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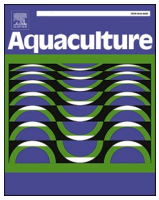

Complete replacement of fish oil by three microalgal products rich in n-3 long-chain polyunsaturated fatty acids in early weaning microdiets for gilthead sea bream (*Sparus aurata*)

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ABSTRACT

The aim of the present study was to evaluate the effect of different n-3 LC-PUFA-rich microalgae replacing FO in larval diets, on performance, biochemical composition and health of gilthead sea bream (*Sparus aurata*). Dietary fish oil (FO) was replaced with oleic acid (OA) or 3 n-3 LC-PUFA-rich algae products (2 commercial products: All G Rich and DHA Gold, and a development product: MO060). The five early weaning microdiets were fed to 20-dah gilthead sea bream larvae for 21 days. Proximate composition and fatty acid profiles of total, neutral and polar lipids of seabream larvae were analysed, as well as hepatocyte morphology and the expression of selected genes related to lipid metabolism. OA diet significantly inhibited larval growth and survival after a stress challenge, whereas FO replacement by DHA Gold and MO60 led to a higher body weight, total length, and stress resistance. Larvae fed the diets containing microalgae had increased DHA content in whole body lipids and showed a down-regulation of expression of *fads2*, indicating an inhibition of LC-PUFA biosynthesis. Relative expression of genes related to lipid (*ppar-α*) or eicosanoids (*cox-2* and *5-lox*) metabolism were unaffected by the replacement of FO by any of the three microalgal products or OA. In conclusion, all the microalgae products tested were effective total replacers of FO in weaning microdiets for gilthead sea bream larvae, providing the necessary dietary n-3 LC-PUFA for optimal growth and survival.

1. Introduction

Fish oil (FO) is rich in long-chain polyunsaturated fatty acids (LC-PUFA), particularly in docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), which have critical functional and physiological roles in fish larval growth, survival and health (Tocher, 2010). Indeed, n-3 LC-PUFA participate in the normal immune function of fish and are particularly important in stress response. Concretely, these FA are substrates for lipoxygenases (LOX) and cyclooxygenases (COX), enzymes that modulate the production of eicosanoids, hormone-like metabolites involved, among others, in inflammatory processes in vertebrates (Rowley et al., 2005). Additionally, n-3 LC-PUFA regulates lipid metabolism by controlling fatty acid synthesis and catabolism to maintain lipid homeostasis. Several enzymes are involved in both metabolic pathways, but peroxisome proliferator-activated receptor α (PPAR-α) is a critical enzyme for fatty acid β-oxidation in peroxisomes (Tocher et al., 2003). Furthermore, PPAR is involved not only in energy balance and lipid metabolism, but also in glucose metabolism (Lee et al., 2003). Gluconeogenesis is promoted during starvation, energy-restricted periods or different dietary lipid sources, by increasing

Abbreviations: AGR, All G rich; ARA, arachidonic acid; COX, cyclooxygenases; DHA, docosahexaenoic acid; DHG, DHA Gold; DPA, docosapentaenoic acid; EFA, essential fatty acids; EPA, eicosapentaenoic acid; FADS2, fatty acyl desaturase 2; FO, fish oil; G6P, glucose-6-phosphatase; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acids; LNA, linolenic acid; LOX, lipoxygenases; M60, MO060; MUFA, monounsaturated fatty acids; PL, phospholipids/polar lipids; PPAR-α, peroxisome proliferator-activated receptor α; SFA, saturated fatty acids; VO, vegetable oil.

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gluconeogenic enzymes activities, including that of glucose-6-phosphatase (G6P), converting glucose-6-phosphate to glucose and, consequently, increasing blood glucose levels (Enes et al., 2008; Wang et al., 2019). Therefore, changes in fatty acid composition of tissues are also related to affect PPAR and, directly or indirectly glucose metabolism to maintain homeostasis, through glycolysis/gluconeogenesis pathways (Caseras et al., 2002; Wang et al., 2019) because FA oxidation leads to acetyl-CoA that is used in gluconeogenesis (Enes et al., 2008).

Supply of n-3 LC-PUFA through the diet is particularly important in marine teleosts, since they lack the ability to synthesize these FA from their 18-carbon precursors, such as alpha-linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6). This is due to a reduced activity in Δ4, Δ5 and/or Δ6 desaturase steps, regulated at a molecular and enzymatic level by fatty acyl desaturase 2 (FADS2), an enzyme that in most marine fish has been shown to have a Δ6 activity (Tocher et al., 2003; Tocher et al., 2006; Izquierdo et al., 2008). Therefore, n-3 LC-PUFA are dietary essentials for marine fish such as the gilthead sea bream (*Sparus aurata*). Furthermore, fish larvae have usually a much higher requirement for n-3 LC-PUFA than later life stages due to the pivotal important plastic purposes of these FA, particularly of DHA, for tissue and organs development (Izquierdo, 2005; Izquierdo and Koven, 2011).

