Effect of dietary ARA/EPA/DHA ratios on growth performance and intermediary metabolism of gilthead sea bream (*Sparus aurata*) juveniles


Vegetable oils (VO) are generally accepted as valuable alternatives to fish oils but are devoid of n-3 LC-PUFA (Long-Chain-Polyunsaturated Fatty Acids) and rich in C18-PUFA. Marine fish have limited capacity to express Δ6/Δ5 desaturases (FADS1 and FADS2) and elongases of very-long-chain fatty acids (Elovl), which severely limits their capacity to produce LC-PUFA from C18-PUFA. This study aimed to evaluate the effects of dietary essential fatty acids (EFA) ratios, arachidonic (ARA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids on growth performance, feed utilization, whole-body composition, hepatic and intestinal activity of key-enzymes of lipid and carbohydrate metabolism, and expression of lipid metabolism-related genes. Dietary EFA ratios did not affect growth performance and whole-body composition. Nevertheless, feed and protein utilization were higher with diet B than diets C or D. Except for ARA, muscle EFA profile followed that of the diets. Plasma triglycerides were higher with diet B and C than A, and plasma cholesterol decreased in fish fed the higher DHA diet. Hepatic ELOVL5 and phospholipase A2 were upregulated in fish fed diet C compared to diet A. In the intestine, ELOVL5 levels were down-regulated in fish fed diet A and D compared to fish fed diet B. Overall, results showed that besides EPA and DHA, adequate dietary ARA level needs to be provided to optimize feed utilization efficiency of gilthead sea bream juveniles.

1. Introduction

Aquaculture growth has been so high in the last decades that from 2014 onwards human population consumed more fish from aquaculture than wild-caught fish (FAO, 2016). This increase has been paralleled with the increased use of aquafeeds where fish meal and fish oil (FO) still are major dietary ingredients, particularly for carnivorous fish species (NRC, 2011). As the use of fisheries-based feedstuffs in aquafeeds is not environmentally or economically sustainable, for a steady increase of aquaculture it is, therefore, necessary to find sustainable alternatives to these fisheries-based ingredients.

Marine fish evolved in an environment where natural diets were rich in long-chain polyunsaturated fatty acids (LC-PUFA). Over the years, marine fish lost or only has limited capacity to elongate and desaturate their precursors, linoleic (LA, 18:2n-6) and linolenic acid (ALA, 18:3n-3) to LC-PUFA (Jin et al., 2017; NRC, 2011; Torrecillas et al., 2018; Xue et al., 2014). Thus, arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) are essential fatty acids (EFA) for marine fish. Therefore, marine fish rely on dietary sources of LC-PUFA for normal growth, development, and survival (Izquierdo, 2005; Jin et al., 2017; Rombenso et al., 2016; Xu et al., 2018). Further, LC-PUFA are also important for physiological functions related to lipid metabolism (Jin et al., 2017; Torrecillas et al., 2018), antioxidant status (Jin et al., 2017; Luo et al., 2012), skeletogenesis (Boglino et al., 2012; Lie et al., 2016), pigmentation (Land et al., 2010; Villalta et al., 2008), stress tolerance (Liu...
et al., 2002; Rezek et al., 2010), and resistance to disease (Xu et al., 2016; Zuo et al., 2012a).

Over the last years, new sources of LC-PUFA, especially from microalgae and genetically modified Camelina sativa and yeast, reached the market giving the possibility to include the necessary EPA dietary content to optimise marine fish health and welfare (Tocher et al., 2019). Moreover, besides providing adequate levels of each EPA to meet requirements, it is also important to optimize the dietary balance of EPA. For instance, while no differences were observed in growth performance of juvenile black sea bream (Acanthopagrus schlegelii) fed diets with DHA/EPA ratios between 0.65 and 2.67, the highest ratios (2.03 and 2.67) decreased LC-PUFA biosynthesis and lipid anabolic pathway while increasing catabolic pathway and oxidative stress (Jin et al., 2017). In large yellow croaker (Larimichthys crocea), fed diets with similar lipid and LC-PUFA content, high dietary DHA/EPA ratios of 2.17–3.88 resulted in higher growth rates, nonspecific immunity, and immune-related gene expression than a dietary DHA/EPA ratio of 0.61 (Zuo et al., 2012b). In Atlantic salmon (Salmo salar), performance was improved with dietary inclusion of ARA/EPA at 0.8 ratio than with diets richer in ARA or EPA (ARA/EPA ratio of 2.4 and 0.1) (Norambuena et al., 2016). Also, in freshwater species, such as yellow catfish (Peleobagrus fulvidraco), higher growth performance was found with moderate dietary inclusion levels of ARA (0.6 and 0.9%) when compared to fish fed low ARA (0.03 and 0.3%) levels (Ma et al., 2018). On the other hand, high dietary ARA levels (1.2 and 1.5%) increased liver oxidative stress. The diverse physiological responses to altered dietary n-6/n-3 ratios among species highlight the need to optimize these ratios for each species.

