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

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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. Diets were formulated to include ARA/EPA/DHA ratios of 2.0/0.2/0.1 (Diet A); 1.0/0.4/0.4 (Diet B); 0/0.6/0.6 (Diet C); and 0/0.3/1.5 (Diet D) and were fed to triplicate groups of gilthead sea bream juveniles for 56 days.

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 $ELOVL_5$ and phospholipase A_2 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 (Lund et al., 2010; Villalta et al., 2008), stress tolerance (Liu

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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 EFA dietary content to optimize marine fish health and welfare (Tocher et al., 2019). Moreover, besides providing adequate levels of each EFA to meet requirements, it is also important to optimize the dietary balance of EFA. 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 croacker (*Larmichthys 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 0.7 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 (*Pelteobagrus 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 EFA 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 larvae fed diets with dietary EPA/DHA levels of 0.7/1.3% (Izquierdo, 2005) or 4/5% (Atalah 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 EFA 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 plasmatic, 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/n-3 LC-PUFA ratios. The first diet was rich in ARA and had very low EPA + DHA content; the second diet was assumed to meet the EFA (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 isoproteic (47% crude protein) and isolipidic (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 EFA 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 ARA: 0.6 EPA: 0.6 DHA (Diet C); and 0 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-weighed 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 anesthesia (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. Thereafter, 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 Beutler (1984), and hepatic glycogen 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 methylate 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).

2.6. Enzymatic activities

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 (GK, EC 2.7.1.2), hexokinase (HK, EC 2.7.1.1), L-type pyruvate kinase (PK, EC 2.7.1.40), 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.

2.7. Gene expression

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/8 μl H_20. 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 15 s for denaturing, and at 60 °C for 30 s 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 ELF1 α 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 (ln 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 1
Growth performance, feed utilization and nitrogen and energy retention of gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Initial body weight (g)	15.0	15.0	15.0	15.0	0.0
Final body weight (g)	45.5	50.0	51.2	45.4	1.2
Weight gain (g kg ABW^{-1} day^{-1})	18.0	19.2	19.5	17.9	0.3
Daily growth index[a]	2.0	2.2	2.2	2.0	0.1
Feed intake (g kg ABW^{-1} day^{-1})	31.7	30.8	38.4	35.9	1.3
Feed efficiency[b]	0.57^{ab}	0.64^{b}	0.52^{a}	0.50^{a}	0.2
Protein efficiency ratio[c]	1.2^{ab}	1.4^{b}	1.1^{a}	1.1^{a}	0.0
Survival (%)	96.7	96.7	91.7	95.0	1.1
Nitrogen retention (% NI)[d]	21.0	23.4	19.0	19.2	0.8
Energy retention (% EI)[e]	26.3	28.1	24.8	23.9	0.9

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 ($P < 0.05$).
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. ABW: average body weight = ((initial body weight + final body weight)/2).
[a] DGI: daily growth index = ((final body $weight^{1/3}$ – initial body $weight^{1/3}$)/time in days) × 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 IBE)/EI) x 100.

Table 2
Whole-body, liver, and muscle composition (% wet weight), hepatosomatic (HSI) and Visceral Somatic Index (VSI) of gilthead sea bream juveniles fed the experimental diets.

Diets	Initial	A	B	C	D	SEM
(ARA/EPA/DHA ratio)		2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
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
Lipids	6.1	10.5	10.0	11.5	10.0	0.4
Ash	5.5	4.3	4.0	3.8	4.2	0.2
Gross energy (kJ g^{-1})	5.9	8.6	8.3	9.0	8.7	0.1
Liver composition						
Lipid (%)	–	7.6^{a}	8.8^{ab}	10.9^{b}	11.2^{b}	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[a] (%)	–	1.3	1.3	1.3	1.2	0.3
VSI[b] (%)	–	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 ($P < 0.05$).

[a] Hepatosomatic index: (liver weight/body weight) × 100.

[b] Visceral Somatic Index: (Viscera weight/body weight) × 100.

index (HSI) and visceral somatic index (VSI) were not affected by dietary treatment (Table 2). Hepatic lipid content was higher in fish fed diets C and D than diet A, while hepatic glycogen content was not affected by diet composition (Table 2).

