Title

The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain polyunsaturated fatty acid content

Authors

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Shortened title

Responses of gilthead seabream to LC-PUFA

Keywords

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Abstract

The replacement of fish oil (FO) with vegetable oil (VO) in feed formulations reduces the availability of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) to marine fish such as gilthead seabream. The aim of this study was to examine compositional and physiological responses to a dietary gradient of n-3 LC-PUFA. Six isoenergetic and isonitrogenous diets (D1-D6) were fed to seabream, with the added oil being a blend of FO and VO to achieve a dietary gradient of n-3 LC-PUFA. Fish were sampled after four months feeding, to determine biochemical composition, tissue fatty acid concentrations and lipid metabolic gene expression. The results indicated a disturbance to lipid metabolism, with fat in the liver increased and fat deposits in the viscera reduced. Tissue fatty acid profiles were altered towards the fatty acid compositions of the diets. There was evidence of endogenous modification of dietary PUFA in the liver which correlated with the expression of fatty acid desaturase 2 (*fads2)*. Expression of sterol regulatory element-binding protein 1 (*srebp1*)*, fads2* and fatty acid synthase increased in the liver, while peroxisome proliferator-activated receptor alpha 1 pathways appeared to be supressed by dietary VO in a concentration-dependent manner. The effects in lipogenic genes appear to become measurable in D1-D3, which agrees with the weight gain data suggesting that disturbances to energy metabolism and lipogenesis may be related to performance differences. These findings suggested that suppression of beta-oxidation and stimulation of *srebp1*-mediated lipogenesis may play a role in contributing toward steatosis in fish fed n-3 LC-PUFA deficient diets.

1. Introduction

Sustainable expansion of aquaculture requires reduction in the use of fishmeal (FM) and fish oil (FO) in aquafeed formulations [1-4]. Both raw materials, particularly FO, are rich in the two key n-3 (or omega-3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), recognised as essential fatty acids (EFA) for the majority of marine fish species [5]. DHA is an essential component of neural and retinal membranes [6] and both EPA and DHA are precursors for an extensive range of autocrine signalling molecules (e.g. eicosanoids, resolvins, protectins, etc.) [7]. Dietary deficiency of n-3 LC-PUFA has impacts on the health [8], metabolism [9, 10] , composition [11, 12] and growth [13] of marine fish.

Typically, an aquafeed for a given marine fish species contains a combination of FO to supply essential n-3 LC-PUFA and vegetable oils (VO) that, while devoid of LC-PUFA, supply dietary energy [14, 15]. Marine fish lack sufficient activity of the LC-PUFA biosynthesis pathway to satisfy requirements [5]. In terms of fatty acid composition, the key effects of high inclusion levels of VO are an increase in C_{18} unsaturated fatty acids (α-linolenic acid, linoleic acid and oleic acid) in the fish tissues at the expense of LC-PUFA that is reflective of the altered composition of dietary fatty acids [16]. With regards to lipid metabolism, studies have reported that inclusion of dietary VO leads to reduced fatty acid catabolism and the accumulation of lipid in liver of Atlantic salmon (*Salmo salar*) and gilthead seabream, [17-19]. Studies examining the influence of VO on lipid biosynthesis have yielded conflicting results with some reporting increased gene expression in Atlantic salmon and black seabream (*Acanthopagrus schlegelii*) [18, 19]

and others reporting decreased enzyme activity in gilthead seabream [20] although, in mammals, EPA has been shown to supress lipogenesis [21].

Lipid homeostasis is maintained in animals through a balance of catabolic and anabolic processes. Fatty acids and cholesterol can be synthesised *de novo* by pathways that are activated by sterol regulatory element binding proteins (Srebp) 1 and 2, respectively. Srebp are transcription factors involved in energy homeostasis and have many target genes with examples of those in lipid metabolism including fatty acid synthase (*fas*) and fatty acid desaturase 2 (*fads2*), the latter being key enzymes in the LC-PUFA biosynthesis pathway [22, 23]. Fatty acids are catabolised by the β-oxidation pathway in either mitochondria or peroxisomes, and expression of genes encoding proteins involved in these pathways are regulated by, among others, peroxisome proliferator-activated receptors (Ppar) [24]. Upon binding ligands and retinoid X receptor, Ppars bind to peroxisome proliferator response elements in the promoter regions of target genes, many of which are involved in β-oxidation, such as carnitine palmitoyl transferase I (*cpt1a*) and liver-type fatty acid binding protein (*fabp1*), both proteins involved in the intracellular transport of fatty acids destined for catabolism [24, 25].

Despite recent advances in our knowledge regarding the impacts of dietary VO some questions remain, for instance, are dietary differences in gene expression dependent on precise concentrations of dietary nutrients or are genes activated/deactivated at particular levels of FO substitution? Therefore, the present study aimed to examine the impact of modern (high lipid, low fish meal) aquafeed formulations across a gradient of n-3 LC-PUFA, achieved by blending commercially

available oils (FO, rapeseed oil and palm oil), on the biochemical composition of body compartments, and fatty acid compositions and gene expression in the liver and mid-intestine of a marine teleost, the gilthead seabream (*Sparus aurata*). The liver being a key metabolic tissue in vertebrate metabolism and the mid-intestine being a primary site of nutrient absorption and processing.

2. Experimental methods

2.1 Fish husbandry and diets

The nutritional trial was carried out at the BioMar Feed Trial Unit (Hirtshals, Denmark) between April and August 2014. Seabream juveniles of approximately 3 g were purchased from a commercial hatchery (Les Poissons du Soleil, Balaruc-les-Bains, France) and randomly distributed among 18 x 1 m^3 tanks. The tanks were part of a Recirculation Aquaculture System (RAS) with photoperiod, temperature and salinity maintained at 12:12 h L:D, 24 °C and 32 ppm, respectively. Initially, the fish were fed with commercial fry feeds rich in FM and FO until they reached ~24 g. After acclimation, each tank was assigned one of six iso-energetic and iso-nitrogenous diets for 18 weeks, initially as a 3 mm pellet (8-weeks) and then with a 4.5 mm pellet to the end of the trial. Fish were fed to satiation twice per day using automatic feeders and waste feed was collected to accurately measure feed consumption. The six diets were formulated to deliver specific levels of LC-PUFA by progressively replacing FO with blends of rapeseed and palm oil, whereas the other dietary ingredients were selected to meet the known nutrient requirements of seabream [26] (Table 1). Diets were produced by extrusion at the BioMar Tech-Centre (Brande, Denmark). The experimental diets were numbered to reflect the VO/FO inclusion so that diet D1 contained the VO blend as sole exogenously added oil source, diet D6 contained only FO, and diets D2 - D5

contained graded levels of VO and FO as described in Table 1. The fatty acids that increased with dietary FO were: 16:1n-7, 24:1n-9, 20:3n-6, 20:4n-6, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3, while 20:0, 22:0, 18:1n-9, 18:2n-6 and 18:3n-3 increased with dietary VO (Table 2).

Experimental animals were maintained under the current European legislation on handling experimental animals. In addition, all research performed by the Institute of Aquaculture, University of Stirling (UoS) is subject to thorough ethical review carried out by the UoS Animal Welfare and Ethical Review Board (AWERB) prior to any work being approved. This involves all projects, irrespective of where they are carried out, to be submitted to AWERB for approval using detailed Ethical Approval forms that require all aspects of the experimentation to be described including all animal health and welfare issues as well as other ethical considerations. The present research was assessed by the UoS AWERB and passed the ethical review process of the University of Stirling.

2.2 Sampling

Fish were sampled at the initiation of the trial and at termination after being euthanised with a lethal dose of benzocaine (Centrovet, Kalagin, Santiago, Brazil). Fish were not fed the day before sampling. Five whole fish and three eviscerated carcasses, liver and viscera (the entire contents of the body cavity minus the liver) were sampled from each tank for compositional analysis. Three fish per tank were also sampled for gene expression and fatty acid composition taking samples of liver and mid-intestine. Samples for RNA analysis were incubated with 1 mL RNA*later*® at 4 °C for 24h (Sigma-Aldrich, UK) before storage at -70°C, while samples for fatty acid analysis were

138 immediately frozen and stored at -20 °C before shipment on dry ice to the Institute of
139 Aquaculture, University of Stirling.

140 *2.3 Proximate composition*

141 Feed samples were ground prior to analyses. Whole fish, carcass and viscera
142 samples were homogenised in a blender (Waring Laboratory Science, Winsted, CT,
143 USA) to produce pates. Proximate compositions of feeds and fish were determined
144 according to standard procedures [27]. Moisture contents were obtained after drying in an
145 oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h.
146 Crude protein content was measured by determining nitrogen content (N × 6.25) using
147 automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington,
148 U.K) and total lipid content determined as described below.