Gilthead sea bream (Sparus aurata) is the main farmed marine fish species in the Mediterranean region (Eurostat, 2019). Despite this, current knowledge on the EPA requirements and optimal dietary n-3/n-6 ratio for this species is limited. For juveniles, EPA and DHA requirements were estimated to be 0.7 and 0.6% of dry matter (DM), respectively (Izquierdo, 2005), while total n-3 LC-PUFA requirement was estimated to be around 0.9 or 1.9% of DM, depending on the dietary DHA/EPA ratio being 1 or 0.5, respectively (Houston et al., 2017; Ibeas et al., 1994; Kalogeropoulos et al., 1992; Tocher, 2010). Regarding ARA, no differences were observed in growth performance or feed utilization of sea bream juveniles with dietary ARA inclusion ranging from 0.2% to 11.2% of total fatty acids (FA) (Fountoulaki et al., 2003). However, for larval fed diets with dietary EPA/DHA levels of 0.71/1.3% (Izquierdo, 2005) or 4/5% (Atalal et al., 2011), the inclusion of dietary ARA at levels of 1–1.2% increased growth and survival compared to fish fed ARA devoid diets. Therefore, it is important to better understand the comparative effects of n-6 and n-3 EPA in marine fish species. Thus, the aim of this study was to evaluate the effect of different dietary ARA, EPA, and DHA ratios on growth performance, whole-body composition, muscle lipid content and FA profile, and relevant plasma, hepatic, and intestine parameters related to lipid metabolism in gilthead sea bream juveniles. For that purpose, four diets were tested with distinct n6 LC-PUFA/n3 LC-PUFA ratios. The first diet as w = r ich in A RA an d h ad v ery low EPA + DHA content; the second diet was assumed to meet the EPA (n6 and n3 FA) requirements of gilthead seabream juveniles, the third diet meet n3-EFA requirements (with EPA and DHA ratio of 1:1) but was devoid of ARA; and the fourth diet also the meet n3-EFA requirements, but contained mainly DHA (EPA and DHA ratio of 0.2:1).

2. Material and methods

2.1. Diets composition

Four isoprotein (47% crude protein) and isoilpidic (18% crude lipids) diets were formulated containing fish meal (FM) and vegetable feedstuffs (VF) as protein sources (26% protein from FM and 74% from VF) and a vegetable oil (VO) blend as lipid source (20:50:30 ratios from rapeseed, linseed, and palm oils). Diets were supplemented with purified sources of EPA to obtain LC-PUFA ratios (% DM) of 2.0 ARA: 0.2 EPA: 0.1 DHA (Diet A); 1.0 ARA: 0.4 EPA: 0.4 DHA (Diet B); 0.0 ARA: 0.6 EPA: 0.6 DHA (Diet C); and 0.9 ARA: 0.3 EPA: 1.5 DHA (Diet D). ARA levels were achieved with a commercially available ARA oil obtained from Mortierella alpina (Vevodar®; DSM Food Specialties, the Netherlands); EPA levels with krill oil (Euphausia superba; SuperbaKrill™ Oil, Solchem*) and DHA with tuna oil (70% DHA; BrudyTechnology*). All dietary ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3.0 mm die. All diets were supplemented with lysine, taurine, and dicalcium phosphate to avoid essential amino acid and phosphorus deficiencies. Pellets were dried in an oven at 40 °C for 48 h and then stored in airtight bags in a freezer until use. Ingredients and proximate composition of the experimental diets are presented in the additional file 1 - Table S1, and dietary FA composition is presented in the additional file 1 - Table S2.

2.2. Growth trial

The trial was performed in CIIMAR, Matosinhos, Portugal. Gilthead sea bream (Sparus aurata) juveniles were acquired from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain). The quarantine period lasted 1 month, and after that fish were moved to the experimental system and allowed to adapt to the experimental conditions for 15 days. The experimental system consisted of a recirculating water system equipped with 12 cylindrical fiberglass tanks of 100 L water capacity and thermo-regulated to 23.0 ± 1.0 °C. Tanks were supplied with a continuous flow of filtered seawater (2.5–3.5 L min−1; salinity 35 ± 1 g L−1; dissolved oxygen: 7 ± 1 mg L−1) and controlled photoperiod of 12 h light and 12 h dark. At the beginning of the trial, 240 gilthead sea bream juveniles with an initial mean body weight of 15 g were grouped into 12 tanks. Each experimental diet was tested in triplicate and fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation for 8 weeks. Utmost care was taken to avoid feed losses.