Muscle FA composition is presented in Table 3. Fish fed diet D had the highest ALA level while fish fed diet C had the lowest LA level. EPA and DHA levels varied according to the dietary inclusion level; the higher values were observed in fish fed diets C and D, respectively. Saturated fatty acids (SFA) content was lowest in fish fed diet D, while monounsaturated fatty acids (MUFA) content was higher with diet A, followed by diets B, C, and D, and this trend was mainly related to muscle C22:1 n-9 concentration. Fish fed diet D had the highest polyunsaturated fatty acids (PUFA) content, followed by fish fed diets C, B, and A, and this trend followed that of DHA content. Muscle ARA content was very low in all groups, but it was higher in fish fed the low ARA diets. Total n-3 FA content was highest in fish fed diet D, followed by C, B, and A while total n-6 FA content was higher in fish fed diets A and B than in fish fed diet C.

No differences in the activity of intermediary metabolism enzymes were observed in the intestine (Table 4), while in the liver, FAS activity was lower in fish fed diet D and HOAD activity was lower in fish fed diet C than diet A and B (Table 5).

No differences were found in plasma glucose and total protein content (Table 6). However, plasma triglycerides and cholesterol were higher in fish fed diets B and C than diets A and D, respectively.

Hepatic expression of $Elovl_5$ was higher in fish fed diets C than diet A, while expression of PLA_2 was higher in fish fed diet C than diet A (Table 7). No further differences were observed in the hepatic expression of other genes measured in relation to diet composition. In the intestine, only the expression of $Elovl_5$ was affected by dietary treatments, being higher in fish fed diet B than diets A and D (Table 8).

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-3 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 Izquierdo (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

Table 3
Muscle fatty acid composition (% of total fatty acids) of gilthead sea bream fed the experimental diets.

Fatty acid	A 2.0/0.2/0.1	B 1.0/0.4/0.4	C 0/0.6/0.6	D 0/0.3/1.5	SEM
SFA					
C14:0	1.25^{b}	1.55^{c}	2.19^{d}	1.08^{a}	0.09
C15:0	0.14^{a}	0.15^{ab}	0.17^{b}	0.13^{a}	0.00
C16:0	15.0^{a}	16.2^{b}	17.3^{c}	15.2^{a}	0.22
C17:0	0.17^{b}	0.17^{ab}	0.15^{ab}	0.13^{a}	0.01
C18:0	4.49	4.43	4.37	4.09	0.08
C20:0	0.82^{c}	0.45^{b}	0.21^{a}	0.15^{a}	0.06
C22:0	1.11^{c}	0.69^{b}	0.22^{a}	0.16^{a}	0.08
C24:0	0.38^{a}	0.32^{a}	0.07^{b}	0.06^{b}	0.03
MUFA					
C16:1 n-7	1.93^{a}	2.23^{b}	2.75^{c}	1.89^{a}	0.08
C17:1 n-7	0.08^{ab}	0.09^{cb}	0.10^{c}	0.06^{a}	0.00
C18:1 n-9 c	29.7^{a}	30.0^{a}	31.6^{b}	30.5^{a}	0.19
C20:1n-9	0.72	0.71	0.74	0.78	0.02
C22:1 n-9	8.35^{c}	4.83^{b}	0.30^{a}	0.24^{a}	0.71
C24:1 n-9	0.24	0.25	0.25	0.22	0.01
PUFA					
C18:2 n-6 c (LA)	16.9^{b}	16.8^{ab}	16.3^{a}	16.9^{b}	0.08
C18:3 n-3 (ALA)	14.1^{a}	14.2^{a}	13.9^{a}	16.0^{b}	0.24
C18:3 n-6	0.26^{c}	0.25^{bc}	0.23^{ab}	0.20^{a}	0.01
C20:2 n-6	0.34	0.37	0.35	0.35	0.01
C20:3 n-3	0.27^{a}	0.36^{ab}	0.36^{ab}	0.47^{b}	0.02
C20:3 n-6	0.27^{c}	0.22^{b}	0.12^{a}	0.12^{a}	0.02
C20:4 n-6 (ARA)	0.10^{a}	0.10^{ab}	0.17^{c}	0.14^{bc}	0.01
C20:5 n-3 (EPA)	1.00^{a}	1.63^{b}	2.62^{c}	1.55^{b}	0.13
C22:6 n-3 (DHA)	2.30^{a}	3.92^{b}	5.52^{c}	9.65^{d}	0.58
ΣSFA	23.4^{b}	24.0^{b}	24.6^{b}	20.9^{a}	0.33
ΣMUFA	41.0^{d}	38.1^{c}	35.7^{b}	33.7^{a}	0.58
ΣPUFA	35.6^{a}	37.9^{b}	39.6^{c}	45.4^{d}	0.77
Σn3	17.7^{a}	20.1^{b}	22.4^{c}	27.7^{d}	0.78
Σn6	17.9^{b}	17.8^{b}	17.2^{a}	17.7^{ab}	0.09

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 ($P < 0.05$). FA: fatty acid; LA: Linoleic acid; ALA: α-Linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

Table 4
Specific activities of intestine intermediary metabolism related enzymes (mU/mg protein) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
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
FBPASE	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 = 6) and pooled standard error of the mean (SEM). Absence of superscript letters indicate no significant differences between dietary treatments ($P > 0.05$); – undetected enzymatic activity.