149 *2.4 Lipid extraction*

150 Total lipid for fatty acid analyses was extracted from seabream tissues according
151 to Folch *et al.* [28]. Briefly, liver and mid-intestine samples (~ 0.5 g) were homogenised
152 in 20 mL chloroform/methanol (2:1, v/v) and incubated for 1 h on ice. Subsequently, 5
153 mL of 0.88 % KCl (w/v) was added, samples were vortexed and centrifuged at 400 g to
154 separate organic and aqueous fractions. The aqueous fraction was discarded and the
155 organic layer (infranatant) was then filtered (Whatman No. 1) and solvent evaporated
156 under a stream of oxygen-free nitrogen. After desiccation *in vaccuo* overnight, lipid
157 content was determined gravimetrically. Total lipid samples were stored at 10 mg ml^{-1}
158 in chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) butylated hydroxytoluene
159 (BHT) as antioxidant.

160 *2.5 Fatty acid analysis*

Fatty acids were quantified by gas-liquid chromatography (GC) after preparation of fatty acid methyl esters (FAME) by acid-catalysed transesterification of total lipid [29]. Briefly, 1 mg of total lipid and 0.1 mg of heptadecanoic acid (17:0) as internal standard were incubated with 1 ml toluene and 2 ml 1 % sulphuric acid in methanol (v/v) for 16 h at 50 °C. FAME were extracted and purified by thin-layer-chromatography as described by Tocher and Harvie [30] and resuspended in isohexane before GC analysis using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). FAME were identified by comparison to known standards (Supelco 37-FAME mix; Sigma-Aldrich Ltd., Poole, UK) and published data [30]. Fatty acid contents were expressed as mg g^{-1} of tissue and estimated using the response of the internal standard. The coefficient of variation estimated using mg DHA g^{-1} over a subset of 20 samples was 2.80 ± 2.51 %.

2.6 RNA extraction

Total RNA was extracted from ~100 mg of liver and mid-intestine by homogenisation in 1 mL of TriReagent (Sigma-Aldrich, Dorset, UK) using a Mini-Beadbeater 24 (Biospec, Bartlesville, Oklahoma, USA). Phase separation was achieved by the addition of 100 µl of 1-bromo-3-chloropropane (BCP, Sigma) and centrifugation at 20,000 g. Subsequently 400 µl of the supernatant were recovered and RNA precipitated by the addition of 200 µl isopropanol (Fisher, UK) and 200 µl RNA precipitation solution (1.2 M sodium chloride and 0.8 M sodium citrate sesquihydrate) followed by centrifugation at 20,000 g. The resulting pellet was washed twice with 75

% ethanol, air-dried and resuspended in 50 µl nuclease-free water. The concentration and quality were verified spectrophotometrically (NanoDrop ND-1000, Spectrophotometer, Sussex, U.K.) and by agarose gel electrophoresis to visualise the presence of 18S and 28S ribosomal subunits. Extracts were stored at -70 °C until cDNA synthesis.

2.7 cDNA synthesis

Reverse transcription was performed according to the kit manufacturer's protocol (High Capacity Reverse Transcription kit, Applied Biosystems, Warrington, UK). A no template control (NTC) reaction and reverse transcriptase-free reactions were prepared as blank and negative controls. Each 20 µl reaction included: 10x reverse transcription buffer (2 µl), 100 mM dNTP mix (0.8 µl), 10 µM random primers (1.5 µl), 10 µM oligo dT primers (0.5 µl), reverse transcriptase (1.0 µl), 2 µg of total RNA as template and nuclease-free water to make up the volume. RNA was denatured at 95 °C for 10 min prior to addition of master mix containing all other reagents. Reverse transcription was performed on a Biometra Thermocycler (Analytik Jena, Goettingen, Germany) using the following program: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and then terminated at 4 °C. A pool of cDNA samples was created for serial dilutions, calibrator samples and primer validations. Samples of cDNA were diluted 20-fold with nuclease-free water as template for qPCR, and stored at -20 °C.

2.8 Gene expression analysis

Gene expression was determined for candidate genes involved in key pathways by quantitative real time PCR (qPCR). Primers for qPCR were designed using Primer3 through the NCBI database's "Primer-BLAST" against known gene sequences

208 including sequences from the *S. aurata* expressed sequence tag (EST) NCBI database
209 that were confirmed to be the gene of interest by BLAST searches. Primer sequences for
210 genes in the present study are given in Table 3. Primers were tested to confirm that they
211 functioned optimally at annealing temperatures of 60 °C and that a single amplicon of
212 appropriate length was visualised on agarose gel. A serial dilution of the cDNA pool
213 was analysed by qPCR (Luminaris Color HiGreen qPCR Master Mix, Thermo
214 Scientific, Hemel Hempstead, UK) to determine primer efficiency (rejected at < 1.85).
215 Duplicated qPCR reactions were carried out on 96-well plates, each reaction contained:
216 Luminaris Color HiGreen (10 µl), 10 µM primers (1 µl each), 1/20 diluted cDNA
217 sample (5 µl and 2 µl for target and reference genes, respectively) and nuclease-free
218 water up to 20 µl. As there were 54 samples per tissue (6 treatments, n = 9) two plates
219 were run per gene with treatments equally represented on both plates, a 1/20 dilution of
220 the cDNA pool was used as a calibrator and a serial dilution included on one plate. A
221 single master mix was used for all the reactions required per gene and both plates were
222 run consecutively on a Biometra TOptical Thermocycler (Analytik Jena). The
223 thermocycling program was 50 °C for 2 min, 95 °C for 10 min, 35 cycles of 95 °C for
224 15 s (denaturation), 60 °C for 30 s (annealing) and 72 °C (extension) for 30 s, followed
225 by a melting curve to check for non-specific products. Data were acquired through the
226 software package qPCRsoft 3.1 (Analytik Jena) and calculations for sample expression
227 ratios were carried out according to Pfaffl [31]:

228 $$Expression\ ratio = \frac{E(ref)^{Ct(Sample)}}{E(goi)^{Ct(Sample)}} \div \frac{E(ref)^{Ct(Calibrator)}}{E(goi)^{Ct(Calibrator)}}$$
229

where *E* is the determined efficiency, *ref* is the geometric mean of four reference genes, *goi* is the gene of interest and *Ct* is the threshold cycle. The genes of interest were normalised to the geometric mean of four reference genes, elongation factor 1α (*ef1α*), beta-actin (*β-act*), alpha-tubulin (*tuba1α*) and ribosomal protein P0 (*rplp0*), whose expression was not influenced by dietary treatment. Gene expression data are presented as log2 expression ratios [32] of genes related to lipid metabolism: *srebp1* (sterol regulatory element binding factor 1), *srebp2* (sterol regulatory element binding factor 2), *pparα1* (peroxisome-proliferator activated receptor alpha 1), *fas* (fatty acid synthase), *fads2* (fatty acid desaturase 2), *cpt1α* (carnitine palmitoyltransferase I) and *fabp1* (fatty acid binding protein 1) and *elovl5* (elongation of very long chain fatty acid 5 protein). The average intra assay coefficient of variation was 0.44 ± 0.12 % at the level of quantification cycle (C_q).

2.9 Statistical analysis

Final weights, specific growth rate (SGR), proximate composition and tissue fatty acids were analysed by between groups ANOVA, rejecting null hypotheses at $P < 0.05$. Absence of within treatment effects (i.e. tank effects) were first confirmed using tank as a nested variable in the ANOVA formula. F-tests were verified by Bartlett's test and Shapiro-Wilk for variance homogeneity and distribution.

Three individuals from each tank (9 per treatment) were randomly sampled giving 54 fish in total, this is the minimum number of fish to detect medium effect sizes ($f^2 = 0.15$; power = 0.8) by ordinary least squares regression. Proximate composition data were analysed using linear regression to identify the existence of trends across the experimental diets. Trends were reported as significant if the slope was significantly

253 different ($P < 0.05$) to 0. For fatty acid profiles of tissues ($n = 54$) the first step of
254 analysis was to reduce the dimensions of the data by principal component analysis
255 (PCA), which enabled the identification of fatty acids that were correlated with each
256 other and that should be analysed further. The level of dietary FO was supplied as a
257 supplementary variable to PCA. Fatty acids with several no detects (effectively zero)
258 were removed from the data set, because zero values are problematic with PCA
259 analysis, and the C_{20} and C_{22} monounsaturated fatty acids were combined into two
260 single variables (20:1 and 22:1, respectively) as these peaks do not always separate in
261 the GC analysis. Further analyses of selected fatty acids were performed using
262 regression of the tissue fatty acid concentration as a function of the diet fatty acid
263 concentration. Tissue fatty acid levels were regressed with absolute data (mg g^{-1} tissue).
264 Where a range was reported, this was derived from the fitted values of the model
265 applied to the data, not the mean values reported in the Supplementary tables. Where
266 appropriate, percentage data (% of total fatty acids) were used to support the analyses.
267 When a fatty acid did not have a dietary component it was regressed against dietary VO,
268 for instance the fatty acid 18:2n-9, and the expression of genes. Analyses and plots were
269 produced in the statistical package R [33] (version 3.1.3, Vienna, Austria). PCA were
270 performed using the FactoMineR package [34] and regression diagnostics using the Car
271 package [35].