2.3. Sampling

At the end of the trial, fish in each tank were slightly anesthetized with 0.3 mL L−1 of 2-phenoxyethanol and bulk-weighted after one day of feed deprivation. For whole-body composition analyses, ten fish from the initial stock population and three fish from each tank at the end of the trial were randomly sampled, euthanized by excess anaesthesia (0.6 ml L−1), and stored at −20 °C until analysis. Fish, liver, and viscera weights were recorded for measurement of hepatosomatic and visceral indices. The rest of the fish were then fed for two more days to allow recovery from final weighing manipulation stress. Therefore, 4 h after the morning meal, three fish from each tank were randomly sampled for blood collection using heparinized syringes. Blood was collected from the caudal vein and immediately centrifuged at 10 000 × g for 10 min. The supernatant plasma was stored in aliquots and frozen at −80 °C for metabolites analysis. Thereafter, fish were euthanized and intestines of two and liver of three fish were dissected on ice-cold trays and stored for analysis of key intermediate metabolism enzymes. Two other fish were euthanized and dissected, the digestive tract discharged from adjacent adipose and connective tissues, and a small portion of the intestine and liver were sampled for gene expression analysis. The remaining liver was stored for quantification of liver glycogen and lipid content. The muscle of these fish was also sampled for total lipids and fatty acids profile quantification. All tissue samples were stored at −80 °C until used.

2.4. Chemical analysis

Chemical analysis of whole-fish and diets was performed following
the Association of Official Analytical Chemists methods AOAC, 2000. Dietary starch was determined according to Beulter (1984), and hepatic glycerogen as described by Plummer (1972). Hepatic and muscle lipid content was determined by the method of Folch et al. (1957) using dichloromethane instead of chloroform for muscle lipid content determination.

Fatty acid methyl esters were prepared by adding sodium methylaate reagent and sequentially esterification with boron trifluoride in methanol according to Bondia-Pons et al. (2007) and analyzed in a Shimadzu GC-2010 gas chromatograph (Kyoto, Japan) equipped with a FID and a Shimadzu AOC-20i autoinjector.

2.5. Plasma metabolites analysis

Plasma cholesterol, glucose, triglycerides (TAG), and total protein were analyzed using enzymatic colorimetric kits from Spinreact, Girona, Spain (cholesterol kit, code 1001091; glucose kit, code 1001191; triglycerides kit, code 1001312; total proteins kit, code 1001291) as described in Magalhães et al. (2017). Liver and intestinal samples were homogenized (dilution 1:7 and 1:5, respectively) in ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. The resulting homogenates were centrifuged at 30 000 × g for 30 min at 4 °C, and aliquots of the supernatants were separated and stored at −80 °C. All enzymatic assays were performed at 37 °C in a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China). To ensure maximal activity for each enzyme, the optimal substrate and protein concentrations were accessed by preliminary assays. The molar extinction coefficient used for NADPH was 6.22 mM−1 cm−1. Glucokinase concentrations were accessed by preliminary assays. The molar extinction coefficient used for NADPH was 6.22 mM−1 cm−1. Glucokinase (GK, EC 2.7.1.2), hexokinase (HK, EC 2.7.1.1), L-type pyruvate kinase (PK, EC 2.7.1.4), fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), fatty acid synthetase (FAS; EC 2.3.1.38) multienzymes complex activities, glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) were determined according to Guerreiro et al. (2014). β-hydroxyacyl-CoA dehydrogenase (HOAD, EC 1.1.1.35) activity was determined according to Pérez-Jiménez et al. (2009). Protein concentration in the homogenates was quantified following the Bradford method using Bio-Rad Protein Assay Dye Reagent (ref. 5000006) with bovine serum albumin as standard. Enzyme activity was expressed as milliunits per mg of hepatic or intestinal soluble protein.

2.6. Enzymatic activities

Analyses of mRNA levels were performed on liver and intestine samples (two fish per tank). Total RNA was extracted using TRIzol reagent (Direct-zolTM RNA MiniPrep, Zymo Research) according to manufacturer recommendations, and RNA quality and quantity were assessed by 1% agarose gel electrophoresis and spectrophotometry (μDrop™ plate, ThermoScientific). The resulting total RNA concentration was adjusted to 0.5 μg/μl. cDNA was produced using the NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12501, Lisbon, Portugal). Gene expression was determined by real-time quantitative PCR (q-PCR) using the Bio-Rad, California, USA, CFX Connect Real-Time System. Analysis was carried out using 0.4 μl diluted cDNA (1:3 for liver samples and 1:2 for intestine samples) mixed with 0.2 μl of each primer (10 μM), 5 μl of SsoAdvanced™ Universal SYBR® Green SuperMix, Bio-Rad Laboratories*, and 4.2 μl DNaSe/RNase/Protease-free water (Sigma-Aldrich), in a total volume of 10 μl. Primers were obtained from literature (see additional file 1- Table S3). The slope of a standard curve using serial dilutions of cDNA measured PCR primers efficiency. Thermal cycling was initiated with incubation at 95 °C for 30s for hot-start iTaqTM DNA polymerase activation. A total of forty PCR cycles were then performed, each one consisting of heating at 95 °C for 15s for denaturing, and at 60 °C for 30s for annealing and extension. Following the final PCR cycle, melting curves were systematically monitored (65 °C temperature 0.5 °C 10 s −1 from 65 to 95). Each PCR run included duplicates of reverse transcription for each sample and negative controls. The PCR run for the reference gene included duplicates for each sample and negative controls. Quantification of the target gene transcripts was performed using elongation factor 1α (EF1α) gene expression as a reference, as previously used for gilthead sea bream by Castro et al. (2016a). Relative quantification of the target gene transcript with ELF1α reference gene transcript was performed using the mathematical model described by Pfaffl (2001). The relative expression ratio of a target gene was calculated based on real-time PCR efficiency and the cycle quantification value (CT) deviation of the unknown sample compared with the ELF1α reference gene.