Table 5
Specific activities of liver intermediary metabolism related enzymes (mU/mg protein) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
ME	5.3	5.0	4.5	3.9	0.25
FAS	5.4^{b}	5.2^{b}	5.4^{b}	2.9^{a}	0.31
HOAD	25.6^{c}	20.5^{b}	15.9^{a}	19.2^{ab}	0.77
HK	3.9	3.5	3.2	3.1	0.17
GK	4.2	3.5	3.3	3.5	0.15
FBPASE	23.9	20.7	19.8	20.6	0.64
PK	19.7	17.4	16.3	18.8	0.74

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 ($P < 0.05$).

Table 6
Plasma metabolites of gilthead sea bream juveniles fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Glucose (mg dl^{-1})	66.0	75.1	67.5	63.7	2.29
Total protein (g dl^{-1})	3.2	3.6	3.5	3.2	0.54
Triglycerides (mg dl^{-1})	317.7^{a}	662.7^{b}	681.6^{b}	492.5^{ab}	49.63
Total cholesterol (mg dl^{-1})	149.2^{ab}	154.2^{b}	154.5^{b}	128.4^{a}	3.50

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 ($P < 0.05$).

Table 7
Hepatic expression of genes involved in LC-PUFA biosynthesis ($Elovl_5$, $FADS_2$), lipoprotein assembly ($ApoA_1$), transcription factor ($Srebp_1$), lipolysis (LPL), TAG (DGAT), lipogeneses (FAS), lipid digestion (PLA_2), lipid transport ($FABP_2$), and cholesterol transport ($ABGC_5$) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
$Elovl_5$	0.54^{a}	1.33^{ab}	2.02^{b}	1.79^{ab}	0.22
$FADS_2$	1.41	1.26	1.28	1.12	0.21
$ApoA_1$	539.5	503.2	788.7	458.7	74.1
$Srebp_1$	0.26	0.43	0.81	0.52	0.10
LPL	1.61	2.28	3.09	1.77	0.43
DGAT	6.23	6.58	11.1	14.3	2.06
FAS	2.22	2.53	2.46	1.14	0.54
PLA_2	13.3^{a}	20.6^{ab}	65.4^{b}	34.3^{ab}	8.17
$FABP_2$	0.01	0.02	0.02	0.02	0.00
$ABGC_5$	0.04	0.03	0.06	0.04	0.01

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.

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).

Diets used in this study were formulated to have very different n6/n3 EFA 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 presented 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.

Table 8
Intestine expression of genes involved in LC-PUFA biosynthesis (Elovl5, FADS2), lipoprotein assembly (ApoA1), transcription factor (Srebp1), lipolysis (LPL), TAG (DGAT), lipogeneses (FAS), lipid digestion (PLA2), lipid transport (FABP2) and cholesterol transport (ABGC5) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
$Elovl_5$	0.039^{a}	0.071^{b}	0.051^{ab}	0.043^{a}	0.05
$FADS_2$	0.014	0.009	0.024	0.011	0.00
$ApoA_1$	0.103	0.082	0.084	0.119	0.04
$Srebp_1$	0.038	0.026	0.048	0.031	0.01
LPL	0.019	0.024	0.025	0.027	0.00
DGAT	0.022	0.017	0.028	0.043	0.01
FAS	0.007	0.007	0.008	0.006	0.00
PLA_2	0.002	0.001	0.002	0.001	0.00
$FABP_2$	0.104	0.063	0.116	0.043	0.04
$ABGC_5$	0.007	0.009	0.008	0.005	0.00

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.

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 EFA 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 EFA 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 ARA:EPA:DHA ratio of 0:0.6:0.6. Thus, FE and PER are affected by dietary n-6:n-3 LC-PUFA ratios.