272 **3. Results**

273 *3.1 Growth rates and proximate composition of gilthead seabream*

274 After feeding the experiment diets for 18 weeks mean weights ranged between
275 200 - 250 g and SGR's between 1.64 – 1.81, with significant differences ($P < 0.001$)

276 between dietary groups that showed increasing the dietary VO content beyond the level
277 of that in diet D4 led to reduced growth (Table 4).

278 Significant effects of diet on proximate compositions of gilthead seabream were
279 observed in liver, mid-intestine and viscera. In liver and mid-intestine, total lipid
280 contents increased from 18.6 – 31.8 % diet ($R^2 = 67.8$, $P < 0.001$) and 8.6 – 13.9 % (R^2
281 = 23.9, $P < 0.001$), respectively, as dietary VO increased from 0 – 15.6 % of diet. In
282 viscera (minus liver), total lipid content decreased from 53.5 – 45.2 % ($R^2 = 24.0$, $P <$
283 0.001) as dietary VO increased in the diet. The lipid contents of whole fish and carcass
284 were unaffected by dietary treatment ($P > 0.05$). Protein and ash contents were not
285 affected in any body compartment examined in the present study ($P > 0.05$). A summary
286 of these data is presented in Supplementary table S1.

287 *3.2 Fatty acid composition of liver and mid-intestine*

288 Fatty acid profiles of total lipid of two major lipid metabolic sites, namely liver
289 and mid-intestine, were determined and further analysed by PCA. In both the liver and
290 mid-intestine the first principal component (PC1) was correlated to dietary FO and
291 explained 57.5 % and 60.1 % of the variance in fatty acid compositions, respectively
292 (Figs. 1 and 2). The fatty acids associated with FO, namely 14:0, 16:1n-7, 20:4n-3,
293 20:5n-3, 22:5n-3 and 22:6n-3, had positive correlations to PC1, and those associated
294 with VO, specifically 18:1n-9, 18:2n-6 and 18:3n-3, a negative correlation to PC1 in
295 liver and mid-intestine Thus, PC1 separated the fatty acid profiles according to diet, D1
296 had low values and D6 had high values for PC1. PC2 accounted for 15.3 % and 15.5 %
297 of the variance in liver and mid-intestine fatty acid profiles, respectively. The
298 contributions of the fatty acids towards PC2, which separated the profiles vertically in

299 Figs. 1 and 2, differed between liver and mid-intestine. In liver, 20:2n-6 and 20:1 had a
300 positive influence on PC2, and 18:2n-9, 20:2n-9, 18:3n-6, 20:3n-6 a negative influence,
301 with these fatty acids thus being important to separate individual fatty acid profiles once
302 the variability due to PC1 (diet) had been accounted for. In the case of mid-intestine
303 samples, 18:0, 20:0 and 22:0 had a positive influence, and 18:1n-7 a negative influence
304 on PC2 while 18:2n-9 and 20:2n-9 were not detected, again it can be interpreted that
305 these are the fatty acids that vary the most between individual fatty acid profiles once
306 the effect of PC1 had been removed. It was also apparent from biplots that there was
307 considerable overlap between the fatty acid profiles of liver and mid-intestine of fish
308 consuming diets D1 - D4, although fatty acid profiles from individuals fed on diets
309 containing the highest inclusion levels of FO (D5 and D6) formed clearly separated
310 groups (panels A in Figs. 1 and 2). PCA indicated that the n-3 LC-PUFA (20:3n-3,
311 20:4n-3, EPA, DPA and DHA) were all correlated with each other in both liver and
312 mid-intestine and therefore these fatty acids were summed as a single variable reflecting
313 their origin from FO. Absolute levels of tissue n-3 LC-PUFA were strongly related to
314 dietary n-3 LC-PUFA in liver ($R^2 = 0.75$, $P < 0.001$) and mid-intestine ($R^2 = 0.87$, $P <$
315 0.001) (Fig. 3). Absolute levels of monounsaturates (MUFA) responded positively to
316 dietary MUFA in liver ($R^2 = 0.57$, $P < 0.001$) and mid-intestine ($R^2 = 0.76$, $P < 0.001$)
317 (Fig. 4). Contents of MUFA in liver were typically 75 - 175 mg g^{-1} in fish fed diets D1 -
318 D4, and < 50 mg g^{-1} in fish consuming the FO diet (D6). High variability is evident in
319 MUFA levels with the major contributing factor being variation in the total lipid content
320 of the tissues. Two fatty acids, namely 18:2n-9 and 20:2n-9, were identified in liver in
321 spite of their absence in diets and mid-intestine, so they were plotted against dietary
322 VO. Levels of 18:2n-9 increased with the dietary VO ($R^2 = 0.81$, $P < 0.001$) (Fig. 5A).

323 The level of 18:2n-9 was correlated (R^2 = 0.41, r = 0.64, P < 0.001) to the $\log_2$
324 expression of *fads2* (Fig. 5B), the latter data requiring to be transformed to satisfy the
325 assumptions of Pearson's correlation. Dietary levels of saturates were relatively stable
326 across the experimental diets (32 - 36 g kg^{-1}). Despite this low range, livers of fish fed
327 diet D1 (VO rich) contained 50 - 70 mg g^{-1} saturates and those of fish fed diet D6 (FO
328 rich) ranged between 10 - 40 mg g^{-1}, and there was a significant relationship with
329 dietary saturates (R^2 = 0.27, P < 0.001) (data not shown). Examination of percentage
330 data indicated that absolute levels of saturates was mainly associated with the increasing
331 lipid level of the liver, lower levels of saturates were observed in the mid-intestine (15 –
332 35 mg g^{-1}). Quantitatively, palmitic acid was the dominant saturated fatty acid in both
333 tissues and only 14:0 increased with dietary FO. Liver and mid-intestine fatty acid data
334 are summarised in Supplementary Tables, S2 and S3, respectively, where they are
335 analysed by ANOVA, with the results being in agreement with those reported above.

336 *3.3 Gene expression*

337 Genes representing lipid metabolic pathways were assayed by qPCR, and the
338 expression of target genes plotted against dietary VO and, although variability (R^2 = 19
339 – 52 %), between individuals was high, significant trends showed that the diets had an
340 impact on the regulation of lipid metabolism. Subtle, negative trends were found in the
341 liver between dietary VO and the expression of *pparα1* (R^2 = 0.32, P < 0.001) and its
342 target genes *cpt1α* (R^2 = 0.26, P < 0.001) and *fabp1* (R^2 = 0.19, P < 0.001) (Fig. 6). The
343 highly variable within treatment data indicated that diet is not the sole source of this
344 variation in the expression of these genes. The level of variation in the mid-intestine
345 prevented the application of suitable models to the data. In the liver, positive trends,
346 fitted with quadratic functions were found between VO and *srebp1* (R^2 = 0.37, P <

347 0.001) and its target genes *fas* (R^2 = 0.42, P < 0.001) and *fads2* (R^2 = 0.52, P < 0.001)
348 (Fig. 7). Effects of VO on the expression of *srebp2* and *elovl5* in liver were not detected
349 (data not shown). However, in mid-intestine, the effect on *srebp1* expression was not as
350 strong and linear (R^2 = 0.23, P < 0.001), but *srebp2* showed a strong up-regulation in
351 fish fed diets D1 and D2 (R^2 = 0.49, P < 0.001) and *elovl5* was responsive to dietary VO
352 (R^2 = 0.26, P < 0.001) (Fig. 8). Effects of VO on the expression of *fads2* were not
353 detected in mid-intestine (data not shown).

354 **4. Discussion**

355 Replacement of FO by alternative oils in aquafeeds has been an extensively
356 investigated research topic over the last two decades [16]. The most common FO
357 alternatives are VOs, for example rapeseed oil or soya bean oil, which are devoid of
358 essential LC-PUFA and, consequently, their use has important implications, not only on
359 the nutritional value of the product for consumers [36-38], but also effects on metabolism
360 and fish health [1]. We employed a dietary gradient of VO to span the EFA requirements
361 reported for a commercially relevant teleost, the gilthead seabream [26] and show how
362 this gradient modifies the composition and the expression of lipid metabolic and
363 regulatory genes in gilthead seabream juveniles.