Given the global limited availability of fish oils for aquaculture feeds and the successive increase in the use of alternative lipid sources, mostly vegetable oils (VO), meeting adequately the dietary n-3 LC-PUFA requirements of fish becomes difficult to achieve. Ensuring increased production in aquaculture is dependent on sustainable feeding strategies and the use of new sources of n-3 LC-PUFA as alternatives to FO and VO. Microalgae appear to be the most promising alternative to the traditional marine derived ingredients, since they are the primary producers of EPA and DHA in the marine ecosystems, and can become a cost competitive product alternative to FO over the next years (Ganuza and Izquierdo, 2007; Chauton et al., 2015; Sprague et al., 2017). The field of microalgae intended for aquaculture is now attracting considerable attention and a number of commercial products are available, particularly as a source of n-3 LC-PUFA for larval nutrition (Glencross et al., 2020; Pratiwy and Pratiwi, 2020). Algal products have been successfully used to replace FO in farmed fish diets. Microalgal meals such as ALL-G RICH™ (Alltech Inc., Nicholasville, KY, USA), obtained from the marine heterotroph *Schizochytrium limacinum*, have been tested as dietary lipid sources and proven effective in longfin yellowtail *Seriola rivoliana* (Kissinger et al., 2016), giant grouper *Epinephelus lanceolatus* (García-Ortega et al., 2016), rainbow trout *Oncorhynchus mykiss* (Lyons et al., 2017), tilapia (Sarker et al., 2016) and red drum *Sciaenops ocellatus* (Perez-Velazquez et al., 2018). Another commercial algal product, DHA GOLD®, produced from *Crypthecodinium cohnii* and based on powder algal fermentation has demonstrated its potential as a supplementary lipid source resulting in similar growth performance to that of a FO based-diet (Betiku et al., 2016). Furthermore, the development microalgae product based on a new fermentation process of *Schizochytrium limacinum* to further enrich in EPA and DHA, (MO060, Adisseo France SAS) has also been tested. However, despite the great potential of microalgal products as a rich source of n-3 LC-PUFA, especially DHA, they generally contain lower levels of EPA than FO, besides having relatively higher contents of other FA that are not commonly found on FO, for instance the n-6 docosapentaenoic acid (DPA, 22:5n-6) (Atalah et al., 2007; Ganuza et al., 2008)

Thus, the purpose of this study was to examine the effects of different commercially available microalgal products (ALL-G RICH™ and DHA GOLD®) or in development (MO060), as complete replacers of dietary FO in early weaning diets for gilthead sea bream. The three products selected were high in n-3 LC-PUFA but differed particularly in their specific fatty acid composition, particularly in DHA, EPA, ARA or n-6 DPA contents. Besides their effects on larval survival, growth performance and body composition, metabolism and health-related genes expression were also assessed.

2. Materials and methods

2.1. Diets and feeding trial

Five practical microdiets (pellet size <250 μm), based on defatted squid meal (defatted 3 consecutive times in our laboratories with a chloroform:methanol ratio of 3:1) as protein source, were formulated and tested in quadruplicate. A positive control diet was based on fish oil (FO diet), and a negative control diet, replaced FO by oleic acid (OA diet). Additionally, three experimental diets were formulated to test three commercial or on-development n-3 LC-PUFA-rich algal products: the AGR diet that included ALL G RICH™ (with 70% fat content, of which 19% of total FA is DHA; Alltech Inc., Nicholasville, KY, USA), the DHG diet that included DHA GOLD (56% of fat of which 39.5% of total FA is DHA; DSM, Basel, Switzerland) and the M60 diet that included MO060 (73% fat, of which 49% of total FA is DHA; Adisseo, France SAS). For diets preparation, squid powder and water-soluble components were firstly mixed, followed by mixing the fat-soluble vitamins and, finally, gelatin, which was dissolved in warm water. The microdiet paste was pelleted and dried in an oven at 38 °C for 24 h. The following day, pellets were grounded and sieved to particle sizes below <250 μm. Diet formulation, proximate composition and fatty acid contents of microalgae meals and microdiets are described in Tables 1–3.

Fish larvae were manually fed with the experimental diets (each 45 min from 8:00 to 20:00), for 21 days by a trained researcher. After 5 days of the beginning of the feeding trial, larvae were observed under the binocular microscope to determine feed acceptance by removing (after feeding) 5 larvae per tank, which were kept in distilled water on a slide microscope to observe the presence of the feed inside the digestive tube.

2.2. Larval performance

Quadruplicate groups of 20 days after hatching (dah) gilthead sea bream larvae, with an initial total length of 8.04 ± 0.1 mm and initial dry body weight of 0.48 ± 0.1 mg, were allocated in a total of 24 tanks of 200 L with a water flow of 150 L hour^{-1}, at a density of 600 larvae tank^{-1}. Temperature along the trial was 22.6 ± 0.12 °C, dissolved oxygen 5.56 ± 0.05 mgL^{-1}, pH 8.2 ± 0.02 and salinity 37 mg L^{-1}. After the feeding period, larval growth was determined by measuring body weight of 30 larvae per tank, that were dried at 105 °C until constant weight (~24 h). Additional 30 larvae per tank were removed, placed on ice, and transferred to a microscope slide with distiller water to measure individual total length (Profile Projector V-12A; Nikon, Tokyo, Japan). Final survival was calculated by individually counting all the alive larvae that survived to the whole feeding period.

