exclusion and an oxygen consumption test prior to metabolic assays. In the Trypan blue dye exclusion method, 50µl cell suspensions were diluted with 200 µL 0.4% Trypan blue solution, stained for 5 min. at room temperature, and viable cells (dye excluded) counted using a Neubauer haemocytometer. Data were expressed as mean percentage of total viable cells. This measure was also taken after the incubation period in both, the control cells and cells with radiolabelled fatty acids.

Oxygen consumption was measured on freshly isolated cells in an O_2-saturated closed system using a Clark electrode connected to a computerized data acquisition system, and processed with RESPI software (Jeulin SAV, France). Approximately 120×10^6 cells in 3 mL M199 solution were added to the incubation chamber and maintained at 20 °C. After a 5 min stabilization period, O_2 concentrations were linear with time and plotted on-line for 10-15 min. Experimental zero O_2 concentration was obtained by adding sodium dithionite. The rate of O_2 consumption (R_{Oxygen}) was calculated as the slope of the linear relationship in the plots [O_2] versus time, and expressed in nmol O_2/mg protein/hour (nmol $O_2 \cdot$mg pp$^{-1} \cdot h^{-1}$) (Dópido et al., 2004).

2.4. Enterocyte and hepatocyte incubation with ^{14}C-labelled fatty acids.

Six mL of each cell preparation were incubated as monolayers in plastic tissue culture flasks for

2 h at 20ºC with 70 µL (0.35 µCi) of either [1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-3. The radiolabelled fatty acids were added to the medium as their potassium salts bound to FAF-BSA (Ghioni et al., 1997). A third, control group of cells was also maintained under the same conditions, but without adding fatty acid to the incubation media, for subsequent lipid analysis.

2.5. Enterocyte and hepatocyte lipid composition.

After incubation, the 6 mL samples of control cells were centrifuged (700 x *g*, 15 min) and washed with Hanks balanced salt solution (HBSS). Chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, was added to extract total lipids (TL) as described by Christie (1982). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically. The TL extracts were stored in chloroform/methanol (2:1) with BHT at -20º C until analysis. The lipid class composition of TL from the control cells was determined by HPTLC using single-dimension double-development as previously described (Tocher and Harvie, 1988). The classes were quantified by charring followed by calibrated densitometry using a dual-wavelength flying spot scanner CS-9001PC (Shimadzu) (Olsen and Henderson, 1989) and identified according to known lipid class standards. Samples of TL were subjected to acid-catalyzed transmethylation in 1 mL toluene and 2 mL 1% sulfuric acid (v/v) in methanol, for 16 h at 50 ºC, and the resultant fatty acid methyl esters (FAME) purified by TLC (Christie, 1982). The FAME were separated and quantified using a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector (250 ºC) and a fused silica capillary column, Supelcowax TM 10 (30 m x 0.32 mm I.D.), as described in Díaz et al. (2009).

2.6. Enterocyte and hepatocyte fatty acid metabolism

Fatty acid metabolism was determined in the cells incubated with radiolabelled fatty acids.

2.6.1. Beta-oxidation

The assay of fatty acid oxidation in intact cells requires the determination of acid-soluble radioactivity as previously described (Frøyland et al., 2000). Briefly, after incubation, 1 mL of cells (1/6 of cell suspensions) was homogenized and centrifuged at 16000 *g* for 5 min, and 500 µl supernatant placed into a microcentrifuge tube and 100 µL of 6% HBSS/FAF-BSA solution added. After mixing, protein was precipitated by the addition of 1 mL of ice-cold 4 M perchloric acid. After vortexing, tubes were centrifuged (16000 *g*, 10 min), 500 µL supernatant transferred to scintillation vials, and radioactivity determined in a RackBeta 1214 liquid scintillation β-counter (LKB, Wallac, USA). Radioactive in dpm was transformed into pmol/mg protein/hour

(pmol·mg $pp^{-1}h^{-1}$), taking into account specific activity of each substrate and cell protein contents (Lowry et al., 1951).

2.6.2. Incorporation into total lipids

Lipid was extracted from the remaining 5 mL of cells as described in section 2.5 and 1/10 of the TL extracts taken for determining radioactive incorporation as described above. Data of total uptake of radiolabelled fatty acids was calculated as the sum of β-oxidation and incorporation into TL.

2.6.3. Fatty acyl desaturation/elongation.

The remainder of the TL extracts were subjected to acid-catalyzed transmethylation as described above (section 2.5), and FAME then applied as 3-cm streaks to TLC plates previously impregnated with 2 g silver nitrate in 20 mL acetonitrile and preactivated at 110 ºC for 30 min. The plates were fully developed in toluene/acetonitrile (95:5, v/v) to separate the labelled fatty acids according to chain length and degree of unsaturation (Wilson and Sargent, 1992). The developed TLC plates were placed in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen for 7 days (Imagen Screen-K, Biorad, Madrid, Spain). The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad), and the bands quantified first in percentage by the "Quantity One" image software and then transformed into pmol·mg $pp^{-1}h^{-1}$ taking into account incorporation into total lipids of each sample. Identification of labelled bands was confirmed by radiolabelled standards run on the same plates (Rodríguez et al., 2002).