364 Fish consuming diets D4-D6 (LC-PUFA: 1.20-3.54 % of diet) gained significantly
365 more weight than the other dietary groups, a likely result that these diets were supplied
366 EPA and DHA in excess of the nutrient requirement. The results of the present study
367 show that dietary provision of n-3 LC-PUFA below the reported EFA requirement, 0.9
368 % EPA+DHA dry weight, for gilthead seabream [15] led to alterations in lipid
369 metabolism as indicated by increased lipid content in liver and decreased lipid in

viscera, the latter being regarded as the normal lipid storage site in this species [39]. These results were in agreement with previous studies regarding increased hepatic lipid content as a result of dietary deficiency of EPA and DHA in gilthead seabream [13, 17, 40] and other fish species [1, 41]. Interestingly, increased lipid contents in liver have been also described when dietary lipid was increased to boost the energy content of the diet [42]. The range of values for hepatic total lipid was 17 - 32 % (wet wt) and these were higher than those reported previously, 15 - 25 % (wet wt), in gilthead seabream fed graded levels of soya bean oil [13]. This may be due to the larger size of the fish in the present trial (24-230g) whereas the previous study used fry (1.2-12.4g), the longer feeding period of four months in the present study, or the higher crude lipid levels in the diets. In the previous study there was a threshold level of soya bean oil (~ 50 % of oil) that increased hepatic lipid content whereas data in the present study suggested VO increased hepatic lipid in a concentration-dependant manner (linear increase).

Quantitatively, the main fatty acids driving the increased liver lipid were 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid), all major constituents of the VO used in the experimental diets. Such accumulation of dietary fatty acids observed in liver, occurring as well in mid-intestine, has been commonly reported in FO replacement studies in gilthead seabream [12, 13, 43]. Interestingly, two fatty acids, namely 18:2n-9 and 20:2n-9, were found in the liver, but not mid-intestine, of fish fed VO that were not present in the diets. The presence of 18:2n-9 is likely to be the result of Δ6 desaturation of 18:1n-9, with 20:2n-9 being the elongation product of 18:2n-9. Fads2 is typically a Δ6 desaturase in marine teleosts [23] and, although its activity towards 18:1n-9 has not been demonstrated in gilthead seabream [44], it is likely that Fads2 activity was

393 responsible for observed production of 18:2n-9 in liver. Additional data in the form of
394 enzyme protein levels or activity assays could lend support to this conclusion. Indeed,
395 this is consistent with increased expression of *fads2* in liver of fish fed high VO diets
396 and n-3 LC-PUFA deficient diets, a regulatory mechanism often reported in literature
397 not only on desaturases, as observed herein, but also elongases such as *elovl5* [(19)].
398 Interestingly, an up-regulation of hepatic *elovl5* was not observed, that would support
399 the production of 20:2n-9 mentioned above. Instead, a moderate but detectable increase
400 in *elovl5* expression was observed in the mid-intestine with dietary VO, in agreement
401 with previous studies on seabream [(45)], and it is likely that this decrease is a response to
402 declining availability of n-3 LC-PUFA. Hepatic *fads2* expression was variable between
403 individual fish fed diets with high VO inclusion (D1 - D4). The PCA analysis showed
404 that this was also the case for the fatty acid profiles of fish consuming these diets and
405 that 18:2n-9 and 20:2n-9 were important fatty acids in driving this variability.
406 Furthermore, the level of 18:2n-9 was strongly correlated to the level of *fads2*
407 transcripts in the liver. It may be possible to exploit this individual variability in
408 response to VO to select seabream that are better adapted to diets that are rich in VO, as
409 has been described previously in Atlantic salmon [(18)]. The fatty acids, 18:2n-9 and
410 20:2n-9, are metabolites of 18:1n-9 derived from its metabolism via the LC-PUFA
411 biosynthesis pathway. Marine fish are able to perform the first two steps, delta-6
412 desaturation (*fads2*) and elongation (*elovl5*), but are unable to perform the subsequent
413 step of the pathway due to the absence of an active delta-5 desaturase [(46)]. The above
414 result shows that the delta-6 in seabream can operate on 18:1n-9.

415 In addition to the distinctive patterns of *fads2* and *elovl5* expression described
416 above, the regulatory mechanisms by which dietary fatty acids modulate metabolic

417 responses in liver and mid-intestine appear to differ. In liver, *srebp1*, but not *srebp2*,
418 was increased in gilthead seabream fed diets D1 - D3, with a threshold between diets
419 D3 and D4, interestingly this at the point where effects on growth were also observed.
420 In mid-intestine, both *srebp1* and *srebp2* expression were increased with dietary VO.
421 Srebp signalling is responsible for maintaining lipid levels in balance and, although
422 there is some overlap between the functions of Srebp1 and Srebp2, the former is mainly
423 associated with fatty acid/lipid synthesis, whereas the latter is associated with
424 cholesterol synthesis in mammals [22, 47, 48] and fish [49]. While the up-regulation of
425 *srebp1* has been often associated with increased expression of *fads2* in response to VO-
426 rich diets [18, 19, 50], the up-regulation of *srebp2* in mid-intestine suggested putative
427 activation of cholesterol biosynthesis. Cholesterol was added to the experimental diets
428 to balance the supply of this key nutrient, but VOs are known to contain a range of
429 phytosterols that may have stimulated upregulation in the mid intestine [25, 51-53].

430 The *fas* gene is also regulated by Srebp1 [22] and its product, Fas, is an enzyme
431 complex responsible for *de novo* synthesis of saturated fatty acids [54, 55]. Despite the
432 inclusion of 20 % lipid in the diets, the inclusion of VO resulted in up-regulation of *fas*
433 in liver. There have been reports of VO increasing *fas* expression in Atlantic salmon [18]
434 and black seabream [19]. Nevertheless, this is not always supported by measurements of
435 Fas activity in gilthead seabream fed diets with 80 % of dietary FO replaced with
436 linseed oil [20] however, in turbot (*Scophthalmus maximus*), Fas activity was stimulated
437 by dietary VO although the differences were not significant [56]. It is unclear what
438 exactly is responsible for the apparent discrepancy between these results. However, the
439 increased expression of *srebp1* in response to dietary VO, particularly notable in liver,
440 suggested increased regulatory activity of Srebp1 towards potential target genes

441 including *fas* and thus increasing their transcription. The patterns of dietary regulation
442 of *srebp1* and *fas* share a similar shape in liver (modelled by quadratic functions)
443 suggesting co-regulation. Their up-regulation in response to dietary VO indicated that
444 *de novo* lipogenesis may contribute to increased lipid deposition, as suggested
445 previously by Morais *et al.* [18] when studying diet/genotype interactions in Atlantic
446 salmon.

447 Beyond the anabolic processes described above, the impact of dietary VO was
448 further evidenced in lipid catabolic processes such as β-oxidation. Generally, the
449 expression of catabolic genes (*pparα1* and *cpt1α*) in liver and, to some extent, in mid-
450 intestine, decreased with increasing dietary VO mid-intestine. The data were variable as
451 illustrated by low R^2 values, indicating that the diet was not the sole source of variation
452 in the expression of these genes. The reduction in catabolic gene expression is coherent
453 with the observed increased hepatic lipid levels associated with dietary VO.
454 Furthermore, the results were consistent with previous studies in rats [57, 58] and Atlantic
455 salmon [18] that revealed that dietary FO increased the expression of *pparα* and the
456 activity of β-oxidation enzymes. In contrast, a recent study demonstrated expression of
457 *pparα1* and its target *cpt1α* were increased in liver of gilthead seabream fed diet
458 containing both wild-type *Camelina sativa* oil (a low LC-PUFA diet) and containing
459 genetically-modified camelina oil (containing n-3 LC-PUFA), although both these diets
460 contained sufficient FM to satisfy EFA requirements [59]. Dong *et al.* [60] have recently
461 shown that *pparα1* expression in response to VO was different in three species of fish.
462 The authors observed, dietary VO increased expression of *pparα1* and *pparα2* in
463 rainbow trout (*Oncorhynchus mykiss*) and decreased expression of *pparα2* in Japanese
464 seabass (*Lateolabrax japonicus*), but had no effect in livers of yellow croaker

(*Larimichthys crocea*). This finding complicates the regulation of lipid homeostasis but means that lipid catabolic processes can be regulated by a broad range of endogenous stimuli.