Additionally, to test larval stress resistance, at the end of the feeding period, an air-exposure stress test was conducted with 20 larvae per tank that were placed in a scoop net out of the water for 2 min. Subsequently, larvae were allocated in a new tank supplied with clean seawater and aeration, and survival was determined after 24 h.

Whole-larvae samples were also, washed with distilled water after starvation of 16 h and sampled for analysis (biochemical, histological and molecular analysis).

2.3. Biochemical analysis

Moisture (AOAC, 1995), protein by nitrogen determination N x 6.25 (Kjeldahl, 1883) and crude lipid (Folch et al., 1957) contents of diets and larvae were analysed. Neutral (NL) and polar lipid (PL) fractions of larvae were separated for characterising FA profile of each fraction. For that, total lipids were filtered with a Sep-Pack NH_2 cartridge, firstly eluting NL with 30 ml of chloroform and 20 ml of chloroform/methanol (49:1 *v*/v) and secondly eluting PL with 30 ml of methanol (Juaneda and Rocquelin, 1985). Eluents were added to sample in series of 10 ml. Then, each lipid fraction was evaporated in rotary evaporators and finalizing under N_2 atmosphere for subsequent transmethylation. Fatty acid

Table 1
Ingredients and proximate composition (% dry weight) of the experimental microdiets with different oils fed to gilthead sea bream larvae for 21 days.

Ingredients (%)	Diets				
	OA	FO	AGR	DHG	M60
Squid meal[1] (defatted)	69.2	69.2	68.2	68.2	68.2
Gelatin[2]	3.0	3.0	3.0	3.0	3.0
Soy lecithin[3]	8.0	8.0	8.0	8.0	8.0
Sardine oil[4]	0	5.0	0	0	0
Oleic acid[5]	5.0	0	0	0	0
All G Rich[6]	0	0	6.0	0	0
DHA Gold[7]	0	0	0	6.0	0
MO060[8]	0	0	0	0	6.0
Mineral premix[9]	4.5	4.5	4.5	4.5	4.5
Vitamin premix[10]	5.8	5.8	5.8	5.8	5.8
Taurine	1.5	1.5	1.5	1.5	1.5
Attractants[11]	3.0	3.0	3.0	3.0	3.0
Analysed proximate composition (% dw)					
Moisture	6.9	8.4	7.2	6.6	7.8
Proteins (N x 6.25)	67.5	68.5	69.3	69.0	69.5
Total lipids	12.6	13.3	11.6	11.4	11.6
Neutral lipids (% total lipids)	82.8	50.0	60.8	53.3	42.6
Polar lipids (% total lipids)	17.2	50.0	39.3	46.7	57.4
Ash	8.0	8.0	7.8	8.5	7.7

[1] Rieber & Son, Bergen, Norway.
[2] Sigma-Aldrich, St.Louis, USA.
[3] Novadiet, S.A., Burgos, Spain.
[4] Agramar S.A., Spain.
[5] Merck KGaA, Darmstadi, Germany.
[6] Alltech Inc., Nicholasville, KY, USA.
[7] DSM Nutritional Products, Basel, Switzerland.
[8] Adisseo, France SAS.
[9] Mineral premix supplied (in mg per 100 g diet): NaCl 215.13, $MgSO_47H_2O$ 677.55, $NaH_2PO_4H_2O$ 381.45, K_2HPO_4 758.95, $Ca(H_2PO_4)2H_2O$ 671.61, $FeC_6H_5O_7$ 146.88, $C_3H_5O_31/2Ca$ 1617.21, $Al_2(SO_4)_36H_2O$ 0.69, $ZnSO_47H_2O$ 14.84, $CuSO_45H_2O$ 1.25, $MnSO_4H_2O$ 2.99, KI 0.74, $CoSO_47H_2O$ 10.71; Sigma-Aldrich, Madrid, Spain.
[10] Vitamin premix supplied (in mg per 100 g diet): cyanocobalamine 0.030, astaxanthin 5.00, folic acid 5.44, pyridoxine-HCl 17.28, thiamine 21.77, riboflavin 72.53, ca-pantothenate 101.59, p-aminobenzoic acid145.00, nicotinic acid 290.16, inositol 1450.90, retinol acetate 0.24, ergocalcipherol 3.65, menadione 17.28, alpha-tocopherol acetate 150.00, ascorbyl poliphosphate 180.00, choline chloride 2965.8; Sigma-Aldrich, Madrid, Spain.
[11] Attractants premix supplied (in mg per 100 g diet): inosine-5-monophosphate 500.0, betaine 660.0, L-serine 170.0, *L*-phenylala-nine 250.0, DL-alanine 500.0, L-sodium aspartate 330.0, L-valine 250.0, glycine 170.0; Sigma-Aldrich, Madrid, Spain.

Table 2
EPA and DHA contents (% total identified fatty acids) of the microalgae meals and fish oil used for formulating the experimental microdiets.

	AGR	DHG	M60	Fish oil
Protein	15.63	15.23	10.37	–
Lipid	70	56	73	100
Ash	2.63	11.17	3.26	–
DHA	18.74	39.5	49	4.64
EPA	0.43	0.96	1.73	2.44

methyl esters were obtained by transmethylation of lipids (Christie, 1982), separated by gas-liquid chromatography (GLC), quantified by FID (GC-14A; Shimadzu, Tokyo, Japan) (Izquierdo et al., 1992), and identified by comparison with previously characterized standards (Supelco 37 Component FAME Mix, Sigma Aldrich) and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific).