2.8. Statistical analysis

Data are presented as mean and pooled standard error of the mean. Normality and homogeneity of variances were tested by the Shapiro-Wilk and Levene tests, respectively, and normalized when appropriate (In or log transformation). Statistical evaluation of the data was done by one-way ANOVA. When p-values were significant (p < 0.05), means were compared with Tukey’s multiple range test. All statistical analyses were performed using SPSS 24.0 software package for Windows (IBM* SPSS® Statistics, New York, USA).

3. Results

Fish promptly accepted the experimental diets and survival was high and not affected by dietary treatment (Table 1). Growth performance and feed intake were not affected by diet composition, but feed efficiency and protein efficiency ratio were higher in fish fed diet B than diets C and D. However, nitrogen and energy retention (% intake) were also not affected by diet composition (Table 1).

Whole-body composition, muscle lipid content, hepatosomatic

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Growth performance, feed utilization and nitrogen and energy retention of gilthead sea bream fed the experimental diets.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diets</td>
<td>A</td>
</tr>
<tr>
<td>(ARA/EPA/DHA ratio)</td>
<td>2.0/0.2/</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>15.0</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>45.5</td>
</tr>
<tr>
<td>Weight gain (g kg ABW−1 day−1)</td>
<td>18.0</td>
</tr>
<tr>
<td>Daily growth indexa</td>
<td>2.0</td>
</tr>
<tr>
<td>Feed intake (g kg ABW−1 day−1)</td>
<td>31.7</td>
</tr>
<tr>
<td>Feed efficiencyb</td>
<td>0.57ab</td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>1.2ab</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>96.7</td>
</tr>
<tr>
<td>Nitrogen retention (% NI)c</td>
<td>21.0</td>
</tr>
<tr>
<td>Energy retention (% EI)d</td>
<td>26.3</td>
</tr>
</tbody>
</table>

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

a DGI: daily growth index = ((final body weight − initial body weight)/initial body weight) × 100.
b FE: feed efficiency = wet weight gain/dry feed intake.
c PER: protein efficiency ratio = wet weight gain/crude protein intake.
d Nitrogen retention = ((FBWxFBN − IBW x IBN)/NI) x 100.
e Energy retention = ((FBWxFBE − IBW x FBE)/EI) x 100.
Whole-body, liver, and muscle composition (% wet weight), hepatosomatic (HSI) and Visceral Somatic Index (VSI) of gilthead sea bream juveniles fed the experimental diets.

<table>
<thead>
<tr>
<th>Diets</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ARA/EPA/DHA ratio)</td>
<td>2.0/0.2/0.1</td>
<td>1.0/0.4/0.4</td>
<td>0.0/0.6/0.6</td>
<td>0/0.3/1.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**Whole-body composition**

- Dry matter (%): 25.0, 30.5, 30.0, 31.0, 30.7, 0.5
- Protein: 14.3, 16.3, 16.5, 16.6, 17.0, 0.2
- Ash: 6.1, 10.5, 10.0, 11.5, 10.0, 0.4
- Gross protein (g kg⁻¹): 5.5, 4.3, 4.0, 3.8, 4.2, 0.2

**Liver composition**

- Lipid (%): –, 7.6, 8.8, 10.9, 11.2, 0.5
- Glycogen (%): –, 8.2, 8.3, 7.7, 7.8, 0.2
- Muscle lipids (%): –, 6.5, 4.4, 4.6, 4.5, 0.3
- HSI (%) –, 1.3, 1.3, 1.2, 1.3, 0.3
- VSI (%) –, 7.6, 7.4, 7.6, 7.3, 0.4

Values presented as means (n = 3 for body composition; n = 6 for liver composition and muscle lipids) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

- Hepatosomatic index: (liver weight/body weight) × 100.
- Visceral Somatic Index: (viscera weight/body weight) × 100.