2.7. Chemical and reagents

TLC (20x20 cm, Ø 0.25 mm) and HPTLC (10x10 cm, Ø 0.15 mm) plates, precoated with silica gel (without fluorescent indicator), were from Machery-Nagel (Düren, Germany). Fish oil standard (PUFA N-3) was supplied by SUPELCO (Supelco PARK, Bellefonte, PA, USA). All organic solvents used were of reagent grade and were from Panreac (Barcelona, Spain). OptiPhase "HiSafe" 2 scintillant liquid and radiolabelled fatty acids, [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 were from PerkinElmer (USA). HBSS (Hanks Balanced Salt Solution), M199 with Hanks salts, collagenase type IV, FAF-BSA (bovine serum albumin essentially fatty acid free), butylated hydroxytoluene, potassium chloride and potassium bicarbonate were from Sigma (Spain).

2.8. Statistical analysis

Results are presented as means ± S.D (n=42 for growth parameters and n=6 for the rest of data). The data were checked for normal distribution by the one-sample Kolmogorov-Smirnoff test as well as for homogeneity of the variance with the Levene test and, when necessary, arcsine transformation was applied. Effect of treatment was determined using the Student t-test (2 variables) and $P<0.05$ was considered statistically different. Total lipid content of enterocytes and hepatocytes was analyzed by two-way ANOVA. The percentages of lipid classes and main fatty acids of enterocytes and hepatocytes were included as variables in a principal components analysis (PCA) model. Factor scores were subsequently analyzed by two-way ANOVA to study the combined effects of both: dietary treatments and type of cell, and their interactions. Statistical analysis was performed using SPSS (versions 15.0 and 17.0, SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Diet composition

Total lipid contents of the diets were equal and close to 21% (DWB) (Table 1). Total saturated fatty acids, particularly 16:0, were higher in the FO diet than in the EO diet. Monounsaturated fatty acids other than 16:1 were higher, and n-6 PUFA, 18:2n-6 and 18:3n-6, 3-fold higher in the EO diet compared to the FO diet. Total n-3 PUFA fatty acids were similar in the two diets, but EPA and DHA were 2- to 3-fold lower in the EO diet compared to the FO diet, whereas 18:4n-3 and, especially, 18:3n-3, were much higher in the EO diet (Table 1).

3.2. Growth and survival

No significant differences were observed in fish final weight or length among dietary treatments, with values of 351.0 ± 60.0 g and 25.7 ± 1.5 cm for FO-fed fish and 343.3 ± 51.1 g and 25.5 ± 1.3 cm for EO-fed fish, and with an average specific growth rate (SGR) of 0.3 from the second month until the end of the experimental period, for both dietary regimes. Mortality over the experimental period was less than 1% for both treatments.

3.3. Enterocyte and hepatocyte viability

Viability of isolated enterocytes and hepatocytes was not significantly ($P<0.05$) different between the two dietary groups, with values of oxygen consumption of 42.6 ± 15.3 and 47.8 ± 12.9 nmol/ mg pp for enterocytes from FO- and EO-fed fish, respecitively, and 28.6 ± 15.7 and 37.9 ± 13.2 nmol/ mg pp for FO and EO-fed fish hepatocytes, respectively. Similarly, viability rate obtained by the Trypan-blue cell exclusion test was not significantly ($P > 0.05$) different

between the dietary groups with more than 91 % viability achieved even when radioactive substrates were added.

3.4. Enterocyte and hepatocyte lipid composition.

Two-way ANOVA of total lipid contents showed they were not affected by diet (p=0.745) but differed according to cell type (p=0.004) (Table 2). Similarly, lipid class profiles were generally not significantly affected by the dietary regime with the exception of cholesterol and steryl esters (SE) that were lower and higher, respectively, in hepatocytes from EO-fed fish (Table 2). There was a trend in EO enterocytes for TAG to be lower and structural lipids including phospholipids and cholesterol to be higher. The PCA (Fig 1) and two-way ANOVA of PCA highlights the significant differences between the lipid class profiles of the two cell types reflected in principal component 1 (PC1, p=0,000). PC1 was positively related with TAG and negatively with phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM) and SE. Phospholipids (PC and PE) were more abundant in hepatocytes than enterocytes.

After 4 months of feeding, enterocyte and hepatocyte fatty acid profiles clearly reflected those of diets (Table 3). Fish fed the EO diet showed significantly higher proportions of n-6 PUFA, including LA and GLA and also 20:3n-6, with the latter not being present in the diet. Similarly, LNA and SDA were also higher in cells from EO-fed fish, whereas n-3 HUFAs, trended to be lower. As a result, the n-3/n-6 ratio was significantly reduced in cells of fish fed the EO diet (Table 3). The PCA and two-way ANOVA for CP1 of fatty acids (p=0.000) confirmed these differences between dietary treatments (Fig. 2). The PC1 component was clearly related with the most abundant fatty acids in fish fed EO: LA, GLA, LNA, SDA and one of their elongation products (20:3n-6), so PC1 reflects the characteristic EO profile. However, the fatty acid profile was also affected, in principal component 2 (PC2), by the differences between enterocytes and hepatocytes profiles (p=0.009), which was negatively correlated with HUFA (ARA, 20:3n-3, 20:4n-3, EPA, 22:5n-3 and DHA) and positively with oleic acid. The effect of EO was influenced by the cell type in PC1, shown by an interaction between dietary treatment and cell type (Interaction, p=0.031). Thus, enterocytes were more affected by EO showing higher levels of the characteristic EO-derived C_{18} PUFA compared to hepatocytes. Hepatocytes, in general, had more HUFA and less oleic acid than enterocytes.