Table 3
Fatty acid composition (% total identified fatty acids) of the experimental microdiets with different oils fed to gilthead sea bream larvae for 21 days.

Fatty acids (% total FA)	Diets				
	OA	FO	AGR	DHG	M60
14:0	0.09	0.63	1.18	1.63	1.17
14:1n-7	0.01	0.03	0.02	0.01	0.02
14:1n-5	0.01	0.07	0.02	0.01	0.01
15:0	0.04	0.11	0.65	0.15	0.21
15:1n-5	0.01	0.03	0.03	0.02	0.01
16:0ISO	0.01	0.07	0.04	0.01	0.01
16:0	9.94	12.26	29.51	16.26	33.66
16:1n-7	0.24	1.50	0.26	0.44	0.19
16:1n-5	0.02	0.16	0.04	0.03	0.03
16:2n-4	0.01	0.17	0.03	0.02	0.01
17:0	0.01	0.09	0.02	0.01	0.02
16:3n-4	0.06	0.19	0.05	0.06	0.06
16:3n-3	0.03	0.13	0.04	0.04	0.04
16:3n-1	0.08	0.12	0.11	0.10	0.09
16:4n-3	0.03	0.08	0.02	0.02	0.05
16:4n-1	0.01	ND	0.02	0.01	0.01
18:0	4.23	3.76	3.49	3.07	3.37
18:1n-9	45.25	27.06	7.30	10.90	6.43
18:1n-7	1.29	2.52	1.04	1.24	0.88
18:1n-5	0.05	0.10	0.04	0.05	0.04
18:2n-9	0.02	0.10	0.02	0.05	0.00
18:2n-6	31.99	26.96	22.77	23.62	20.94
18:2n-4	0.01	0.10	0.03	0.02	0.02
18:3n-6	0.05	0.24	0.08	0.15	0.10
18:3n-4	0.03	0.17	0.04	0.03	0.03
18:3n-3	1.94	4.22	2.02	2.73	1.80
18:3n-1	0.02	ND	ND	0.01	0.01
18:4n-3	0.03	0.52	0.08	0.18	0.10
18:4n-1	0.02	0.18	0.06	0.03	0.02
20:0	0.33	0.30	0.31	0.24	0.24
20:1n-9	0.09	0.43	0.09	0.12	0.08
20:1n-7	1.31	3.43	1.42	1.46	1.37
20:1n-5	0.05	0.17	0.05	0.06	0.04
20:2n-9	0.03	0.12	0.05	0.09	0.02
20:2n-6	0.09	0.67	0.09	0.17	0.08
20:3n-9	0.03	0.07	0.08	0.22	0.11
20:3n-6	0.05	0.27	0.06	0.03	0.04
20:4n-6	0.11	0.36	0.76	0.96	0.27
20:3n-3	0.07	0.30	0.20	0.21	0.10
20:4n-3	0.03	0.51	0.31	0.50	0.27
20:5n-3	0.51	2.67	0.84	1.24	0.88
22:1n-1	0.04	1.79	0.08	0.23	0.28
22:1n-9	0.24	0.52	0.25	0.30	0.04
22:4n-6	0.03	0.14	0.07	0.08	0.04
22:5n-6	0.05	0.20	3.48	8.27	5.39
22:5n-3	0.14	0.98	0.18	0.43	0.12
22:6n-3	1.30	5.49	22.63	24.50	21.26
SFA	14.65	17.22	35.21	21.37	38.68
MUFA	48.60	37.81	10.64	14.87	9.43
n-3	4.08	14.91	26.33	29.85	24.63
n-6	32.36	28.84	27.32	33.28	26.85
n-9	45.64	28.30	7.80	11.67	6.69
n-3/n-6	0.13	0.52	0.96	0.90	0.92
n-3 LC-PUFA	2.06	9.95	24.16	26.88	22.65
n-6 LC-PUFA	0.33	1.64	4.47	9.51	5.82
EPA/ARA	4.8	7.4	1.1	1.3	3.2
EPA/DHA	0.4	0.5	0.1	0.1	0.1

SFA: saturated fatty acids; MUFA: Monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids; ND, not detected.

2.4. Larval morphology

At the beginning and at the end of the feeding trial, 30 larvae per tank were sampled and preserved in 4% buffered formaldehyde, dehydrated through graded alcohol and xylene, and finally embedded in paraffin wax for histological study. Paraffin blocks were made and cut with a Leica microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) in 4 μm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) to visualization under a light microscope (BX51TF, Olympus, Tokyo,

Japan). Five larvae were kept per paraffin block. Livers were blinded assessed by three different researchers for cytoplasmic lipid vacuolization infiltration, using a semi-quantitative four graded examination scheme as follows: 0, no lipid infiltration; 1, few lipid vacuolization; 2, medium lipid vacuolization; 3, severe lipid vacuolization.