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 japonicas*) (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; 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 accordantly 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; Torrecillas 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 (Geay et al., 2011). Indeed, the presence of n9-PUFA, particularly 18:1n-9 and 20:1n-9, were 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 (Sissener 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 composition did not affect lipid metabolism at the intestine level, only at hepatic level. Indeed, high dietary ARA levels seemed to increase hepatic HOAD activity, a key enzyme involved in β-oxidation of FA (Wu, 2018). FA catabolism occurs in two organelles, mitochondria, and peroxisomes (Tocher, 2003). In mammals, these two biological pathways have different purposes, with mitochondrial β-oxidation being related to ATP production and peroxisome β-oxidation being involved in chain-shortening of long-chain FA that will be further oxidized in the mitochondria (Wu, 2018). Fish, however, appear to utilize peroxisome β-oxidation system at a significant level, including short-chain fatty acids (Stubhaug et al., 2007). Thus, the observed in-crease of 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 DHA content in diet D promoted a reduction of hepatic FAS activity. Similarly, in mammals DHA 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, DHA 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 DHA diet, but no correlation was found with hepatic transcript levels of $ABCG_5$, a protein involved in cholesterol transport. High dietary n-3 LC-PUFA level has been associated with hypocholesterolemia 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 LDL binding capacity leading to lower plasmatic cholesterol content, as observed in the present work.

$ApoA_1$ 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 $ApoA_1$ expression to be lower in fish fed the high DHA diet, though differences in $ApoA_1$ expression were not statistically different among groups. Previously, Xu et al. (2018) described in Japanese seabass a down-regulation of $ApoA_1$ 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 $Srebp_1$, LPL, and DGAT followed a similar trend. In Japanese seabass, liver downregulation of $Srebp_1$ 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 $SREBP_1$ 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 $SREBP_1$ 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 $Srebp_1$, LPL, and DGAT is still very scarce and contradictory and needs to be further investigated.

As FAS is a target of $Srebp_1$, 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 $Srebp_1$ 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; Limtipsuntorn et al., 2014; Peng et al., 2014).

Hepatic $FABP_2$ 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 $FABP_2$ expression in gilthead sea bream juveniles fed FO or VO-based diets. However, an upregulation of hepatic $FABP_2$ 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 $FADS_2$, typically a Δ6 desaturase, as well as $Elovl_5$, 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 $ELOVL_5$, 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 $ELOVL_5$, but this effect was only significant in the intestine. Similarly, in juvenile black seabream, increasing dietary DHA/EPA ratio decreased hepatic $ELOVL_5$ 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, PLA_2 expression was downregulated in the liver of fish fed diets with high ARA level. A drastic reduction of PLA_2 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 PLA_2 activity in the gills (Bell et al., 1996). ARA is primarily stored in the membrane PL and PLA_2

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 PLA_2 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 PLA_2 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-hydroxyacyl-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
Elovl5	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

Alvarez, M.J., Diez, A., Lopez Bote, C., Gallego, M., Bautista, J.M., 2000. Short-term modulation of lipogenesis by macronutrients in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Br. J. Nutr. 84, 619–628. https://doi.org/10.1017/s0007114500001951. [pii].

Alves Martins, D., Rocha, F., Martínez-Rodríguez, G., Bell, G., Morais, S., Castanheira, F., Bandarra, N., Coutinho, J., Yúfera, M., Conceição, L.E.C.C., Martins, D.A., Rocha, F., Martínez-Rodríguez, G., Bell, G., Morais, S., Castanheira, F., Bandarra, N., Coutinho, J., Yúfera, M., Conceição, L.E.C.C., 2012. Teleost fish larvae adapt to dietary arachidonic acid supply through modulation of the expression of lipid metabolism and stress response genes. Br. J. Nutr. 108, 864–874. https://doi.org/10.1017/S0007114511006143.

AOAC, 2000. Official methods of analysis. 17th edition, the association of official analytical Chemists, gaithersburg, MD, USA. Methods 925 (10) 65.17, 974.24, 992.16.

Atalah, E., Hernández-Cruz, C.M., Benítez-Santana, T., Ganga, R., Roo, J., Izquierdo, M., 2011. Importance of the relative levels of dietary arachidonic acid and eicosapentaenoic acid for culture performance of gilthead seabream (*Sparus aura*ta) larvae. Aquacult. Res. 42, 1279–1288. https://doi.org/10.1111/j.1365-2109.2010.02716.x.

Bandarra, N.M., Rema, P., Batista, I., Pousão-Ferreira, P., Valente, L.M.P.P., Batista, S.M.G.G., Ozório, R.O.A.A., 2011. Effects of dietary n-3/n-6 ratio on lipid metabolism of gilthead seabream (*Sparus aurata*). Eur. J. Lipid Sci. Technol. 113, 1332–1341. https://doi.org/10.1002/ejlt.201100087.

Bell, J.G., Farndale, B.M., Dick, J.R., Sargent, J.R., 1996. Modification of membrane fatty acid composition, eicosanoid production, and phospholipase A activity in Atlantic salmon (*Salmo salar*) gill and kidney by dietary lipid. Lipids 31, 1163–1171. https://doi.org/10.1007/BF02524291.

Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Gordon Bell, J., Kaushik, S., Pérez-Sánchez, J., 2008. High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues. Br. J. Nutr. 100, 992–1003. https://doi.org/10.1017/S0007114508966071.

Betancor, M.B., Sprague, M., Montero, D., Usher, S., Sayanova, O., Campbell, P.J., Napier, J.A., Caballero, M.J., Izquierdo, M., Tocher, D.R., 2016. Replacement of Marine Fish Oil with de novo Omega-3 Oils from Transgenic Camelina sativa in Feeds for Gilthead Sea Bream (*Sparus aurata* L.). Lipids 51, 1171–1191. https://doi.org/10.1007/s11745-016-4191-4.

Beutler, H., 1984. 0.: starch. In: BERGMEYER, H.U. (Ed.), Methods of Enzymatic analysis. 6. Verlag Chemie Weinheim, Basel, pp. 2–10.

Boglino, A., Darias, M.J., Estévez, A., Andree, K.B., Gisbert, E., 2012. The effect of dietary arachidonic acid during the Artemia feeding period on larval growth and skeletogenesis in Senegalese sole, *Solea senegalensis*. J. Appl. Ichthyol. 28, 411–418. https://doi.org/10.1111/j.1439-0426.2012.01977.x.

Bondia-Pons, I., Moltó-Puigmartí, C., Castellote, A.I., López-Sabater, M.C., 2007. Determination of conjugated linoleic acid in human plasma by fast gas chromatography. J. Chromatogr. A 1157, 422–429. https://doi.org/10.1016/J.CHROMA.2007.05.020.

Carmona-Antoñanzas, G., Tocher, D.R., Martinez-Rubio, L., Leaver, M.J., 2014. Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. Gene 534, 1–9. https://doi.org/10.1016/J.GENE.2013.10.040.

Castro, C., Corraze, G., Basto, A., Larroquet, L., Panserat, S., Oliva-Teles, A., 2016a. Dietary lipid and carbohydrate interactions: implications on lipid and glucose absorption, transport in gilthead sea bream (*Sparus aurata*) juveniles. Lipids 51, 743–755. https://doi.org/10.1007/s11745-016-4140-2.

Castro, C., Corraze, G., Firmino-Diógenes, A., Larroquet, L., Panserat, S., Oliva-Teles, A., 2016b. Regulation of glucose and lipid metabolism by dietary carbohydrate levels and lipid sources in gilthead sea bream juveniles. Br. J. Nutr. 116, 19–34. https://doi.org/10.1017/s000711451600163x.

Dias, J., Conceição, L.E.C., Ribeiro, A.R., Borges, P., Valente, L.M.P., Dinis, M.T., 2009. Practical diet with low fish-derived protein is able to sustain growth performance in gilthead seabream (*Sparus aurata*) during the grow-out phase. Aquaculture 293, 255–262. https://doi.org/10.1016/J.AQUACULTURE.2009.04.042.

Eurostat, 2019. Archive:Aquaculture statistics. Stat. Explain .

FAO (Food & Agriculture Organization), 2016. The State of World Fisheries and Aquaculture 2016.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1007/s10858-011-9570-9.

Fountoulaki, E., Alexis, M.N., Nengas, I., Venou, B., 2003. Effects of dietary arachidonic acid (20:4n-6), on growth, body composition, and tissue fatty acid profile of gilthead bream fingerlings (*Sparus aurata* L.). Aquaculture 225, 309–323. https://doi.org/10.1016/S0044-8486(03)00298-9.

Geay, F., Ferraresso, S., Zambonino-Infante, J.L., Bargelloni, L., Quentel, C., Vandeputte, M., Kaushik, S., Cahu, C.L., Mazurais, D., 2011. Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. BMC Genomics 12, 522. https://doi.org/10.1186/1471-2164-12-522.

Gijón, M.A., Leslie, C.C., 1999. Regulation of arachidonic acid release and cytosolic Phospholipase A2 activation. J. Leukoc. Biol. 65 (3), 330–336. https://doi.org/10.1002/jlb.65.3.330.

Guerreiro, I., Peres, H., Castro, C., Pérez-Jiménez, A., Castro-Cunha, M., Oliva-Teles, A., 2014. Water temperature does not affect protein sparing by dietary carbohydrate in Senegalese sole (Solea senegalensis) juveniles. Aquacult. Res. 45, 289–298.

Guerreiro, I., Enes, P., Oliva-Teles, A., 2015. Effects of short-chain fructooligosaccharides (scFOS) and rearing temperature on growth performance and hepatic intermediary metabolism in gilthead sea bream (Sparus aurata) juveniles. Fish Physiol. Biochem. 41 (5), 1333–1344. https://doi.org/10.1007/s10695-015-0089-y.