3.5. Enterocyte and hepatocyte metabolism of radiolabelled substrates

3.5.1. Beta-oxidation, incorporation into total lipid and total absorption

Diet had no effect on β-oxidation of [1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-3 in either enterocytes or hepatocytes (Table 4). Although β-oxidation expressed in absolute terms was similar in both

cellular types, when data were expressed relatively to total uptake, enterocyte β-oxidation was lower than that of hepatocytes (11-15 % in enterocytes vs. 27-37 % in hepatocytes). Incorporation of fatty acids into total lipids as well as total uptake (sum of β-oxidation and incorporation) were also similar for [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3, independent of diet, but fatty acid uptake and incorporation were 2-3 times higher in enterocytes than hepatocytes (Table 4).

3.5.2. Fatty acyl desaturation/elongation.

There were no marked differences between treatments in the production of elongated or desaturated products from the two fatty acid substrates (Table 4). Irrespective of experimental group, cell type, or fatty acid assayed, total radioactivity was almost recovered in a single band (97-99 %) which corresponded to the C_{18} PUFA added to the cells ([1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-3). Bands corresponding to elongation activity were clearer to define than those of desaturation. In this sense, some activity was recovered in 20:2n-6, 22:2n-6, 20:3n-3 and 22:3n-3 whereas no clear bands for 18:3n-6 or 18:4n-3 or any of their elongation-desaturation products were found.

4. Discussion

As reported by other authors (Montero et al., 2003; Izquierdo et al., 2005) a 50% substitution of FO by VO is possible in gilthead seabream without negatively affecting the fish growth.

In agreement with other similar studies performed with enterocytes and hepatocytes from fish fed VOs, cell viability was high (Rodríguez et al., 2002; Almaida-Pagán et al., 2007). Cell oxygen consumption was neither affected by diet showing values that were in the range previously reported for gilthead seabream enterocytes (Dópido et al., 2004).

The inclusion of VOs in carnivorous fish diets is often associated with increased fat contents and lipid droplets in liver and enterocytes, usually when substitution is over 75% (Rodríguez et al., 2002; Caballero et al., 2003; Menoyo et al., 2004; Francis et al., 2007), or at lower levels of substitution when an unbalanced fatty acid profile is provided (Caballero et al., 2003, 2004; Menoyo et al., 2004). Nevertheless, when the substitution level is not too large (50-75 %) and the fatty acid profile of the VO is well balanced, fat deposition in enterocytes and liver is not reported (Caballero et al., 2004; Mourente et al., 2005; Francis et al., 2007). Moreover, in cod, Arctic charr and Atlantic salmon fed up to 100 % EO, TL in muscle and liver did not increase (Bell et al., 2006; Tocher et al., 2006; Miller et al., 2007), in agreement with our previous trial where muscle TL content of gilthead seabream fed EO was even decreased (Díaz-López et al., 2009). Therefore, studies in marine fish should focus on the use of blends of VOs to obtain a

balanced fatty acid profile such as that present in EO to avoid excessive tissue fat deposition. In the same way, the lipid class profile of gilthead seabream enterocytes and hepatocytes was unaffected by EO as reported for other balanced VOs including EO in liver (Mourente et al., 2005; Bell et al., 2006), enterocytes (Tocher et al., 2002) and muscle (Bell et al., 2006; Miller et al., 2007, 2008), of different fish species.

Fatty acid profiles of enterocyte and hepatocyte reflected the dietary VO profile as widely observed for several fish species fed VO. In both cells types, the C_{18} fatty acids were significantly increased, but 22:5n-3 was only significantly reduced in hepatocytes. There was also a trend for DHA and EPA to decrease but not in a significant way. In many other trials, fish fed VOs displayed significant reductions in n-3HUFA together with increased C_{18} fatty acids in liver (Rodríguez et al., 2002; Menoyo et al., 2004; Mourente et al., 2005; Bell et al., 2006; Almaida-Pagán et al., 2007) and enterocytes (Caballero et al., 2003; Mourente et al., 2005; Almaida-Pagán et al., 2007) of different marine fish. Nevertheless, in seabass fed a balanced blend of VOs, enterocytes were capable at regulating fatty acid levels, with C_{18} fatty acids not increasing greatly, and EPA and DHA levels relatively maintained (Mourente et al., 2005). In our previous trial with seabream fed 50% EO, EPA, 22:5n-3 and DHA were reduced in muscle and liver after 4 months of feeding but, surprisingly, DHA was partly recovered in muscle after 7 months of feeding (Díaz-López et al., 2009). In agreement with these findings white muscle of Atlantic salmon parr fed 100% EO, displayed an increment of DHA whereas EPA and 22:5n-3 were maintained although growth rate was relatively low in this trial (Miller et al., 2007). The apparent compensation in the reduction of n-3 HUFA when using EO could be related to the moderated and balanced levels of C_{18} fatty acids of EO and some blends of VOs (Mourente et al., 2005). In this sense, the probability to esterify a n-3 HUFA instead of a C_{18} PUFA during phospholipid turnover may increase when proportions of dietary fatty acids are balanced. However, growth rate and duration of the feeding trial are also factors likely to affect final fatty acid compositions.

The most abundant C_{18} fatty acids of VOs are LA, LNA and 18:1n-9, which were increased in enterocytes and hepatocytes of seabass, sharpsnout seabream and turbot fed VOs (Rodríguez et al., 2002; Mourente et al., 2005; Almaida-Pagán et al., 2007). Nevertheless, with EO other C_{18} PUFA, including GLA (18:3n-6) and SDA (18:4n-3) increased in liver and muscle of freshwater and marine fish (Bell et al., 2006; Tocher et al., 2006; Miller et al., 2008; Díaz-López et al., 2009), and also in enterocytes and hepatocytes in the present trial. The elongation products of these fatty acids, 20:3n-6 and 20:4n-3, are precursors of eicosanoids that have competitive and inhibitory effects on the production of proinflammatory eicosanoids derived from ARA in fish (Bell et al., 2006; Villalta et al., 2007) and in mammals (Chilton et al., 2008; Whelan, 2009). In

this respect, the fatty acid profile of seabream fed EO can be considered beneficial for human consumption.