In conclusion, the present study demonstrated that diets deficient in essential LC-PUFA can cause reduction in growth rates alterations to lipid metabolism, fatty acid composition of liver and the regulation of genes involved in lipogenesis and catabolism. Despite the high variability in the expression of lipogenic genes the effect of their expression appears to be become measurable in D1-3 and this agrees with the analysis of the final weight and SGR data. Therefore, the alteration to the expression of the genes involved in lipogenesis and energy balance appear to be related to the fish growth performance. Therefore, D4-D6 appear to provide sufficient EFA and D1-D3 appear to be deficient. Despite the high energy (high lipid) diets used in the present study, expression of lipogenic genes such as *fas* involved in *de novo* biosynthesis was increased by dietary VO. In contrast, *ppara1* and its target *cpt1α* were down-regulated and the expression was linear and therefore appeared to be modulated in a concentration-dependant manner. However, the expression of *srebp1* and its gene targets was modelled by a curve, which was indicative of a threshold concentration at which gene expression was activated, although it is difficult to determine this point due to high variability between fish. Overall, the results reported in the present study were consistent with those reported in rats [(61)], that the LC-PUFA found in FO have a stimulating effect on β-oxidation and an inhibitory effect on *de novo* lipogenesis. These opposing biochemical activities would be expected to contribute towards the increased hepatic lipid observed in fish fed increasing VO in the present study and others

investigating EFA nutrition. These physiological effects of VO have direct relevance to decisions regarding sustainable and modern feed formulations for marine fish species.

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Conflict of interest

None

Authorship

V.K and J.T. designed and executed the nutritional trial and all authors contributed to planning the analyses. V.K., J.T. and S.J.S.H. carried out the sampling. O.M., D.R.T and S.A.M.M. supervised the lead author. M.B. provided training in molecular biology to S.J.S.H. who carried out all analytical procedures. S.J.S.H. analysed all of the data and prepared the manuscript. Subsequently the manuscript was shared between all authors who made amendments, contributions and recommendations.

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672

673

674 **Tables**

675 Table 1. Diet formulations and proximate analyses of the six experimental diets.

DIET ***Ingredients (%)***	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Fishmeal	12.5	12.5	12.5	12.5	12.5	12.5
Soya Protein Concentrate	21.9	21.9	21.9	21.9	21.9	21.9
Rape seed meal	10.0	10.0	10.0	10.0	10.0	10.0
Wheat Gluten	4.0	4.0	4.0	4.0	4.0	4.0
Corn Gluten	25.0	25.0	25.0	25.0	25.0	25.0
Wheat	7.1	7.1	7.1	7.1	7.1	7.1
Aminoacids[1]	0.8	0.8	0.8	0.8	0.8	0.8
Micro-ingredients[2]	3.1	3.2	3.2	3.3	3.5	3.9
Yttrium	0.03	0.03	0.03	0.03	0.03	0.03
OILS (%)						
Fish Oil (SA)	0.0	1.8	2.6	4.4	8.0	14.9
Rapeseed Oil	10.4	9.2	8.6	7.3	4.8	0.0
Palm Oil	5.2	4.6	4.2	3.6	2.4	0.0
PROXIMATE COMPOSITION (% of diet as fed)						
Protein	41.8	43.0	42.9	41.7	42.4	42.5
Lipid	22.2	21.7	21.7	21.5	21.8	20.8
Ash	6.1	6.1	5.9	5.9	6.1	6.2
Moisture	10.3	9.7	9.4	10.7	8.9	8.9
Energy crude (MJ/kg)[3]	22.0	22.1	22.2	21.8	22.2	22.0

676 [1] Lysine and methionine

677 [2] Vitamin and mineral premix, monocalcium-phosphate (MCP), cholesterol, Emulthin

678 G35, antioxidants

679 [3] Estimated by using the mean values of gross energy for proteins, lipids and

680 carbohydrates 23.6, 39.5 and 17.2 kJ/g, respectively [(15)]

681

682 Table 2. Fatty acid composition of the experimental diets (D1-D6) given as percentage
683 of total fatty acids. Note trace (<0.05) fatty acids are removed. Saturated fatty acids,
684 SFA; monounsaturated fatty acids, MUFA; polyunsaturated fatty acids, PUFA.

Fatty acid (%)	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Lipid (%)	22.16	21.71	21.71	21.47	21.82	20.82
14:0	0.55	1.07	1.47	2.10	3.34	5.73
16:0	15.44	15.62	15.71	16.05	16.69	17.52
18:0	2.53	2.62	2.67	2.80	3.04	3.43
20:0	0.46	0.38	0.43	0.41	0.36	0.26
22:0	0.23	0.23	0.22	0.21	0.19	0.16
ΣSFA	19.35	20.04	20.64	21.70	23.75	27.22
16:1n-7	0.51	1.12	1.48	2.20	3.65	6.71
18:1n-9	47.13	43.91	41.89	37.57	29.37	12.38
18:1n-7	2.47	2.55	2.58	2.69	2.77	2.99
20:1n	0.93	0.97	0.98	1.04	1.11	1.29
22:1n-11	0.08	0.14	0.19	0.28	0.43	0.75
24:1n-9	0.14	0.17	0.19	0.23	0.29	0.47
ΣMUFA	51.32	48.92	47.37	44.10	37.77	24.69
18:2n-6	21.42	20.42	19.69	18.56	16.16	11.22
18:3n-6	0.00	0.03	0.04	0.07	0.12	0.25
20:2n-6	0.06	0.07	0.07	0.08	0.10	0.14
20:4n-6	0.05	0.11	0.15	0.23	0.40	0.78
22:5n-6	0.00	0.04	0.05	0.09	0.16	0.31
Σ n-6 PUFA	21.52	20.67	20.01	19.03	17.01	12.90
18:3n-3	6.05	5.69	5.52	5.03	3.99	1.76
18:4n-3	0.08	0.30	0.44	0.70	1.23	2.40
20:4n-3	0.03	0.08	0.11	0.18	0.32	0.62
20:5n-3	0.70	1.96	2.73	4.32	7.48	14.34
22:5n-3	0.11	0.26	0.35	0.53	0.91	1.71
22:6n-3	0.63	1.47	1.98	3.06	5.23	9.89
Σ n-3 PUFA	7.61	9.76	11.13	13.83	19.16	30.77

685

686 Table 3. Primer sequences used for gene expression analysis by quantitative reverse-
687 transcriptase PCR. Amplicon sizes (base pair) and GenBank accession numbers also are
688 provided.

Transcript	Sequence (5'-3')	Amplicon(bp)	Accession no
fads2	F:GCAGGCGGAGAGCGACGGTCTGTTCC	72	AY055749
	R:AGCAGGATGTGACCCAGGTGGAGGCAGAAG		
elovl5	F:CCTCCTGGTGCTCTACAAT	112	AY660879
	R:GTGAGTGTCCTGGCAGTA		
cpt1α	F:GTGCCTTCGTTCGTTCCATGATC	82	JQ308822
	R:TGATGCTTATCTGCTGCCTGTTTG		
srebp1	F:AGGGCTGACCACAACGTCTCCTCTCC	77	JQ277709
	R:GCTGTACGTGGGATGTGATGGTTTGGG		
pparα1	F:TCTCTTCAGCCCACCATCCC	116	AY590299
	R:ATCCCAGCGTGTCGTCTCC		
fabp1	F:CATGAAGGCGATTGGTCTCC	165	KF857311
	R:GTCTCCAAGTCTGCCTCCTT		
srebp2	F:GCTCACAAGCAAAATGGCCT	240	AM970922.1
	R:CAAAACTGCTCCCTTCCCCA		
fas	F:TGCCATTGCCATAGCACTCA	172	JQ277708.1
	R:ACCTTTGCCCTTTGTGTGGA		
β-act	F:TCCTGCGGAATCCATGAGA	50	X89920
	R: GACGTCGCACTTCATGATGCT		
ef1α	F:ACGTGTCCGTCAAGGAAATC	109	AF184170
	R:GGGTGGTTCAGGATGATGAC		
tuba1α	F:ATCACCAATGCCTGCTTCGA	214	AY326430.1
	R:CTGTGGGAGGCTGGTAGTTG		
rplp0	F:GAACACTGGTCTGGGTCCTG	159	AY550965.1
	R:TTCAGCATGTTGAGGAGCGT		