4. Discussion

Dietary EFA requirements of gilthead sea bream seem to be related both to dietary lipid level and EPA/DHA/ARA ratios. Thus, n-3 LC-PUFA requirements of juveniles were estimated to be 0.9% when EPA/DHA ratio was 1:1 and dietary lipid level was 13% (Kalogeropoulos et al., 1992), while with EPA/DHA ratio of 2:1 and 8% dietary lipids, n-

**Table 3**

Muscle fatty acid composition (% of total fatty acids) of gilthead sea bream fed the experimental diets.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΣSFA</td>
<td>2.0/0.2/0.1</td>
<td>1.0/0.4/0.4</td>
<td>0.0/0.6/0.6</td>
<td>0/0.3/1.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

- SFA | C14:0 | 1.25b | 1.55c | 2.19d | 1.08a | 0.09 |
- C15:0 | 0.14ab | 0.15bc | 0.17b | 0.13a | 0.00 |
- C16:0 | 15.0a | 16.2a | 17.3a | 15.2a | 0.22 |
- C17:0 | 0.17bc | 0.17bc | 0.15bc | 0.13a | 0.01 |
- C18:0 | 4.49 | 4.43 | 4.37 | 4.09 | 0.08 |
- C20:0 | 0.82b | 0.45b | 0.21b | 0.15a | 0.06 |
- C22:0 | 1.11c | 0.69a | 0.22b | 0.16a | 0.08 |
- C24:0 | 0.38b | 0.32b | 0.07b | 0.06b | 0.03 |

- MUFA | C16:1 n-7 | 1.93a | 2.23b | 2.75b | 1.89a | 0.08 |
- C17:1 n-7 | 0.08b | 0.09b | 0.10b | 0.06a | 0.00 |
- C18:1 n-9 c | 29.7a | 30.0a | 31.6a | 30.5a | 0.19 |
- C20:1 n-9 | 0.72 | 0.71 | 0.74 | 0.78 | 0.02 |
- C22:1 n-9 | 8.35c | 4.83b | 0.30a | 0.24a | 0.71 |
- C24:1 n-9 | 0.24 | 0.25 | 0.25 | 0.22 | 0.01 |

**Table 4**

Specific activities of intestine intermediary metabolism related enzymes (mU/mg protein) in gilthead sea bream fed the experimental diets.

<table>
<thead>
<tr>
<th>Diets</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ARA/EPA/DHA ratio)</td>
<td>2.0/0.2/0.1</td>
<td>1.0/0.4/0.4</td>
<td>0.0/0.6/0.6</td>
<td>0/0.3/1.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

- ME | 12.1 | 11.0 | 13.4 | 11.0 | 0.80 |
- FAS | – | – | – | – | – |
- HOAD | – | – | – | – | – |
-HK | 17.1 | 14.0 | 14.4 | 14.5 | 0.83 |
- GK | 23.8 | 19.7 | 18.5 | 14.3 | 1.41 |
- FPBAS | 25.0 | 19.6 | 31.4 | 20.2 | 2.45 |
- PK | 26.1 | 20.1 | 21.6 | 22.1 | 2.13 |

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

4 LC-PUFA requirement was estimated to be 1.9% (Ibeas et al., 1994). Recently, Houston et al. (2017) reported a dietary n-3 LC-PUFA requirement of circa 3% in diets with EPA/DHA ratio of 1:1 and 22% lipids. This is in line with the recommendation of Lazo (2005) that dietary n-3 LC-PUFA should be over 2.5% in actual commercial diets, which have high lipid contents. The effect of ARA on marine fish is less certain. When ARA: EPA ratios were changed from 0.04 to 1.7 in diets with a constant level of 2.7% n-3 LC-PUFA, there was no effect on
ARA(2%) and DHA(0.3%).

Table 4

Table 5

Table 6

Table 7

Table 8

Expression were normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Values presented as means (n = 6) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

Expression were normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Values presented as means (n = 6) and pooled standard error of the mean (SEM); for each gene, different letters indicate significant differences.

Expression were normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

Expression were normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Values presented as means (n = 6) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

juvenile growth performance (Fountoulaki et al., 2003). However, Ibeas et al., (1997) found that higher dietary levels of EPA (1%) than DHA (0.5%) were required for maximum growth of juvenile sea bream, while Betancor et al. (2016) found reduced growth when juveniles were fed diets with high EPA:DHA ratios (2.4 and 0.75% DM, respectively; 18% of dietary lipidic content).

Diet used in this study were formulated to have very different n6/n3 EPA ratios and total n-3 LC-PUFA content. While diet A was rich in ARA (2%) and deficient in EPA:DHA (0.3%), diet B included ARA, EPA, and DHA at levels which at least meet the minimum requirements, and diet C was almost devoid of ARA but had 1.2% EPA:DHA in a 1:1 ratio. Diet D was designed to have a 1.8% LC-PUFA level with a ratio of EPA: DHA of 1:5 and being almost devoid of ARA. The oils used in the present study had their LC-PUFA attached to TAG and phospholipids (PL) mainly at the sn-2 position of glycerol. However, the position of the LC-PUFA was not the aim of the study and was not further analyzed.

Despite these differences in dietary ARA/EPA/DHA ratios and overall LC-PUFA content, the growth performance of gilthead sea bream juveniles was not affected by diet composition. This was unexpected as in the studies mentioned above, fish fed EPA deficient diets for periods similar to that of the present study had reduced performance. Although, according to Izquierdo (2005) juveniles may be able to survive for months on a diet with very low EPA content, their performance is expected to be affected, while in the present study sea bream performance was within or better than that obtained in other studies (Bandarra et al., 2011; Castro et al., 2016b; Guerreiro et al., 2015; Dias et al., 2009).