Another objective of the present study was to determine if the inclusion of EO improves the metabolism of the two main n-3 and n-6 HUFA precursors as has been described for some VOs in salmonids (Tocher et al., 2002, 2004; Fonseca-Madrigal et al., 2006) as studies with marine fish were lacking. The uptake of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 by enterocytes and hepatocytes was unaffected by the EO diet, whereas in rainbow trout enterocytes fed linseed oil, increased uptake of [1-^{14}C]18:3n-3, which was very abundant in this VO, was observed (Geurden et al., 2009). In rainbow trout fed rapeseed oil the uptake of [1-^{14}C]18:3n-3 was maintained (Geurden et al., 2009) whereas it was decreased in hepatocytes of salmon fed a VO blend (Stubhaug et al., 2005). This might be due to a higher uptake of 18:1n-9, more abundant than LNA in these diets. In the same way, the incorporation of both labelled fatty acids into TL was maintained with the EO in the present study and decreased in hepatocytes of salmon fed a VO blend with both fatty acids (Stubhaug et al., 2005).

There was no effect of diet on β-oxidation as occurred in enterocytes and hepatocytes of other marine fish fed VOs (Mourente et al., 2005; Almaida-Pagán et al., 2007). We could expect that oxidation of the C_{18} PUFA, 18:2n-6 and 18:3n-3, abundant in VOs, would be increased in EO-fed fish but this did not happen. However, studies measuring β-oxidation capacity in subcellular fractions, showed that long-chain monoenes were preferred over C_{18} PUFA as substrates in salmonids (Henderson 1996; Frøyland et al., 2000). Therefore, lower levels of long-chain monoenes and higher levels of C_{18} PUFA in VOs than in FO may result in increased β-oxidation of C_{18} PUFA in VO-fed gilthead seabream muscle (Menoyo et al., 2004), salmon enterocytes (Tocher et al., 2002), and sharpsnout seabream hepatocytes (Almaida-Pagán et al., 2007). However, in the present study, the EO diet had higher levels of long-chain monoenes than the FO diet so they were available for β-oxidation. Consistent with this, β-oxidation of the C_{18} PUFA was similar in fish fed FO and VO when the VO blend had a similar level of long-chain monoenes to the FO diet (Mourente et al., 2005). In general, fish cells may use the most abundant fatty acids for β-oxidation but prefer long-chain monoenes to PUFA.

Desaturation of the [1-^{14}C] PUFA in gilthead seabream cells was very low as expected for a marine fish (Rodríguez et al., 2002; Mourente et al., 2005; Almaida-Pagán et al., 2007), and much lower than that reported for salmonids (Tocher et al., 2002, 2004, 2006). Increased HUFA synthesis has been described mainly in freshwater fish and salmonids fed VO diets at different levels of substitution (Bell et al., 2001, 2002; Tocher et al., 2002, 2004; Zheng et al., 2005; Stubhaug et al., 2005; Fonseca-Madrigal et al., 2006). Some of these trials also used EO, or even borage oil from the same plant family in salmonids (Tocher et al., 1997, 2006). These

increments have been related with increased expression of desaturases and elongase genes (Seiliez et al., 2003; Zheng et al., 2004, 2005; Miller et al., 2008). However, in marine fish, the effects of dietary VO appear to depend upon the species and does not increase HUFA synthesis unless the substitution is such that HUFA is under requirement levels which it is the case of Rodríguez et al. (2002) when feeding turbot 100% olive oil. Therefore, desaturation of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 was not increased in cod with 50 % VO substitution (~12% n-3 HUFA) and in our trial with 50% of EO (11% n-3 HUFA), but it was in cod with 75 and 100% (7.9 and ~1.5 % n-3HUFA) (Francis et al., 2007) and with 100 % of EO (7.9 % n-3 HUFA) (Bell et al., 2006). In contrast, 100% VO substitution did not increase HUFA synthesis in the omnivorous marine fish, sharpsnout seabream (Almaida-Pagán et al., 2007). Moreover, studies with gilthead seabream larvae fed different degrees of substitution of FO by VOs did not show any indications of desaturation activity in tissue fatty acid profiles (Robin and Vincent, 2003; Robin and Peron, 2004), as in the present trial. However, molecular studies reported that the expression of the Δ6 desaturase gene was increased when seabream were fed 100% olive oil (Seileiz et al., 2003), similarly to the above mentioned results obtained in turbot fed 100% olive oil (Rodríguez et al., 2002).

Despite the poor desaturation activity displayed for [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3, 20:3n-6 was significantly increased in the EO enterocytes and hepatocytes. This fatty acid was not supplied by the diet, which suggests elongation of the dietary precursor GLA, as happened in larvae seabream fed VO diet supplemented with *Spirulina* algae rich in GLA (Robin and Peron, 2004). This elongation product is probably accumulated in seabream fed VO because Δ5 desaturase activity required to produce 20:4n-6 is absent or very limited in these marine fish species (Tocher and Ghioni, 1999; Seiliez et al., 2003; Zheng et al., 2004). In contrast, there were no differences between the two treatments with respect to the presence of 20:4n-3 in cells. This may simply be a consequence of GLA, the 20:3n-6 precursor, being much higher in the EO diet (22 times fold) than in FO diet. Whereas, SDA, the 20:4n-3 precursor, was only two-fold higher in the EO diet compared to the FO diet, and also 20:4n-3 was much higher in the FO diet than 20:3n-6 and so differences in elongation were only able to be observed with 20:3n-6. The same situation concerning differences in dietary levels and the accumulation of elongation products, 20:3n-6 compared to 20:4n-3, were reported in liver of Arctic char fed EO and FO (Tocher et al., 2006), muscle and liver of cod fed EO and FO (Bell et al., 2006), and muscle and liver of gilthead seabream fed EO or FO in our previous trial (Díaz-López et al., 2009).