2.5. Gene expression analyses

Total RNA from whole larvae samples was extracted using TRI Reagen (Merck KGaA, Darmstadt, Germany) and the RNeasy Mini Kit (Qiagen), following the conditions described previously (Carvalho et al., 2021). The quality of RNA was check by 1.4% agarose gel electrophoresis and the quantity were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA were reverse transcribed to complementary cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:500). Real-time quantitative PCR was performed in an CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using *β-actin* and *ribosomal protein L27* (*rpl-27*) as housekeeping genes in a final volume of 15 μl per reaction well. The PCR conditions were as follows: 95 °C for 3 min 30 s followed by 40 cycles of 95 °C for 15 s, Tm °C for 30 s, and 72 °C for 30 s, and then 95 °C for 1 min, final denaturing step from 58 °C to 95 °C for 10 s. All PCR reactions were carried out in in a final volume of 15 μl, with 7.5 μl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 μl of each primer (10 mM), 5 μl of cDNA (1:10 dilution) and 1.3 μl of MiliQ water. *5-lipoxygenase* (*5-lox*), *cyclooxygenase-2* (*cox*-2), *fatty acyl desaturase 2* (*fads2*), *glucose 6 phosphatase* (*g6p*) and *peroxisome proliferator-activated receptor α* (*ppar-α*) and expressions were determined. Sequences of primers are shown in Table 4. Relative gene expression was estimated by the Δ–Δ method (Livak and Schmittgen, 2001).

2.6. Statistical analyses

All data are presented as mean ± SE and were tested for normality and homogeneity of variances with Shapiro-Wilk and Levene's test, respectively. Each tank was treated as a replicate (n = 4 for each treatment). A one-way ANOVA were applied to data and, when applicable, means comparisons among different experimental groups were compared by Tukey's test (when homogeneity assumed) or Games-Howell test (when homogeneity was not assumed). Statistical differences were considered when $P < 0.05$. Histological evaluation was statistically treated with a Mann-Whitney *U* test. All statistical analyses were done using statistical analyses were done using the SPSS 21.0 software package. In addition, principal component analysis (PCA) were carried out in overall larvae whole-body fatty acid composition for each lipid fraction, using Prism9 software for Windows.

2.7. Ethical statement

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010/63/EU) and Spanish legislation (RD 1201/2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (reference OEBA-ULPGC-21/2018).

3. Results

3.1. Larval performance

All experimental microdiets were well accepted by larvae denoted by the presence of feed inside digestive tube and the good feed intake. After 3 weeks of feeding the experimental diets, larval dry body weight had increased over threefold, and total length had increased over 44% percent (Table 5). Survival during the feeding period was 28% lower in larvae fed OA diet, but not significantly different ($P > 0.05$), than that of larvae fed the other diets (Table 5). Larvae fed OA diet also showed the lowest growth ($P < 0.05$), as well as the lowest survival after the air exposure (Table 5). In contrast, FO, AGR, DHG and M60 diets led to the highest larval growth and survival after the air exposure test ($P < 0.0$; Table 5).

3.2. Diets and larval whole-body proximate and fatty acid composition

Regarding the fatty acid composition of the microalgae meals used, MO060 was the highest in DHA and EPA contents, followed by DHA GOLD and then ALL-G RICH (Table 2). Fatty acid analysis of the diets showed that the diet containing oleic acid oil (OA Diet) was characterized by the highest content in monounsaturated fatty acids (MUFA), particularly, oleic acid (OA, 18:1n-9), and LA (18:2n-6) (Table 3) and

Table 5
Survival and growth performance of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days.

	Diets				
	OA	FO	AGR	DHG	M60
Survival (%)	38 ± 3.2	53 ± 1.76	46 ± 3.55	52 ± 2.02	46 ± 6.35
Body weight (mg dry weight)	1.61 ± 0.11^{b}	2.12 ± 0.33^{ab}	2.03 ± 0.21^{ab}	2.76 ± 0.17^{a}	2.82 ± 0.29^{a}
Total length (mm)	10.54 ± 0.21^{b}	11.31 ± 0.43^{ab}	11.27 ± 0.23^{ab}	12.47 ± 0.27^{a}	12.17 ± 0.38^{a}
Survival after air exposure test (%)	28.33 ± 2.89^{b}	90.00 ± 8.66^{a}	78.33 ± 16.65^{a}	91.25 ± 3.15^{a}	95.00 ± 5.00^{a}

Values (mean ± SE; $n = 4$) with the different superscripts are significantly different.

Table 4
Sequences of primers used to determine gene expression in 20 dah-gilthead sea bream larvae.

Genes	Sequence (5′–3′)		Tm	Gene bank access no	Reference
	Forward	Reverse			
5-lox	CCTGGCAGATGTGAACTTGA	CGTTCTCCTGATACTGGCTGA	61	FP334124	Alves Martins et al., 2012
cox2	GAG TAC TGG AAG CCG AGC AC	GAT ATC ACT GCC GCC TGA GT	61	AM296029	Chaves-Pozo et al., 2008
fads2	GCAGAGCCACAGCAGCAGGGA	CGG CCT GCG CCT GAG CAG TT	61	AY055749	Izquierdo et al., 2015
g6p	CGCTGGAGTCATTACAGGCGT	CAGGTCCACGCCCAGAACTC	61	AF151718.1	Enes et al., 2008
ppar-α	TCT CTT CAG CCC ACC ATC CC	ATC CCA GCG TGT CGT CTC C	58.1	AY590299	Benedito-Palos et al., 2014
β-actin	GACCAACTGGGATGACATGG	GCATACAGGGACAGCACAGC	58.1	X89920.1	Minghetti et al., 2011
rpl-27	ACAACTCACTGCCCCACCAT	CTTGCCTTTGCCCAGAACTT	58.1	AY188520	Domínguez et al., 2020

ppar-α, peroxisome proliferator-activated receptor α; cox-2, cyclooxygenase-2; 5-lox, 5-lipoxygenase; fads2, fatty acid desaturase; g6p, glucose-6-phosphatase; rpl-27, ribosomal protein L27.