Thus, diet B seemed to meet EFA requirements of gilthead sea bream juveniles, while including only 0.73% DM of n-3 LC-PUFA in 18% lipid diet. This n-3 LC-PUFA content is slightly lower than that previously considered necessary to meet the n-3 LC-PUFA requirements of juveniles of this species (Kalogeropoulos et al., 1992) and well below recommendations of Izquierdo (2005) for high dietary lipid diets.

Despite the lack of effect on growth performance, this study showed that a balanced dietary n-6:n-3 LC-PUFA ratio was required to maximize feed utilization. Thus, higher FE and PER were observed in fish fed diet B, with ARA:EPA:DHA ratio of 1:0.4:0.4, than with diet C with 1.8% LC-PUFA level with a ratio of EPA:DHA of 1:5 and being almost devoid of ARA. This may be due to the balance of dietary EPA and DHA, which are considered necessary to meet the n-3 LC-PUFA requirements of juvenile sea bream (Kalogeropoulos et al., 1992) and well below the recommendations of Izquierdo (2005) for high dietary lipid diets.

The importance of appropriate dietary levels of ARA to improve feed utilization as demonstrated in this study for gilthead sea bream, has previously been highlighted for Japanese sea bass (Lateolabrax japonicus) (Xu et al., 2010), grass carp (Ctenopharyngodon idellus) (Tian et al., 2014), and Japanese eel (Anguilla japonica) (Shahkar et al., 2016).

Fish whole-body composition showed no differences between dietary treatments. This was expected, as previous studies in this and other species showed no major effects of dietary LC-PUFA ratios on whole-body composition (Jin et al., 2017; Norambuena et al., 2016; Fountoulaki et al., 2003). On the contrary, increasing dietary DHA and EPA levels decreased whole-body lipid content in Japanese sea bass (Xu et al., 2016). Further, in other fish species, it was also reported that whole-body lipids decreased in ARA supplemented diets (Norambuena et al., 2016).
et al., 2016; Tian et al., 2014; Shahkar et al., 2016), though an excessive ARA supplementation resulted in lipid accumulation (Xu et al., 2010). Similarly, in the present study liver lipid content was also decreased in ARA supplemented diets.

It is known that dietary FA composition strongly influences muscle FA composition (Benedito-Palos et al., 2008; Izquierdo et al., 2003). In gilthead sea bream, when anchovy oil was replaced by soybean, rapeseed or linseed oil, muscle and liver content of EPA, DHA, and total n-3 LC-PUFA were accordingly reduced (Izquierdo et al., 2003). In the same way, FO substitution at 33%, 66%, or 100% by a VO blend (rapeseed, linseed, and palm oil) promoted a sequential reduction of muscle EPA and DHA content (Benedito-Palos et al., 2008).

Similarly, in this study muscle fatty acid n3-PUFA content, namely EPA and DHA content, strongly reflected dietary FA composition. On the contrary, no such effect was observed for muscle n6-PUFA content, namely for ARA content, which was very low and independent of dietary content. This was unexpected and contradicts previous results in this and other species (Fountoulaki et al., 2003; Shahkar et al., 2016; Torreillas et al., 2018). Interestingly, muscle concentration of 22:1n-9 was correlated to that of dietary ARA. An unexpected result that needs to be further evaluated. In fact, fish and other vertebrates can produce 18:1n-9 from C18 FA by the action of stearoyl-CoA desaturase in response to n-3 LC-PUFA deficiency (Gey et al., 2011). Indeed, the presence of n9-PUFA, particularly 18:1n-9 and 20:1n-9, was suggested to be a good indicator of EFA insufficiency in gilthead sea bream and grouper (Epinephelus malabaricus) (Rodriguez et al., 1993; Wu et al., 2002). Further, endogenous production of 22:1n-9 from 18:1n-9 was also observed in Atlantic salmon (Sissember et al., 2018). Accordingly, in the present study, high 22:1n-9 muscle content, 20:1n-9 product of elongation, was to be expected in fish fed diet A, which was almost devoid of EPA and DHA, but not in fish fed diet B, which seemed to include sufficient amounts of n3 LC-PUFA.

Lipogenesis and lipolysis pathways are known to be regulated by dietary EFA (Castro et al., 2016a; Stubhaug et al., 2007). In the present study, dietary FA c composition did not affect lipid m metabolism at the n tiniest level, only at hepatic level. Indeed, high dietary ARA levels seemed to increase hepatic HOAD activity, a key e enzyme involved i n β-oxidation of FA (Wu, 2018). FAcatabolismoccurs in n two organelles, mitochondria, and peroxisomes (Tocher, 2003). In mammals, these two biological pathways have different purposes, with m iochondrial β-oxidation being related to ATP production and peroxisomal β-oxidation being involved in the chain shortening of long-chain FA that will be further oxidized in the m iochondria (Wu, 2018). Fish, however, appear to utilize peroxisomal β-oxidation system at a significant level, including short-chain fatty acids (Stubhaug et al., 2007). Thus, the observed increase in hepatic HOAD may be related to increased catabolism of ARA and may explain the low ARA levels deposited in the muscle.