In summary, enterocytes and hepatocytes TL contents and lipid class composition were not affected by 50 % substitution of FO by EO. The cells clearly reflected the fatty acid profile of the EO showing increments of healthy fatty acids including SDA, GLA and the elongation

product 20:3n-6. Metabolism of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 was unaffected in terms of uptake, incorporation, β-oxidation and elongation-desaturation activities.

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Legends

Fig 1. a) Factor loadings plot for the lipid classes data. b) Plot of factor scores of the lipid classes for each fish categorized by dietary treatment (□: fish oil. ■: *Echium* oil); c) Plot of factor scores of the lipid classes for each fish categorized by type of cell (Δ: enterocytes. ▲: hepatocytes). SM: Sphingomyelin. PC: Phosphatidylcholine. PS: Phosphatidylserine. PI: Phosphatidylinositol. PG: Phosphatidylglycerol (may also include phosphatidic acid and cardiolipin). PE: Phosphatidylethanolamine. DAG: Diacylglycerol. CHO: Cholesterol. FFA: Free fatty acids. TAG: Triacylglycerols. SE: Sterol esters. UK: Unknown.

Fig 2. a) Factor loadings plot for the fatty acids data. b) Plot of factor scores of the fatty acids for each fish categorized by dietary treatment (□: fish oil. ■: *Echium* oil); c) Plot of factor scores of the fatty acids for each fish categorized by type of cell (Δ: enterocytes. ▲: hepatocytes). UK: Unknown.

Table 1. Proximate composition (%DWB) and fatty acid composition (weight %) of dietary treatments.

	FO diet	EO diet
Moisture	9.0 ± 1.1	8.9 ± 0.3
Ash	7.3 ± 0.2	8.7 ± 0.4
Crude fibre	3.0 ± 0.1	3.1 ± 0.1
Protein	41.6 ± 0.6	43.4 ± 0.9
Fat	20.7 ± 1.6	20.7 ± 0.8
Neutral lipid	19.1 ± 0.1	18.8 ± 0.1
Polar lipid	1.6 ± 0.1	1.9 ± 0.1
16:0	18.7 ± 0.4	10.8 ± -
18:0	3.6 ± 0.2	2.2 ± -
Total saturates	30.5 ± 0.3	17.0 ± -
16:1 [1]	6.7 ± -	3.5 ± -
18:1 n-9	9.8 ± 0.3	12.3 ± -
20:1 [2]	2.1 ± -	6.5 ± -
22:1 [2]	1.8 ± 0.2	7.3 ± -
Total monoenes	25.9 ± 0.1	32.9 ± 0.2
18:2 n-6	**4.4 ± 0.1**	**13.4 ± -**
18:3 n-6	**0.2 ± -**	**4.7 ± -**
20:2 n-6	0.2 ± -	0.2 ± -
20:3 n-6	0.1 ± 0.1	0.0 ± -
20:4 n-6	**0.8 ± -**	**0.1 ± 0.2**
Total n-6 PUFA	6.1 ± 0.2	18.5 ± 0.3
18:3 n-3	**1.4 ± 0.1**	**14.0 ± -**
18:4 n-3	**2.8 ± 0.1**	**5.4 ± -**
20:3 n-3	0.2 ± -	0.3 ± 0.1
20:4 n-3	**0.7 ± -**	**0.1 ± 0.2**
20:5 n-3	**12.3 ± 0.5**	**4.3 ± -**
22:5 n-3	**1.4 ± 0.1**	**0.4 ± -**
22:6 n-3	**13.1 ± 0.2**	**5.6 ± -**
Total n-3 PUFA	32.9 ± 0.7	30.6 ± 0.3
Total n-3 HUFA	28.2 ± 0.7	11.0 ± 0.3
UK	1.5 ± 0.3	0.5 ± 0.1
n-3/n-6	5.4 ± 0.3	1.7 ± -
18:1/n-3 HUFA	0.4 ± -	1.3 ± -
DHA/EPA	1.1 ± -	1.3 ± -
ARA/EPA	0.1 ± -	0.0 ± -

Results represent means ± SD (n=3), SD < 0.1 is represented as "-". Totals include some minor components not shown. DWB: Dry weight basis. pp: protein. FO: Fish oil. EO: *Echium* oil. UK: Unknown. [1] May contain n-9 and n-7 isomers. [2] May contain n-11 and n-9 isomers.

Table 2. Total lipid contents (µg lipid·mg pp^{-1}) and lipid class composition (% of total lipid) of enterocytes and hepatocytes of gilthead seabream fed fish oil or *Echium* oil.