the lowest n-3 LC-PUFA content. In contrast, the diet containing FO was characterized by the highest content in EPA (20:5n-3), and consequently the highest levels of EPA/ARA and EPA/DHA, as well as the highest LNA (18:3n-3) content (Table 3). The diets containing algal products were higher in n-3 LC-PUFA, particularly DHA (22:6n-3) and n-6 DPA (22:5n-6), as well as a higher n-3/n-6, than FO and OA diet. Among them, diet DHG showed the highest n-6 DPA and n-3 LC-PUFA levels. AGR diet was slightly high in n-3/n-6 fatty acids and M60 was highest in saturated fatty acids (SFA), particularly palmitic acid (16:0) (Table 3). Thus, compared to FO diet, the inclusion of M60 or AGR doubled the contents in SFA and all the microalgal products increased the DHA content by almost four-fold (Table 3).

Regarding fatty acid profiles of larval total lipids, they generally reflected the fatty acid composition of the diets, with significantly higher ($P > 0.05$) n-3 LC-PUFA, DHA, and n-6 DPA contents in fish fed the algal diets (Table 6). Besides, EPA/ARA and EPA/DHA followed a similar pattern to the diet. However, despite 18:1n-9 was 7 times higher in OA diet with respect to M60 diet, the contents of this fatty acid in larvae fed these two diets were not significantly ($P > 0.05$) different (Table 6). Similar results were also found for 18:2n-6 and 18:3n-3 (Table 6). On the contrary, the contents in 18:3n-6, 18:2n-9 or 20:2n-9, products of desaturation and elongation from 18:2n-6 and 18:1n-9, respectively, were significantly ($P < 0.05$) increased in OA larvae, followed by FO larvae, in comparison to those fed the algal products (Table 6). The PCA results showed that PC1 explained 40.85% of the total variation in fatty acid composition of total lipids in sea bream larvae, whereas PC2 explained 20.70% (Fig. 1). PC1 was positively correlated with 20:4n-6, 17:0, 16:0 ISO and 16:3n-3 contents, while negatively correlated with n-6, 16:0 and 18:0, tending to separate M60 from DHG larvae. In contrast, PC2 was positively correlated with 18:3n-6, n-9, MUFA, 18:2n-9, 20:2n-9, 16:1n-7 and 18:2n-6, while negatively correlated with 22:5n-6, n-3/n-6, n-3, n-3 LC-PUFA and 22:6n-3, clearly separating FO and OA from AGR and DHG.

In neutral lipids, most fatty acids also followed the dietary profile (Table 7). However, the levels of 18:3n-6, 18:2n-9 or 20:2n-9, products of desaturation and elongation from 18:2n-6 to 18:1n-9, were significantly ($P < 0.05$) increased in OA, FO and M60 larvae, in comparison to those fed the algal products (Table 7), regardless the dietary levels. The lowest levels of these fatty acids were found in DHG larvae (Table 7). Besides, both ARA (20:4n-6) and DHA contents in OA larvae were high and similar to those of FO larvae, despite the lower contents of those fatty acids in the OA diet (Table 7). The PCA results showed that PC1 explained 36.31% of the total variation in fatty acid composition of neutral lipids in sea bream larvae, whereas PC2 explained 17.80% (Fig. 2). PC1 was positively correlated with MUFA, n-9 and, particularly, 20:2n-6, 18:2n-9, 20:2n-9, as well as 18:4n-3 contents, while negatively correlated with SFA, 16:0, 18:0, n-3, n-3 LC-PUFA, 22:5n-6 and 22:6n-3, clearly differentiating FO and OA diets from those containing algal products. PC2 was positively correlated with 20:5n-3 and n-3/n-6 while negatively correlated with n-6 and 18:2n-6, separating FO from OA and DHG from M60.

Fatty acid profiles of polar lipids from larvae (Table 8) fed the different diets was more homogeneous than that of neutral lipids and reflected the dietary profiles in a lesser extent than neutral lipids. For instance, the contents of 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and, particularly, 22:6n-3 in larvae fed the different diets were more similar among them (Table 8), than among the contents of these fatty acids in the different diets (Table 3). SFA, as well as 16:0 and 18:0, were higher in PL than in NL, and were also higher in larvae fed the algal products than in OA larvae (Table 8). MUFA contents in PL, including 18:1n-9 (Table 8), were lower than in diet (Table 3) and in NL (Table 7), and were higher in OA and FO larvae than in the larvae fed the algal products (Table 8). However, the contents in 18:2n-9, 18:3n-6, 20:2n-9, 20:2n-6, 20:3n-9 or 20:3n-6 in PL (Table 8), were not related with the dietary levels of these fatty acids and were higher than in the diet but lower than in the neutral lipids. Besides, all these fatty acids, products of

Table 6
Total lipids (% dry weight) and respective fatty acid composition (% total fatty acids) of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days.