In the present study, high dietary HDA content in diet D promoted a reduction of hepatic FAS activity. Similarly, in m am m aLSHA is known to be a potent inhibitor of FAS expression (Huang et al., 2017). Also in rainbow trout (Oncorhynchus mykiss) hepatocytes, n3 LC-PUFA inhibited lipogenesis, but while EPA strongly inhibited FAS activity, HDA exerted a stronger effect on inhibiting acetyl-CoA carboxylase and G6PD activities (Alvarez et al., 2000).

Plasma glucose and total protein levels were within the reference values for gilthead sea bream (Peres et al., 2013) and were unaffected by dietary composition. Total plasma cholesterol was reduced in fish fed the high HDA diet, but no correlation was found with hepatic transcript levels of ABCG5, a protein involved in cholesterol transport. High dietary n-3 LC-PUFA level has been associated with hypcholesterolemia in both fish and humans (Hong et al., 2018; Pizzini et al., 2017). In fish, low-density lipoprotein (LDL) receptors are responsible for LDL clearance from plasma, and dietary FA modulates activity and expression of these receptors (Richard et al., 2006; Tocher, 2003). In rainbow trout, FO based diets increased LDL receptors gene expression in the liver, but also increased plasmatic cholesterol and LDL content (Richard et al., 2006). However, the authors suggested that membrane fluidity increment by PUFA enrichment can increase LD binding capacity leading to lower plasmatic cholesterol content, as observed in the present work.

ApoA3 is the main apolipoprotein of plasmatic HDL, being the preferential receptor for free and remaining cholesterol (Xu et al., 2018). In the present study, there was a trend for hepatic ApoA3 expression to be lower in fish fed the high DHA diet, though differences in ApoA3 expression were not statistically different among groups. Previously, Xu et al. (2018) described in Japanese seabass a downregulation of ApoA3 transcription level with low (0.05–0.22%) and high (1.38–2.22%) dietary ARA content, and an upregulation with an intermediary (0.8%) dietary ARA content. However, no such ARA effect was observed in the present study.

Plasma TAG levels were lower in fish fed the highest ARA diet. Accordingly, liver expression levels of Srebp1, LPL, and DGAT followed a similar trend. In Japanese seabass, liver downregulation of Srebp1 and DGAT expression was previously associated with increased dietary ARA levels, but no effect on LPL expression level was reported (Xu et al., 2018). However, in yellow catfish, high dietary ARA (1.2 and 1.5%) levels increased SREBP1, but not LPL gene expression (Ma et al., 2018). Contrary to present results, increasing DHA/EPA ratios in diets for juvenile black sea bream led to a decrease of SREBP1 expression level while, similarly to present results, had no effect on LPL expression (Jin et al., 2017). Thus, present knowledge on the effect of dietary ARA in the regulation of Srebp1, LPL, and DGAT is still very scarce and contradictory and needs to be further investigated.

As FAS is a target of Srebp1, regulation of energy storage as TAG is mediated by this lipid-sensing transcription factor. DGAT is the most important enzyme involved in TAG synthesis and LPL hydrolyzes TAG in plasma lipoproteins providing FA for storage in adipose tissues (Betancor et al., 2016; Castro et al., 2016a; Minghetti et al., 2011). Accordingly, liver lipid deposition was also lower in fish fed the high ARA diet. Previously, upregulation of Srebp1 and LPL in the liver, but not in the intestine, of gilthead sea bream fed VO based diets and supplemented with EPA and DHA compared to FO diets was already associated with more pronounced lipid deposition in the liver (Betancor et al., 2016). Similar results were observed in different fish species fed low n-3 LC-PUFA diets (Carmona-Antoñanzas et al., 2014; Limitusonter et al., 2014; Peng et al., 2014).

Hepatic FABP2 expression, a gene involved in lipid transportation and uptake, showed no differences between dietary treatments. In agreement, Castro et al. (2016a) also found no differences in FABP2 expression in gilthead sea bream juveniles fed FO or VO-based diets. However, an upregulation of hepatic FABP2 expression in gilthead sea bream fed high EPA: DHA diets was reported (Betancor et al., 2016).

Recent studies have already confirmed that gilthead sea bream expresses FADS2, typically a Δ6 desaturase, as well as ElovL5, an elongase highly efficient in elongation of C18 and C20 PUFA (Betancor et al., 2016; Castro et al., 2016b; Houston et al., 2017).