Hong, J.W., Lee, S.H., Moniruzzaman, M., Park, Y., Won, S.H., Jo, H.Y., Hung, S.S.O., Bai, S.C., 2018. Dietary eicosapentaenoic acid requirement of juvenile rock bream, *Oplegnathus fasciatus*. Aquacult. Nutr. 24, 36–46. https://doi.org/10.1111/anu.12530.

Houston, S.J.S., Karalazos, V., Tinsley, J., Betancor, M.B., Martin, S.A.M., Tocher, D.R., Monroig, O., 2017. The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n -3 long-chain PUFA content. Br. J. Nutr. 118, 1010–1022. https://doi.org/10.1017/S0007114517002975.

Huang, L.H., Chung, H.Y., Su, H.M., 2017. Docosahexaenoic acid reduces sterol regulatory element binding protein-1 and fatty acid synthase expression and inhibits cell proliferation by inhibiting pAkt signaling in a human breast cancer MCF-7 cell line. BMC Canc. 17, 890. https://doi.org/10.1186/s12885-017-3936-7.

Ibeas, C., Izquierdo, M.S., Lorenzo, A., 1994. Effect of different levels of n−3 highly unsaturated fatty acids on growth and fatty acid composition of juvenile gilthead seabream (*Sparus aurata*). Aquaculture 127, 177–188. https://doi.org/10.1016/0044-8486(94)90424-3.

Ibeas, C., Cejas, J.R., Fores, R., Badía, P., Gómez, T., Hernández, A.L., 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPADHA) of dietary lipids on growth and fatty acid composition of gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 150, 91–102. https://doi.org/10.1016/S0044-8486(96)01473-1.

Izquierdo, M., 2005. Essential Fatty Acid Requirements in Mediterranean Fish Species, vol. 102. pp. 91–102.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L., Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquacult. Nutr. 9, 397–407. https://doi.org/10.1046/j.1365-2095.2003.00270.x.

Jin, M., Monroig, O., Lu, Y., Yuan, Y., Li, Y., Ding, L., Tocher, D.R., Zhou, Q., 2017. Dietary DHA/EPA ratio affected tissue fatty acid profiles, antioxidant capacity, hematological characteristics and expression of lipid-related genes but not growth in juvenile black seabream (*Acanthopagrus schlegelii*). PLoS One 12, e0176216. https://doi.org/10.1371/journal.pone.0176216.

Kalogeropoulos, N., Alexis, M.N., Henderson, R.J., 1992. Effects of dietary soybean and cod-liver oil levels on growth and body composition of gilthead bream (*Sparus aurata*). Aquaculture 104, 293–308. https://doi.org/10.1016/0044-8486(92)90211-3.

Lie, K.K., Kvalheim, K., Rasinger, J.D., Harboe, T., Nordgreen, A., Moren, M., 2016. Vitamin A and arachidonic acid altered the skeletal mineralization in Atlantic cod (*Gadus morhua*) larvae without any interactions on the transcriptional level. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 191, 80–88. https://doi.org/10.1016/j.cbpa.2015.10.011.

Limtipsuntorn, U., Haga, Y., Kondo, H., Hirono, I., Satoh, S., 2014. Microarray analysis of hepatic gene expression in juvenile Japanese flounder Paralichthys olivaceus fed diets supplemented with fish or vegetable oils. Mar. Biotechnol. 16, 88–102. https://doi.org/10.1007/s10126-013-9535-y.

Liu, J., Caballero, M.J.J., Izquierdo, M., El-Sayed Ali, T., Hernandez-Cruz, C.M., Valencia, A., Fernandez-Palacios, H., Hernández-Cruz, C.M., Valencia, A., Fernández-Palacios, H., 2002. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream *Sparus aurata*. Fish. Sci. 68, 1165–1172. https://doi.org/10.1046/j.1444-2906.2002.00551.x.

Lund, I., Steenfeldt, S.J., Hansen, B.W., 2010. Influence of dietary arachidonic acid combined with light intensity and tank colour on pigmentation of common sole (*Solea solea* L.) larvae. Aquaculture 308, 159–165. https://doi.org/10.1016/J.AQUACULTURE.2010.08.004.

Luo, Z., Tan, X.-Y., Li, X.-D., Yin, G.-J., 2012. Effect of dietary arachidonic acid levels on growth performance, hepatic fatty acid profile, intermediary metabolism and antioxidant responses for juvenile Synechogobius hasta. Aquacult. Nutr. 18, 340–348. https://doi.org/10.1111/j.1365-2095.2011.00906.x.