	Diets				
	OA	FO	AGR	DHG	M60
Total lipids	14.13 ± 0.19	12.91 ± 0.64	14.43 ± 0.33	13.72 ± 0.4	13.14 ± 0.42
Fatty acids					
14:0	0.49 ± 0.11	0.51 ± 0.22	0.51 ± 0.20	0.48 ± 0.16	0.40 ± 0.15
14:1n-7	0.12 ± 0.16	0.19 ± 0.17	0.16 ± 0.14	0.20 ± 0.23	0.01 ± 0.01
14:1n-5	0.15 ± 0.20	0.25 ± 0.21	0.21 ± 0.19	0.24 ± 0.29	0.02 ± 0.01
15:0	0.24 ± 0.08	0.40 ± 0.18	0.32 ± 0.06	0.33 ± 0.30	0.17 ± 0.04
15:1n-5	0.03 ± 0.02	0.19 ± 0.15	0.25 ± 0.14	0.28 ± 0.35	0.03 ± 0.00
16:0ISO	0.12 ± 0.14	0.20 ± 0.16	0.23 ± 0.15	0.19 ± 0.22	0.03 ± 0.01
16:0	13.32 ± 3.66	14.89 ± 4.12	16.52 ± 6.40	12.00 ± 5.20	19.82 ± 3.96
16:1n-7	0.72 ± 0.08	0.95 ± 0.26	0.77 ± 0.28	0.64 ± 0.19	0.85 ± 0.13
16:1n-5	0.23 ± 0.10	0.28 ± 0.13	0.30 ± 0.14	0.26 ± 0.20	0.10 ± 0.01
16:2n-4	0.29 ± 0.05	0.31 ± 0.19	0.37 ± 0.06	0.35 ± 0.17	0.27 ± 0.05
17:0	0.25 ± 0.17	0.28 ± 0.13	0.24 ± 0.10	0.26 ± 0.22	0.08 ± 0.01
16:3n-4	0.26 ± 0.15	0.29 ± 0.11	0.28 ± 0.07	0.31 ± 0.19	0.16 ± 0.02
16:3n-3	0.18 ± 0.24	0.21 ± 0.16	0.25 ± 0.13	0.31 ± 0.28	0.05 ± 0.02
16:3n-1	0.76 ± 0.18	0.66 ± 0.07	0.80 ± 0.06	0.73 ± 0.10	0.77 ± 0.06
16:4n-3	0.64 ± 0.12^{ab}	0.48 ± 0.04^{a}	0.35 ± 0.07^{ab}	0.40 ± 0.23^{ab}	0.30 ± 0.01^{b}
16:4n-1	0.15 ± 0.15	0.22 ± 0.13	0.25 ± 0.11	0.08 ± 0.07	0.08 ± 0.01
18:0	8.01 ± 0.56	7.49 ± 0.75	7.99 ± 1.18	6.95 ± 1.73	9.00 ± 0.15
18:1n-9	18.56 ± 8.86^{ab}	16.95 ± 1.99^{a}	10.26 ± 1.66^{b}	9.74 ± 2.84^{ab}	12.85 ± 2.12^{ab}
18:1n-7	1.53 ± 0.08^{b}	2.34 ± 0.34^{a}	1.54 ± 0.19^{b}	1.49 ± 0.23^{b}	1.82 ± 0.17^{ab}
18:1n-5	0.14 ± 0.11	0.24 ± 0.14	0.25 ± 0.12	0.23 ± 0.24	0.06 ± 0.03
18:2n-9	3.92 ± 0.43^{a}	2.43 ± 0.27^{b}	0.84 ± 0.23^{cd}	0.42 ± 0.08^{d}	1.37 ± 0.11^{c}
18:2n-6	15.39 ± 4.78	14.90 ± 1.54	12.02 ± 2.13	10.88 ± 2.71	16.89 ± 0.32
18:2n-4	0.14 ± 0.15	0.22 ± 0.13	0.21 ± 0.11	0.28 ± 0.23	0.03 ± 0.02
18:3n-6	2.54 ± 0.21^{a}	2.25 ± 0.27^{a}	1.30 ± 0.27^{b}	0.93 ± 0.10^{b}	2.25 ± 0.16^{a}
18:3n-4	0.14 ± 0.13	0.21 ± 0.14	0.24 ± 0.14	0.30 ± 0.30	0.08 ± 0.01
18:3n-3	0.56 ± 0.12^{b}	1.22 ± 0.04^{a}	0.68 ± 0.15^{b}	0.78 ± 0.25^{ab}	1.33 ± 1.05^{ab}
18:4n-3	0.30 ± 0.23	0.43 ± 0.07	0.37 ± 0.16	0.32 ± 0.18	0.26 ± 0.16
18:4n-1	0.16 ± 0.15	0.18 ± 0.16	0.23 ± 0.12	0.23 ± 0.23	0.04 ± 0.02
20:0	0.47 ± 0.18	0.48 ± 0.13	0.38 ± 0.04	0.43 ± 0.15	0.36 ± 0.15
20:1n-9	0.15 ± 0.12	0.34 ± 0.10	0.27 ± 0.15	0.28 ± 0.19	0.13 ± 0.06
20:1n-7	1.04 ± 0.25	1.55 ± 0.31	1.16 ± 0.13	1.20 ± 0.09	1.22 ± 0.58
20:1n-5	0.21 ± 0.21	0.30 ± 0.16	0.26 ± 0.07	0.30 ± 0.20	0.16 ± 0.07
20:2n-9	0.83 ± 0.09^{a}	0.69 ± 0.12^{ab}	0.43 ± 0.15^{bc}	0.28 ± 0.23^{c}	0.47 ± 0.08^{abc}