Present results indicate that high dietary ARA downregulated hepatic ELOVL5, a result similar to that observed in grass carp and yellow catfish (Ma et al., 2018; Tian et al., 2014). High DHA/EPA ratio also decreased ELOVL5, but this effect was only significant in the intestine. Similarly, in juvenile black seabream, increasing dietary DHA/EPA ratio decreased hepatic ELOVL5 expression (Jin et al., 2017). This seems to indicate that maintaining an optimum dietary ratio of LC-PUFA is important for promoting FA elongation, and this will be of relevance in the new VO-rich aquafeeds.

In the present study, PLAA expression was downregulated in the liver of fish fed diets with high ARA level. A drastic reduction of PLAA expression was previously observed in gilthead sea bream larvae fed diets with ARA content above 0.4% (Alves Martins et al., 2012). In addition, salmon fed diets with high dietary n-6 PUFA and low n-3 PUFA levels also had reduced PLAA activity in the gills (Bell et al., 1996). ARA is primarily stored in the membrane PL and PLAA.
preferentially hydrolyzes ARA-containing PL, hydrolyzing the sn-2 ester bond of the cellular PL and generating free ARA (Gijón and Leslie, 1999). Thereby, the downregulation of PLA2 expression in fish fed high ARA diets may be a negative feedback mechanism to maintain free ARA levels and its eicosanoids metabolites within normal physiological values. Moreover, fish fed low ARA and high EPA and DHA diets may present a higher cell-wall content of LC-PUFA and thus a higher vulnerability to peroxidation, leading to higher PLA2 activity, as it is activated by peroxidized FA (Bell et al., 1996).

5. Conclusions

Overall, the results of this study showed that besides EPA and DHA, dietary ARA and ARA:EPA:DHA ratios need to be optimized for improved feed utilization efficiency of gilthead sea bream juveniles and for upregulating the expression of FA elongases. This is of high relevance when considering using low FO aquafeeds for marine fish, and such effect should be further evaluated in future studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

VO: vegetable oil
FO: fish oil
FM: fish meal
VF: vegetable feedstuffs
LC-PUFA: long chain polyunsaturated fatty acid
SFA: saturated fatty acid
MUFA: monounsaturated fatty acid
FA: fatty acid
EFA: essential fatty acid
ARA: arachidonic acid
EPA: eicosapentaenoic acid
DHA: docosahexaenoic acid
LA: Linoleic acid
ALA: α-Linolenic acid
DM: dry matter
ORBEA: Órgão responsável pelo bem-estar animal
TAG: triglycerides
FID: Flame ionization detector
EDTA: Ethylenediamine tetraacetic acid
GK: glucokinase
HK: hexokinase
PK: pyruvate kinase
FBPase: fructose-1,6-bisphosphatase
G6PD: glucose 6-phosphate dehydrogenase
ME: malic enzyme
HOAD: β-hydroxacyl-CoA dehydrogenase
SEM: standard error of the mean
HSI: Hepatosomatic index
VSI: Visceral Somatic Index
ABW: average body weight
DGI: daily growth index
FE: feed efficiency
PER: protein efficiency ratio
IBN/FBN or IBE/FBE: initial and final body nitrogen or energy content
NI: Nitrogen intake
EI: Energy intake
IBW and FBW: initial and final body weight
LDL: low-density lipoprotein
PLA2: phospholipase A2
FABP2: FA binding protein
DGAT: Diacylglycerol acyltransferase
ApoA1: Apolipoprotein A1
LPL: Lipoprotein lipase
FAS: Fatty acid synthase
FADS1: Fatty acid desaturase 1
FADS2: Fatty acid desaturase 2
ELOVL: Elongation of very long chain fatty acids protein
Elov5: Elongation of very long chain fatty acids protein 5
Srebp1: Sterol regulatory element-binding transcription factor 1
ABGC5: ATP binding cassette G5
EF1α: elongation factor 1-alpha

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734644.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

R. E. O., H. P., and A. O.T. designed the study, R. M. and I. G. conducted the growth trial, R. M., H. P., F. C., S.M., S. S., C. D. M., and V.F.D. performed the experimental analysis and analyzed the data. R. M., H. P., and A. O. T. wrote the paper. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA), in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

Consent for publication

Not applicable.
References


Atalah, E., Hernández-Cruz, C.M., Benitez-Santana, T., Gang, R., Roo, J., Izquierdo, M., 2011. Importance of the relative levels of dietary arachidonic acid and eico-


M. J., Diez, M., 2000. A simple method for the isolation and pur-

M.J., Diez, M., 2000. A simple method for the isolation and pur-

M.J., Diez, M., 2000. A simple method for the isolation and pur-


Rombenchos, A.N., Trusthenski, J.T., Jirsa, D., Drawbridge, M., 2016. Docosahexaenoic acid (DHA) and arachidonic acid (ARA) are essential to meet LC-PUFAs requirements of juvenile California Yellowtail (Seriola dorsalis). Aquaculture 463, 123–134. https://doi.org/10.1016/j.aquaculture.2016.05.004.