	Enterocytes		Hepatocytes		
	FO diet	EO diet	FO diet	EO diet	
TL	100.8 ± 38.4	106.8 ± 19.0	156.8 ± 60.1	148.0 ± 35.9	
Sphingomyelin	0.6 ± 0.5	0.9 ± 0.2	1.2 ± 0.4	0.8 ± 0.2	
Phosphatidylcholine	10.9 ± 3.5	14.1 ± 3.3	14.0 ± 2.5	13.2 ± 0.8	
Phosphatidylserine	1.1 ± 0.5	1.9 ± 0.6	0.8 ± 0.5	0.8 ± 0.3	
Phosphatidylinositol	2.2 ± 1.0	3.2 ± 0.7	1.7 ± 1.0	1.5 ± 0.9	
Phosphatidylglycerol[1]	2.3 ± 1.0	2.2 ± 0.9	3.6 ± 0.9	3.1 ± 0.9	
Phosphatidylethanolamine	5.1 ± 1.3	7.3 ± 2.6	11.4 ± 3.1	13.5 ± 3.0	
Diacylglycerol	0.9 ± 1.1	0.2 ± 0.5	0.4 ± 0.7	0.4 ± 0.3	
Cholesterol	17.0 ± 4.8	20.2 ± 2.4	15.6 ± 1.5	13.4 ± 2.0	*
Free fatty acids	5.6 ± 4.0	4.9 ± 5.1	7.0 ± 2.7	4.5 ± 1.9	
Triacylglycerols	47.1 ± 10.6	37.6 ± 6.0	35.7 ± 5.6	37.5 ± 6.7	
Sterol esters	3.8 ± 1.3	4.8 ± 1.4	5.7 ± 1.7	9.1 ± 2.6	*
UK	3.2 ± 2.0	2.7 ± 2.0	2.7 ± 1.6	2.0 ± 1.7	
Total polar lipids	22.3 ± 6.6	29.5 ± 7.0	32.8 ± 3.7	33.1 ± 3.2	
Total neutral lipids	74.5 ± 5.9	67.8 ± 8.8	64.5 ± 4.6	64.9 ± 4.0	

Results represent means ± SD (n=6). Pairs of values within a given cell type which are significantly different ($P<0.05$) are shown (*). pp: protein. FO: Fish oil. EO: *Echium* oil. TL: Total lipid. [1] May also include phosphatidic acid and cardiolipin. UK: Unknown.

Table 3. Total fatty acid contents (µg·mg^{-1}pp^{-1}) and fatty acid composition (weight %) of isolated enterocytes and hepatocytes of gilthead seabream fed fish oil or *Echium* oil.

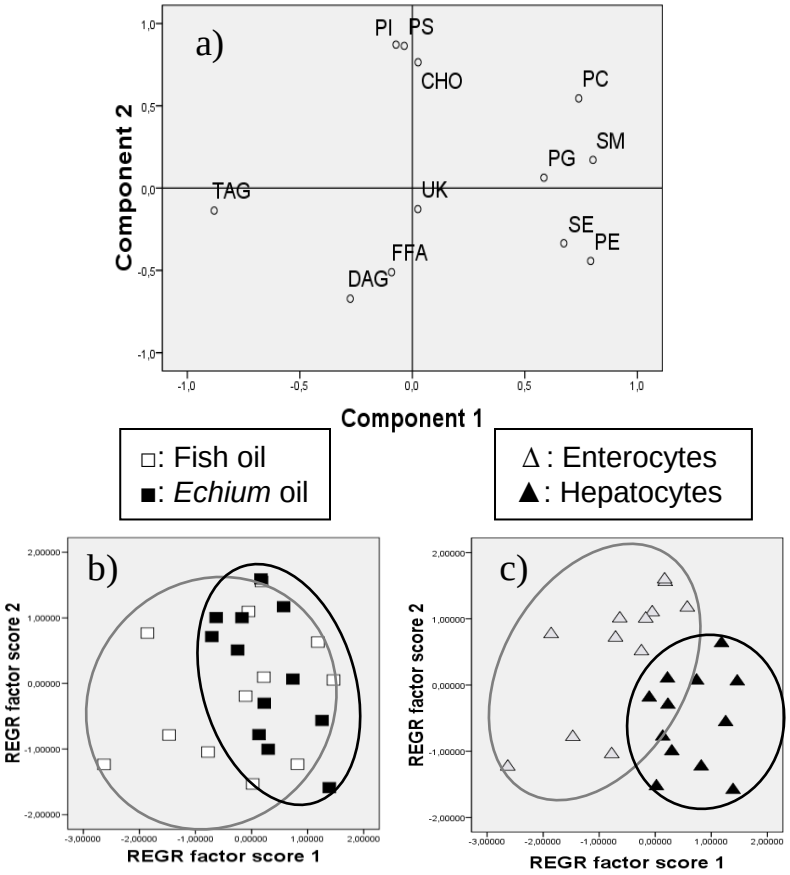
	Enterocytes			Hepatocytes		
	FO diet	EO diet		FO diet	EO diet	
Total FA	47.1 ± 2.7	51.2 ± 5.8		60.5 ± 14.1	67.5 ± 12.2	
14:0	3.7 ± 1.1	2.6 ± 0.7		3.6 ± 0.6	2.8 ± 0.9	
16:0	23.0 ± 3.9	20.1 ± 1.9		21.8 ± 3.8	20.0 ± 2.9	
18:0	7.7 ± 2.3	7.6 ± 0.5		6.2 ± 0.9	7.3 ± 1.8	
Total saturates	36.7 ± 6.4	32.4 ± 3.0		33.3 ± 5.3	31.8 ± 5.0	
16:1 [1]	5.3 ± 1.0	4.8 ± 0.9		6.8 ± 5.9	5.0 ± 1.3	
18:1 n-9	11.8 ± 1.7	13.8 ± 3.3		11.7 ± 2.0	12.2 ± 1.1	
20:1 [2]	3.6 ± 1.2	3.0 ± 1.0		2.4 ± 0.3	2.4 ± 0.3	
22:1 [2]	3.1 ± 1.9	1.6 ± 0.6		2.2 ± 0.5	1.8 ± 0.6	
24:1 n-9	0.8 ± 0.2	1.1 ± 0.6		1.6 ± 0.3	1.8 ± 1.0	
Total monoenes	28.4 ± 4.1	27.5 ± 5.4		27.6 ± 3.4	26.9 ± 3.9	
18:2 n-6	4.4 ± 0.7	8.8 ± 1.1	*	3.8 ± 0.3	7.5 ± 1.5	*
18:3 n-6	0.1 ± 0.1	1.5 ± 0.3	*	0.1 ± 0.1	1.1 ± 0.4	*
20:2 n-6	0.3 ± 0.1	0.3 ± 0.1		0.3 ± -	0.3 ± 0.2	
20:3 n-6	0.1 ± 0.2	0.8 ± 0.4	*	0.1 ± 0.1	0.8 ± 0.3	*
20:4 n-6	1.2 ± 0.2	1.0 ± 0.4		1.2 ± 0.3	1.2 ± 0.6	
Total n-6 PUFA	6.4 ± 1.0	12.4 ± 1.8	*	5.9 ± 0.6	11.3 ± 2.5	*
18:3 n-3	0.8 ± 0.2	4.5 ± 0.8	*	0.8 ± 0.1	3.7 ± 1.5	*
18:4 n-3	0.7 ± 0.4	1.4 ± 0.3	*	0.8 ± 0.1	1.3 ± 0.4	*
20:3 n-3	0.0 ± -	0.1 ± 0.1		0.1 ± 0.1	0.2 ± 0.2	
20:4 n-3	0.6 ± 0.1	0.6 ± 0.2		0.8 ± 0.1	0.8 ± 0.2	
20:5 n-3	5.9 ± 1.9	4.4 ± 1.8		5.4 ± 1.4	3.8 ± 1.1	
22:5 n-3	1.5 ± 0.6	0.8 ± 0.5		2.5 ± 0.5	1.6 ± 0.3	*
22:6 n-3	15.7 ± 2.8	13.3 ± 6.7		19.1 ± 5.9	15.3 ± 4.0	
Total n-3 PUFA	29.6 ± 4.6	27.6 ± 5.9		36.4 ± 6.2	32.2 ± 3.2	
Total n-3 HUFA	23.6 ± 4.4	19.3 ± 8.5		28.0 ± 7.8	21.7 ± 5.3	
UK	1.5 ± 0.4	1.2 ± 0.9		1.5 ± 0.5	1.5 ± 0.9	
n-3/n-6	4.7 ± 0.4	2.3 ± 0.6	*	6.2 ± 0.8	2.9 ± 0.6	*
18:1/n-3 HUFA	0.5 ± 0.2	0.9 ± 0.6		0.5 ± 0.2	0.6 ± 0.2	
DHA/EPA	2.9 ± 0.9	3.0 ± 1.1		3.5 ± 0.3	4.2 ± 0.6	*
ARA/EPA	0.2 ± -	0.2 ± -		0.2 ± -	0.3 ± 0.1	