Ma, H. na, Jin, M., Zhu, T. ting, Li, C. chen, Lu, Y., Yuan, Y., Xiong, J., Zhou, Q. cun, 2018. Effect of dietary arachidonic acid levels on growth performance, fatty acid profiles and lipid metabolism of juvenile yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 486, 31–41. https://doi.org/10.1016/j.aquaculture.2017.11.055.

Magalhães, R., Sánchez-López, A., Leal, R.S., Martínez-Llorens, S., Oliva-Teles, A., Peres, H., 2017. Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). Aquaculture 476, 79–85. https://doi.org/10.1016/j.aquaculture.2017.04.021.

Minghetti, M., Leaver, M.J., Tocher, D.R., 2011. Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1811, 194–202. https://doi.org/10.1016/J.BBALIP.2010.12.008.

Norambuena, F., Rombenso, A., Turchini, G.M., 2016. Towards the optimization of performance of Atlantic salmon reared at different water temperatures via the manipulation of dietary ARA/EPA ratio. Aquaculture 450, 48–57. https://doi.org/10.1016/j.aquaculture.2015.06.044.

NRC, 2011. Nutrient Requirements of Fish and Shrimp. National Academies Press, Washington, D.C.

Peng, M., Xu, W., Mai, K., Zhou, H., Zhang, Y., Liufu, Z., Zhang, K., Ai, Q., 2014. Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. Aquaculture 433, 442–449. https://doi.org/10.1016/J.AQUACULTURE.2014.07.005.

Peres, H., Santos, S., Oliva-Teles, A., 2013. Selected plasma biochemistry parameters in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630–636. https://