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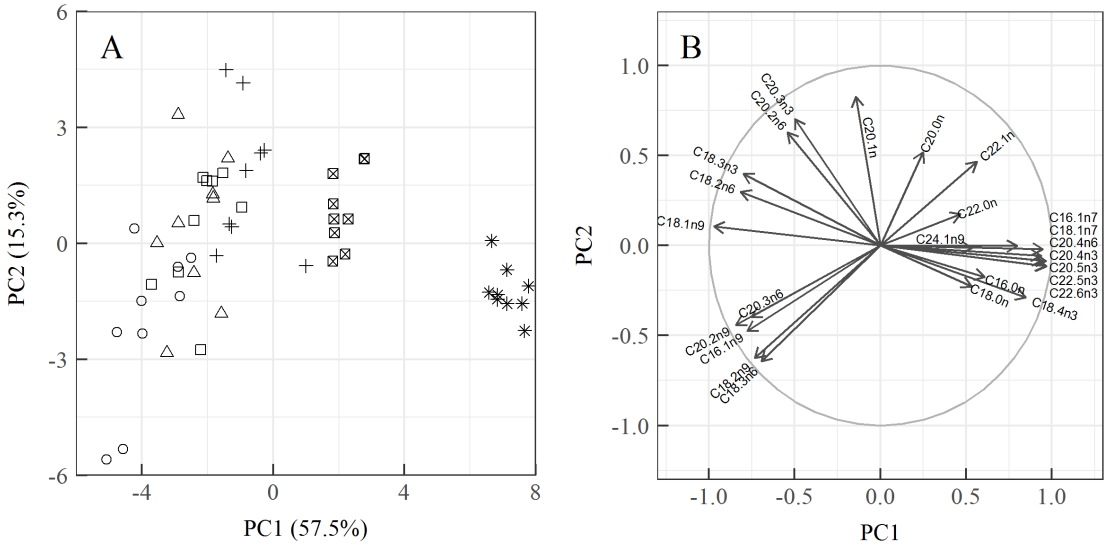
693

694 Table 4. Final weights and specific growth rates of *Sparus aurata* after 18 weeks
695 feeding on diets D1-6. Superscript letters indicate significant between group differences
696 by ANOVA.

Diet	Weight (g)	SD	SGR*	SD
D1	198.6 ^{c}	± 1.3	1.64 ^{d}	± 0.01
D2	219.4 ^{b}	± 6.6	1.72 ^{c}	± 0.02
D3	224.8 ^{b}	± 5.0	1.75 ^{cb}	± 0.01
D4	241.3 ^{a}	± 3.1	1.78 ^{ab}	± 0.00
D5	245.0 ^{a}	± 4.0	1.81 ^{a}	± 0.01
D6	248.3 ^{a}	± 1.5	1.81 ^{a}	± 0.01
ANOVA	$P < 0.001$		$P < 0.001$	

697 *SGR = (ln Final weight – ln Initial weight)/ days x 100

698 **Figures**

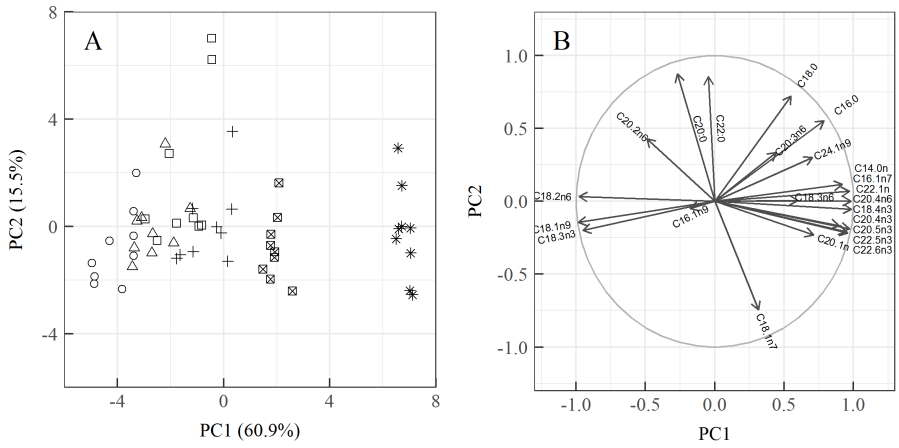


699

700 Figure 1. Graphical representation of principal components analysis (PCA) of fatty acid
701 profiles from liver (n=54) of fish fed experimental diets (D1 - D6). The two panels are
702 complimentary to one another. Panel A is a biplot of the first two principal components
703 (PC1 and PC2) of gilthead seabream liver fatty acid profiles. The fatty acid profiles of
704 fish consuming diets D5 and D6 formed succinct groups to the right of the plot and they
705 were well defined by PC1. The fatty acid profiles of fish consuming diets D1 - D4 were
706 negatively correlated to PC1 and there was considerable overlap (variability) between
707 the dietary treatments. PC2 accounted for 15.3 % of the total variance and was
708 important to distinguish individual liver fatty acid profiles of fish consuming diets D1 -
709 D3. Panel B shows all the variables (fatty acids) used to construct the principal
710 components. The circle in this plot is the correlation circle, the stronger the correlation
711 of a fatty acid to PC1 and, or PC2 the closer its arrowhead to the circle. Arrowheads that
712 do not approach the circle are not well represented by PC1 and PC2 (e.g. 22:0). The
713 arrows indicate how the fatty acids contributed to the formation of PC1 and PC2 and

714 thus the formation of Panel A. Several points can be made to describe the data, the fatty
715 acids derived from fish oil (e.g. 22:6n-3) were strongly correlated to PC1, the main fatty
716 acids from vegetable oil (e.g. 18:1n-9) were negatively correlated to PC1. Several fatty
717 acids were best explained by a combination of PC1 and PC2, including 18:2n-9 and
718 20:2n-9, and it can be said that these fatty acids are important to distinguish liver fatty
719 acid profiles from fish consuming diets D1 - D4. D1 = ○ ; D2 = ∆ ; D3 = □ ; D4 = + ;
720 D5 = ⊠ ; D6 =
721

722

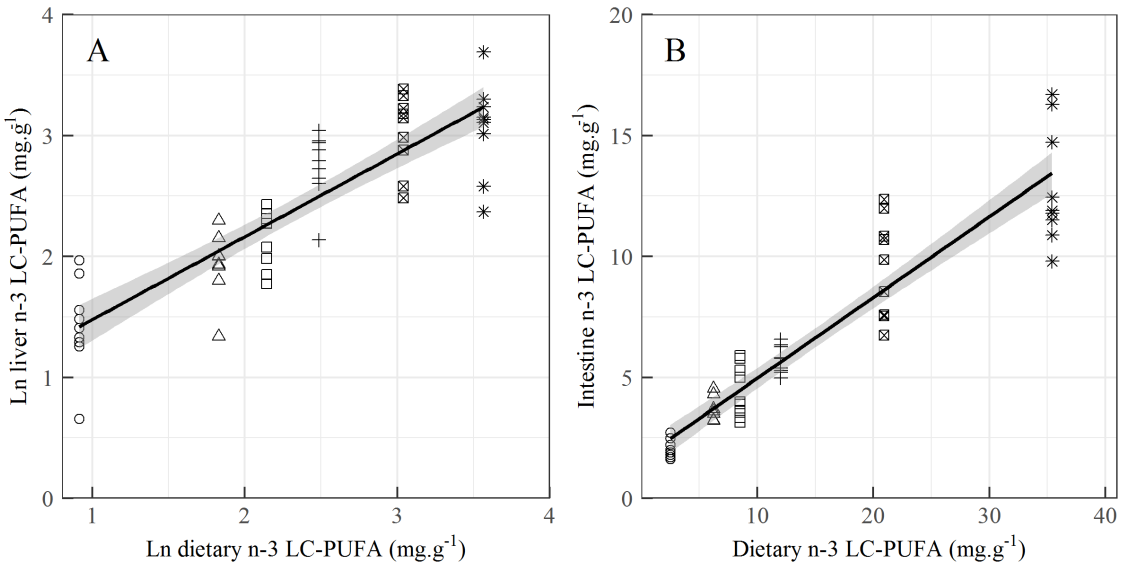


723

724 Figure 2. Graphical representation of principal components analysis (PCA) of fatty acid
725 profiles from mid-intestine (n=54) of fish fed experimental diets (D1 - D6). The two
726 panels are complimentary to one another. Panel A is a biplot of the first two principal
727 components (PC1 and PC2) of seabream mid-intestine fatty acid profiles. The fatty acid
728 profiles of fish consuming diets D5 and D6 formed succinct groups to the right of the
729 plot. The fatty acid profiles of fish consuming diets D1 – D3 were negatively correlated
730 to PC1 and there was considerable overlap in the points. PC2 separated the fatty acid
731 profiles vertically and explained 15.5 % of the variance, two outliers appeared in D3
732 and these samples contained unusually high levels 20:0 and 22:0 and, other than these
733 samples, the variance was quite evenly distributed amongst the diets when compared to
734 liver. Panel B shows all the variables (fatty acids) used to construct the principal
735 components. The circle in this plot is the correlation circle, the stronger the correlation
736 of a fatty acid to PC1 and, or PC2 the closer its arrowhead to the circle. Arrowheads that
737 do not approach the circle were not well represented by PC1 and PC2 (e.g. 16:1n-9).
738 The arrows indicate how the fatty acids contributed to the formation of PC1 and PC2