Results represent means ± SD (n=6) , SD<0.1 is represented as "-". Totals include some minor components not shown. Pairs of values within a given cell type which are significantly different (P<0.05) are shown (*). pp: protein. FO: Fish oil. EO: *Echium* oil. FA: fatty acids. [1] May contain n-9 and n-7 isomers. [2] May contain n-11 and n-9 isomers. UK: Unknown.

Table 4. Metabolic activities: uptake, β-oxidation, incorporation and elongation-desaturation (pmol·mg pp^{-1}·h^{-1}), of enterocytes and hepatocytes of gilthead seabream fed fish oil or *Echium* oil, incubated with of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3.

	Enterocyte		Hepatocyte	
	FO diet	EO diet	FO diet	EO diet
18:2n-6				
Uptake	161.1 ± 46.1	133.4 ± 42.3	57.1 ± 21.0 †	53.5 ± 10.4 †
β-oxidation	20.2 ± 15.4	18.2 ± 5.7	15.5 ± 10.6	19.9 ± 2.4
Incorporation	140.9 ± 41.1	115.3 ± 44.3	41.7 ± 12.5 †	33.6 ± 10.7 †
18:2n-6	138.2 ± 32.7	113.2 ± 38.8	41.0 ± 12.2 †	33.2 ± 10.4 †
20:2n-6	1.0 ± 0.8	0.7 ± 0.6	0.2 ± 0.1	0.0 ± 0.0 *†
22:2n-6	0.8 ± 0.5	0.7 ± 0.4	0.2 ± 0.1 †	0.1 ± 0.2 †
tri-tetraenes	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2	0.2 ± 0.2
pent-hexaenes	0.6 ± 0.4	0.4 ± 0.3	0.0 ± 0.1 †	0.1 ± 0.1 †
elongation	1.8 ± 1.3	1.3 ± 0.8	0.4 ± 0.2 †	0.1 ± 0.3 *†
desaturation	0.9 ± 0.5	0.8 ± 0.6	0.3 ± 0.2 †	0.2 ± 0.2
elongation/desaturation	2.8 ± 1.4	2.1 ± 1.4	0.7 ± 0.4 †	0.4 ± 0.4 †
18:3n-3				
Uptake	136.9 ± 19.2	134.3 ± 42.0	63.3 ± 13.7 †	61.0 ± 16.5 †
β-oxidation	20.4 ± 15.1	16.0 ± 6.3	19.6 ± 13.3	20.4 ± 3.3
Incorporation	116.5 ± 31.8	118.3 ± 43.2	43.7 ± 18.2 †	40.6 ± 16.7 †
18:3n-3	115.6 ± 57.1	116.6 ± 41.4	43.5 ± 18.0 †	40.0 ± 17.7 †
20:3n-3	0.6 ± 0.3	1.0 ± 1.4	0.1 ± 0.1 †	0.3 ± 0.2 *
22:3n-3	0.5 ± 0.4	0.5 ± 0.4	0.2 ± 0.2 †	0.2 ± 0.1
tetraenes	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0
pent-hexaenes	0.6 ± 0.5	0.2 ± 0.4	0.0 ± 0.0 †	0.1 ± 0.1
elongation	1.1 ± 0.7	1.5 ± 1.6	0.2 ± 0.3 †	0.5 ± 0.2
desaturation	0.6 ± 0.6	0.2 ± 0.4	0.0 ± 0.1 †	0.1 ± 0.1
elongation/desaturation	1.7 ± 1.2	1.7 ± 2.0	0.3 ± 0.3 †	0.6 ± 0.3