doi.org/10.1111/j.1439-0426.2012.02049.x.
Pérez-Jiménez, A., Hidalgo, M.C., Morales, A.E., Arizcun, M., Abellán, E., Cardenete, G., 2009. Use of different combinations of macronutrients in diets for dentex (*Dentex dentex*). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 152, 314–321. https://doi.org/10.1016/j.cbpa.2008.11.002.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 45e–45. https://doi.org/10.1093/nar/29.9.e45.
Pizzini, A., Lunger, L., Demetz, E., Hilbe, R., Weiss, G., Ebenbichler, C., Tancevski, I., Pizzini, A., Lunger, L., Demetz, E., Hilbe, R., Weiss, G., Ebenbichler, C., Tancevski, I., 2017. The role of omega-3 fatty acids in reverse cholesterol transport: a review. Nutrients 9, 1099. https://doi.org/10.3390/nu9101099.
Plummer, D.T., 1972. An introduction to practical biochemistry. Biochem. Educ. 1, 14–15.
Rezek, T.C., Watanabe, W.O., Harel, M., Seaton, P.J., 2010. Effects of dietary docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) on the growth, survival, stress resistance and fatty acid composition in black sea bass C*entropristis striata* (Linnaeus 1758) larvae. Aquacult. Res. 41, 1302–1314. https://doi.org/10.1111/j.1365-2109.2009.02418.x.
Richard, N., Mourente, G., Kaushik, S., Corraze, G., 2006. Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). Aquaculture 261, 1077–1087. https://doi.org/10.1016/j.aquaculture.2006.07.021.
Rodriguez, C., Departamento, J.A.P., Animal, D.B., De, U., Palmas, L., 1993. Essential fatty acid requirements of larval gilthead sea bream , Sparus aurata (L .). pp. 295–304.
Rombenso, A.N., Trushenski, J.T., Jirsa, D., Drawbridge, M., 2016. Docosahexaenoic acid (DHA) and arachidonic acid (ARA) are essential to meet LC-PUFA requirements of juvenile California Yellowtail (*Seriola dorsalis)*. Aquaculture 463, 123–134. https://doi.org/10.1016/J.AQUACULTURE.2016.05.004.
Shahkar, E., Yun, H., Lee, S., Kim, D.-J., Kim, S.-K., Lee, B.I., Bai, S.C., 2016. Evaluation of the optimum dietary arachidonic acid level and its essentiality based on growth and non-specific immune responses in Japanese eel, *Anguilla japonic*a. Aquaculture 452, 209–216. https://doi.org/10.1016/J.AQUACULTURE.2015.10.034.
Sissener, N., Ørnsrud, R., Sanden, M., Frøyland, L., Remø, S., Lundebye, A.-K., Sissener, N.H., Ørnsrud, R., Sanden, M., Frøyland, L., Remø, S., Lundebye, A.-K., 2018. Erucic acid (22:1n-9) in fish feed, farmed, and wild fish and seafood products. Nutrients 10, 1443. https://doi.org/10.3390/nu10101443.
Stubhaug, I., Lie, Ø., Torstensen, B.E., 2007. Fatty acid productive value and ?-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period. Aquacult. Nutr. 13, 145–155. https://doi.org/10.1111/j.1365-2095.2007.00462.x.
Tian, J., Ji, H., Oku, H., Zhou, J., 2014. Effects of dietary arachidonic acid (ARA) on lipid metabolism and health status of juvenile grass carp, Ctenopharyngodon idellus. Aquaculture 430, 57–65. https://doi.org/10.1016/j.aquaculture.2014.03.020.
Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11 (2), 107–184. https://doi.org/10.1080/713610925.
Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquacult. Res. 41, 717–732. https://doi.org/10.1111/j.1365-2109.2008.02150.x.
Tocher, D., Betancor, M., Sprague, M., Olsen, R., Napier, J., 2019. Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: bridging the gap between supply and demand. Nutrients 11, 89. https://doi.org/10.3390/nu11010089.
Torrecillas, S., Betancor, M.B., Caballero, M.J., Rivero, F., Robaina, L., Izquierdo, M., Montero, D., 2018. Supplementation of arachidonic acid rich oil in European sea bass juveniles (*Dicentrarchus labrax*) diets: effects on growth performance, tissue fatty acid profile and lipid metabolism. Fish Physiol. Biochem. 44, 283–300. https://doi.org/10.1007/s10695-017-0433-5.
Villalta, M., Estévez, A., Bransden, M.P.P., Bell, J.G.G., 2008. Effects of dietary eicosapentaenoic acid on growth, survival, pigmentation and fatty acid composition in Senegal sole (*Solea senegalensis*) larvae during the Artemia feeding period. Aquacult. Nutr. 14, 232–241. https://doi.org/10.1111/j.1365-2095.2007.00522.x.
Wu, G., 2018. Principles of Animal Nutrition. Purdue University.
Wu, F.-C.C., Ting, Y.-Y.Y., Chen, H.-Y.Y., 2002. Docosahexaenoic acid is superior to eicosapentaenoic acid as the essential fatty acid for growth of grouper, Epinephelus malabaricus. J. Nutr. 132, 72–79. https://doi.org/10.1093/jn/132.1.72.
Xu, H., Ai, Q., Mai, K., Xu, W., Wang, J., Ma, H., Zhang, W., Wang, X., Liufu, Z., 2010. Effects of dietary arachidonic acid on growth performance, survival, immune response and tissue fatty acid composition of juvenile Japanese seabass, Lateolabrax japonicus. Aquaculture 307, 75–82. https://doi.org/10.1016/j.aquaculture.2010.07.001.
Xu, H., Wang, J., Mai, K., Xu, W., Zhang, W., Zhang, Y., Ai, Q., 2016. Dietary docosahexaenoic acid to eicosapentaenoic acid (DHA/EPA) ratio influenced growth performance, immune response, stress resistance and tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus* (Cuvier). Aquacult. Res. 47, 741–757. https://doi.org/10.1111/are.12532.
Xu, H., Wang, C., Zhang, Y., Wei, Y., Liang, M., 2018. Moderate levels of dietary arachidonic acid reduced lipid accumulation and tended to inhibit cell cycle progression in the liver of Japanese seabass *Lateolabrax japonicus*. Sci. Rep. 8, 10682. https://doi.org/10.1038/s41598-018-28867-z.
Xue, X., Feng, C.Y., Hixson, S.M., Johnstone, K., Anderson, D.M., Parrish, C.C., Rise, M.L., 2014. Characterization of the fatty acyl elongase (elovl) gene family, and hepatic elovl and delta-6 fatty acyl desaturase transcript expression and fatty acid responses to diets containing camelina oil in Atlantic cod (Gadus morhua). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 175, 9–22. https://doi.org/10.1016/j.cbpb.2014.06.005.
Zuo, R., Ai, Q., Mai, K., Xu, W., Wang, J., Xu, H., Liufu, Z., Zhang, Y., 2012a. Effects of dietary n-3 highly unsaturated fatty acids on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (Cryptocaryon irr. Fish Shellfish Immunol. 32, 249–258. https://doi.org/10.1016/j.fsi.2011.11.005.
Zuo, Rantao, Ai, Q., Mai, K., Xu, W., Wang, J., Xu, H., Liufu, Z., Zhang, Y., 2012b. Effects of dietary docosahexaenoic to eicosapentaenoic acid ratio (DHA/EPA) on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of paras. Aquaculture 334–337, 101–109. https://doi.org/10.1016/j.aquaculture.2011.12.045.