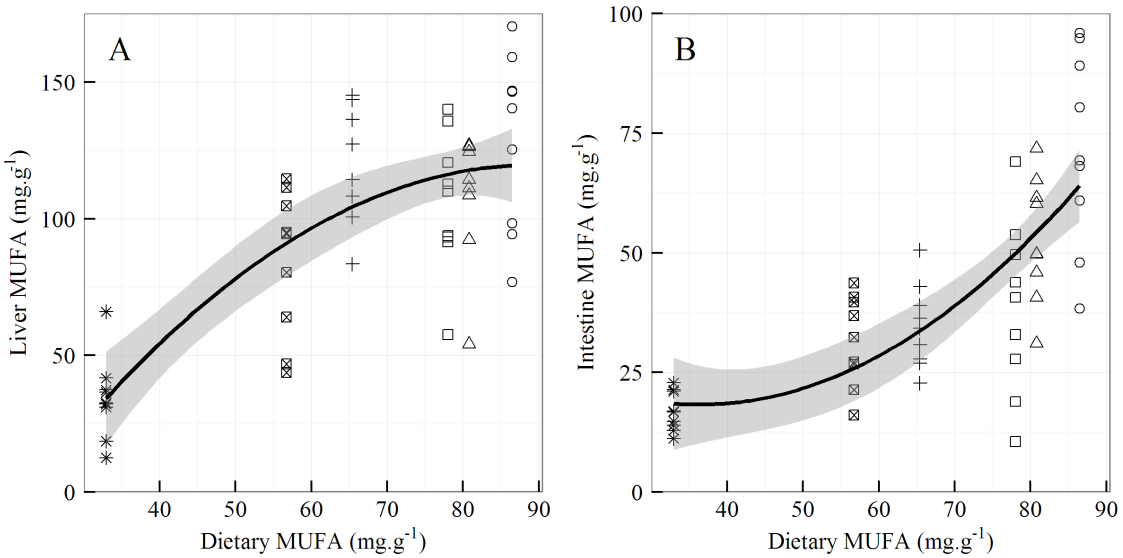
739 and thus the formation of plot A. D1 = ○ ; D2 = ∆ ; D3 = □ ; D4 = + ; D5 = ⊠ ; D6 =

740 .



741

742 Figure 3. Levels of n-3 long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA) in
743 gilthead seabream liver (A) and mid-intestine (B) against the dietary levels of n-3 LC-
744 PUFA. Note the natural logarithm (ln) transformation applied to the data in panel A
745 indicating that in liver this relationship was not linear. Both models were linear ordinary
746 least squares fits with the standard error shaded in grey (n=54). Diet 1 = ○ ; Diet 2 = ∆ ;
747 Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 =



748

749 Figure 4. Levels of monounsaturated fatty acids (MUFA) in gilthead seabream liver (A)
750 and mid-intestine (B) against the dietary levels of MUFA. Both are quadratic ordinary
751 least squares fits with the standard error shaded in grey (n=54). Diet 1 = ○ ; Diet 2 = △ ;
752 Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 =

753

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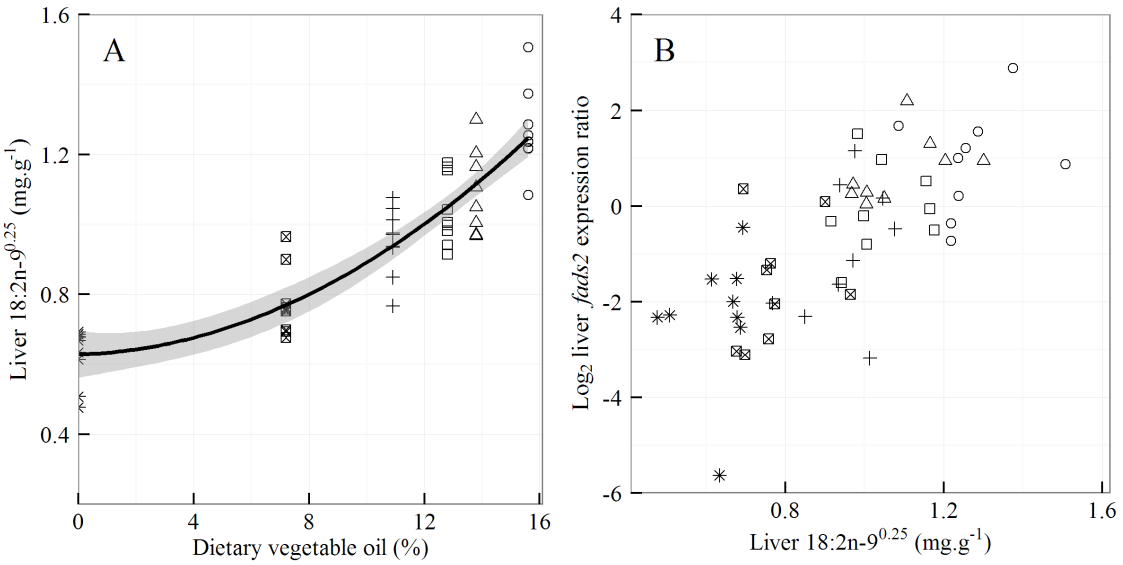
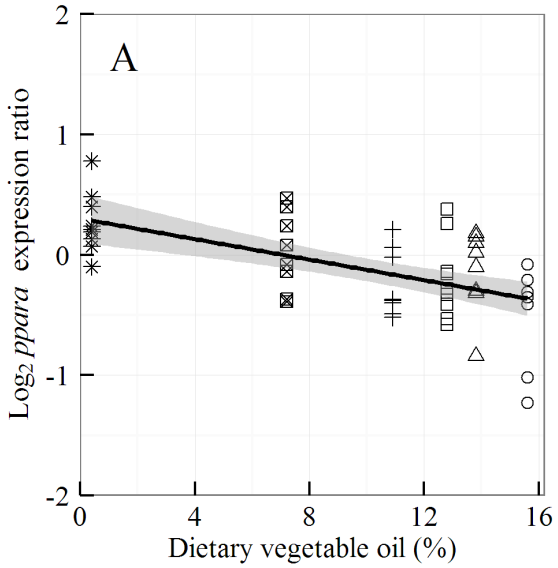
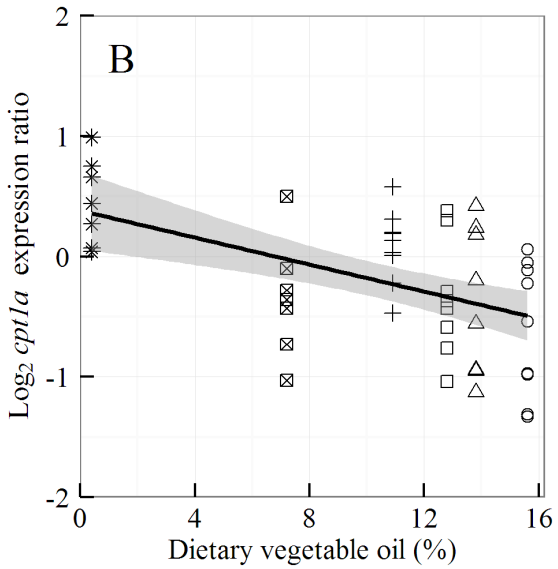
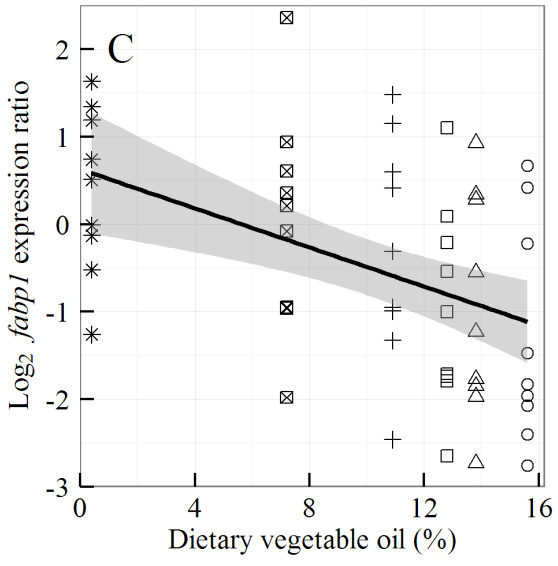


755

756 Figure 5. Levels of 18:2n-9$^{0.25}$, which is not present in the diets, in gilthead seabream
757 liver against dietary vegetable oil (A), and the level of *fads2* expression correlated (r =
758 0.64; P < 0.001) with the levels of 18:2n-9$^{0.25}$ in liver (B). The model applied in A is a
759 quadratic ordinary least squares fit with the standard error shaded in grey (n=54). Note
760 the transformation ($x^{0.25}$) applied to the 18:2n-9 data. Diet 1 = ○ ; Diet 2 = △ ; Diet 3 =
761 □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 =