Results represent means ± SD (n=6). Pairs of values within a given cell type which are significantly different (P<0.05) are shown (*). Pairs of values within a given dietary treatment which are significantly different (P<0.05) are shown (†). pp: protein. h: hour. FO: Fish oil. EO: *Echium* oil. Elo/des: the sum of elongation and desaturation activity.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PI
PS
CHO
PC
SM
PG
TAG
UK
SE
PE
FFA
DAG
Component 2
Component 1
1,0
0,5
0,0
-0,5
-1,0
□: Fish oil
■: *Echium* oil
Δ : Enterocytes
▲: Hepatocytes
b)
c)
REGR factor score 2
REGR factor score 1
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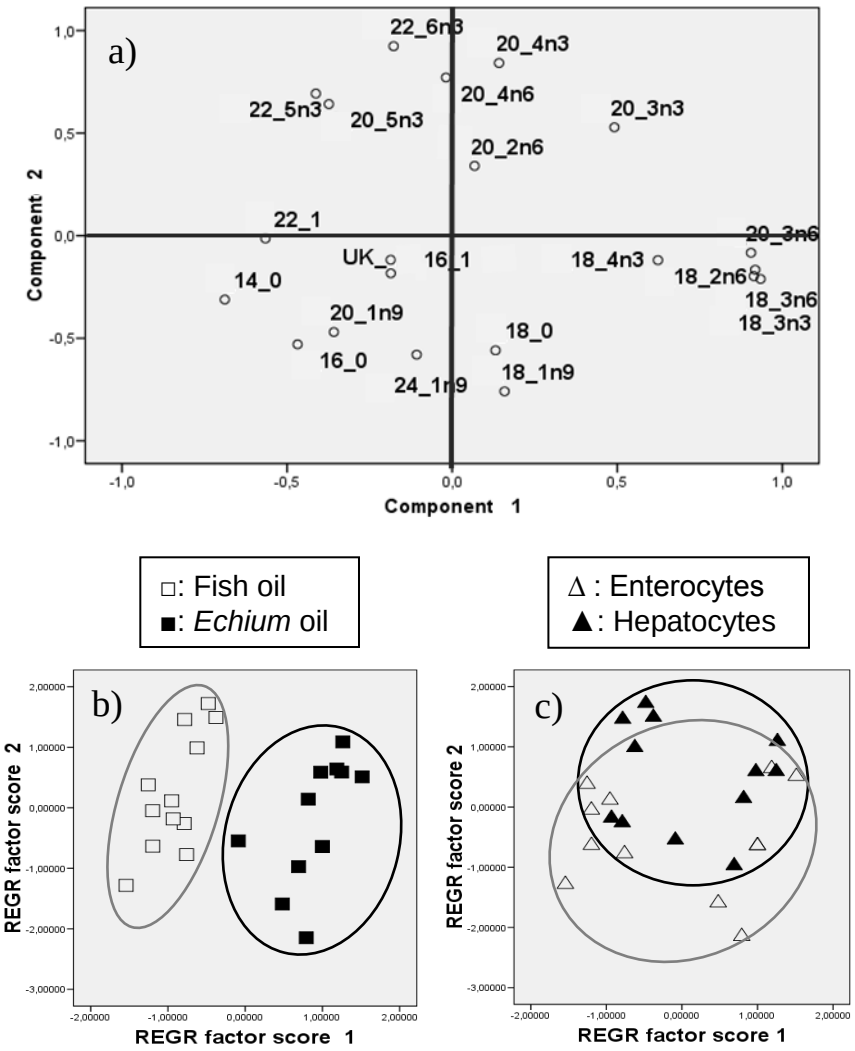
1,00000
0,00000
-1,00000
-2,00000
-3,00000


a)
22_6n3
20_4n3
20_4n6
22_5n3
20_5n3
20_3n3
20_2n6
22_1
20_3n6
UK_
16_1
18_4n3
18_2n6
14_0
18_3n6
20_1n9
18_3n3
18_0
16_0
18_1n9
24_1n9
Component 2
Component 1
1,0
0,5
0,0
-0,5
-1,0
□: Fish oil
■: *Echium* oil
Δ : Enterocytes
▲: Hepatocytes
b)
c)
REGR factor score 2
REGR factor score 1
2,00000
1,00000
0,00000
-1,00000
-2,00000
-3,00000