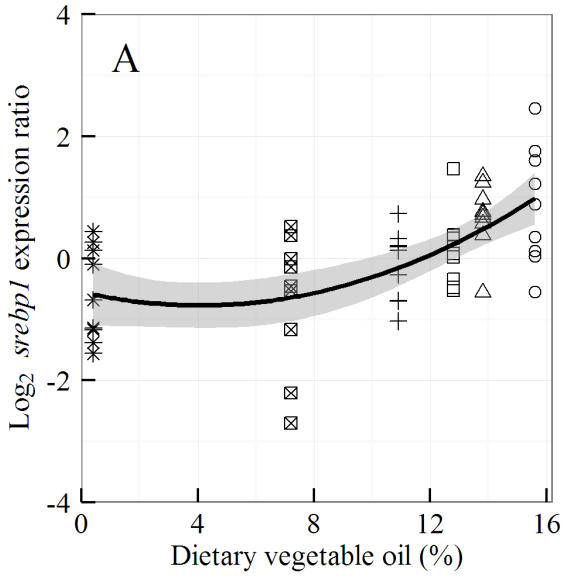

A
Log2 ppara expression ratio
2
1
0
-1
-2
0
4
8
12
16
Dietary vegetable oil (%)

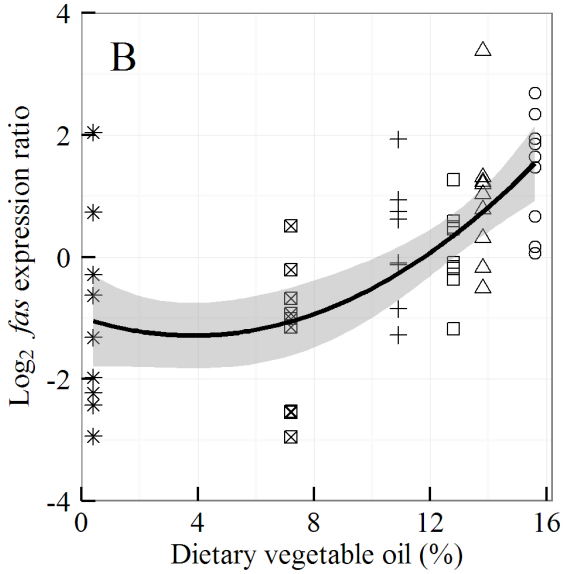

B
Log2 cpt1a expression ratio
2
1
0
-1
-2
0
4
8
12
16
Dietary vegetable oil (%)

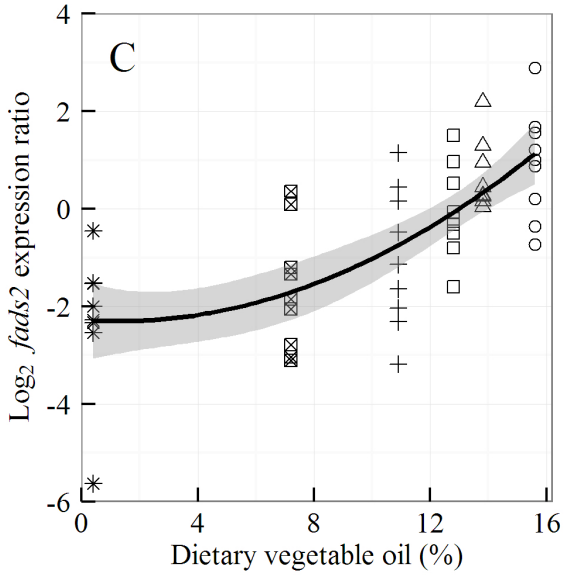

C
Log2 fabp1 expression ratio
2
1
0
-1
-2
-3
0
4
8
12
16
Dietary vegetable oil (%)

762

763 Figure 6. Hepatic gene expression of *ppara1* (A), *cpt1a* (B) and *fabp1* (C) against
764 dietary vegetable oil. Data are $\log_2$ (expression ratios) normalised to four reference
765 genes and then to the calibrator sample. Fitted lines are linear functions with the
766 standard error highlighted in grey (n=54). Diet 1 = ○ ; Diet 2 = ∆ ; Diet 3 = □ ; Diet 4
767 = + ; Diet 5 = ⊠ ; Diet 6 =

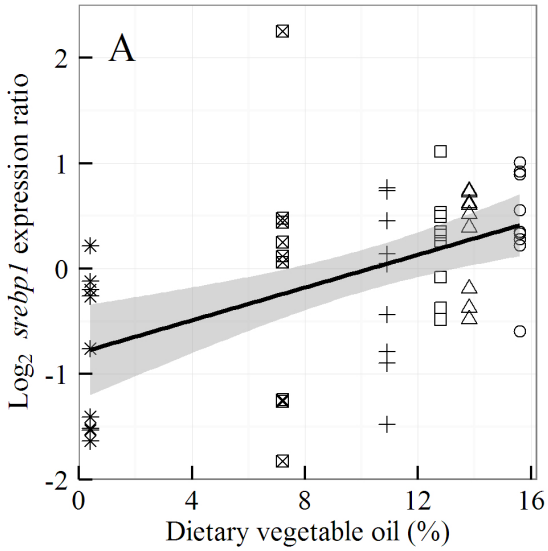

A
Log2 srebp1 expression ratio
Dietary vegetable oil (%)

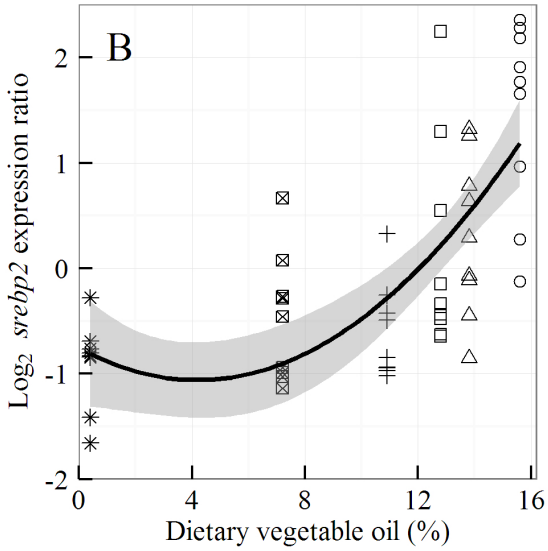

B
Log2 fas expression ratio
Dietary vegetable oil (%)

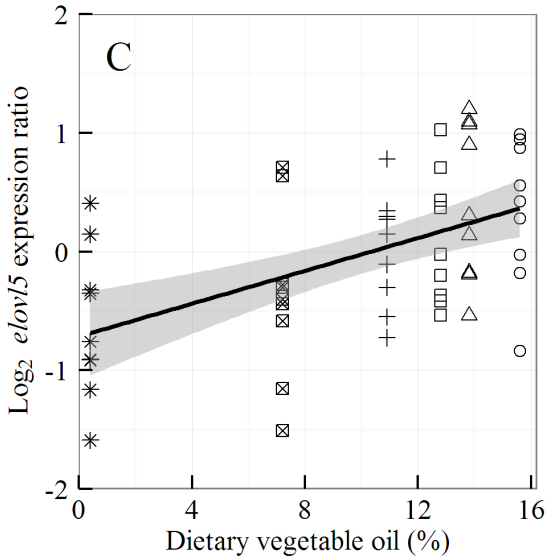

C
Log2 fads2 expression ratio
Dietary vegetable oil (%)

768

769 Figure 7. Hepatic gene expression of *srebp1* (A), *fas* (B) and *fads2* (C) against dietary
770 vegetable oil. Data are $\log_2$ (expression ratio) normalised to four reference genes and
771 then to the calibrator sample. Fitted lines are second order quadratic functions with the
772 standard error highlighted in grey (n=54). The similarity between the responses is
773 striking. Diet 1 = ○ ; Diet 2 = △ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 =


A
Log2 srebp1 expression ratio
Dietary vegetable oil (%)


B
Log2 srebp2 expression ratio
Dietary vegetable oil (%)


C
Log2 elovl5 expression ratio
Dietary vegetable oil (%)

774

775 Figure 8. Mid-intestine gene expression of *srebp1* (A), *srebp2* (B) and *elovl5* (C)
776 against dietary vegetable oil. Data are $\log_2$ (expression ratio) normalised to four
777 reference genes and then to the calibrator sample. Fitted lines are linear fits for *srebp1*
778 and *elovl5* and a second order quadratic function is fitted to *srebp2*. The model standard
779 errors are highlighted in grey (n=54). Diet 1 = ○ ; Diet 2 = ∆ ; Diet 3 = □ ; Diet 4 = + ;
780 Diet 5 = ⊠ ; Diet 6 =