(*continued on next page*)

Table 6 (*continued*)

	Diets				
	OA	FO	AGR	DHG	M60
20:2n-6	0.66 ± 0.21	0.79 ± 0.22	0.61 ± 0.18	0.53 ± 0.10	0.46 ± 0.20
20:3n-9	0.16 ± 0.14	0.21 ± 0.17	0.40 ± 0.39	0.29 ± 0.31	0.03 ± 0.03
20:3n-6	1.10 ± 0.21	1.23 ± 0.26	0.88 ± 0.28	0.90 ± 0.45	0.99 ± 0.18
20:4n-6	1.60 ± 0.08^{b}	1.65 ± 0.25^{ab}	1.74 ± 0.15^{ab}	2.11 ± 0.06^{a}	1.64 ± 0.72^{ab}
20:3n-3	0.27 ± 0.31	0.41 ± 0.16	0.56 ± 0.49	0.43 ± 0.28	0.24 ± 0.15
20:4n-3	0.26 ± 0.27	0.59 ± 0.13	0.26 ± 0.18	0.47 ± 0.32	0.38 ± 0.29
20:5n-3	1.08 ± 0.04^{b}	3.01 ± 0.64^{a}	1.37 ± 0.23^{ab}	1.35 ± 0.15^{ab}	1.81 ± 1.20^{ab}
22:1n-1	0.45 ± 0.25	0.67 ± 0.30	0.51 ± 0.34	1.77 ± 2.51	0.23 ± 0.19
22:1n-9	0.87 ± 0.49	0.97 ± 0.44	0.84 ± 0.32	1.56 ± 1.71	0.38 ± 0.22
22:4n-6	0.31 ± 0.15	0.50 ± 0.30	0.50 ± 0.30	0.52 ± 0.55	0.21 ± 0.17
22:5n-6	1.61 ± 0.22^{b}	1.24 ± 0.44^{b}	4.44 ± 1.30^{ab}	7.21 ± 4.12^{a}	3.62 ± 1.80^{ab}
22:5n-3	0.49 ± 0.16	1.74 ± 0.76	0.79 ± 0.33	0.60 ± 0.30	1.07 ± 1.24
22:6n-3	9.08 ± 0.38^{c}	14.38 ± 5.71^{bc}	26.78 ± 8.82^{ab}	29.96 ± 6.17^{a}	17.43 ± 4.74^{abc}
SFA	22.90 ± 3.52	24.25 ± 4.84	26.17 ± 7.60	20.64 ± 6.32	29.86 ± 4.09
MUFA	24.22 ± 8.73	25.23 ± 2.03	16.77 ± 2.58	18.19 ± 4.85	17.87 ± 3.14
n-3	12.59 ± 0.80^{b}	22.07 ± 7.11^{ab}	30.84 ± 9.05^{ab}	34.19 ± 6.52^{a}	22.63 ± 2.66^{a}
n-6	23.20 ± 4.85^{ab}	22.62 ± 0.81^{b}	21.59 ± 0.72^{b}	23.14 ± 4.50^{ab}	26.09 ± 0.51^{a}
n-9	24.50 ± 8.41^{ab}	21.60 ± 1.81^{a}	13.06 ± 1.90^{b}	12.57 ± 2.43^{b}	15.23 ± 2.28^{ab}
n-3/n-6	0.57 ± 0.15	0.98 ± 0.33	1.43 ± 0.42	1.56 ± 0.51	0.87 ± 0.09
n-3 LC-PUFA	10.91 ± 0.24^{b}	19.72 ± 7.^{17ab}	29.20 ± 9.02^{ab}	32.37 ± 6.29^{a}	20.69 ± 2.96^{a}
n-6 LC-PUFA	5.27 ± 0.81	5.42 ± 1.18	8.16 ± 1.94	11.27 ± 3.47	6.92 ± 0.88
EPA/ARA	0.68 ± 0.06^{c}	1.82 ± 0.16^{a}	0.79 ± 0.12^{bc}	0.64 ± 0.09^{c}	1.02 ± 0.19^{b}
EPA/DHA	0.12 ± 0.01^{a}	0.23 ± 0.05^{a}	0.06 ± 0.02^{b}	0.05 ± 0.01^{b}	0.14 ± 0.14^{ab}

SFA: saturated fatty acids; MUFA: Monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids.
Values (mean ± SE; n = 4) with the different superscripts are significantly different.

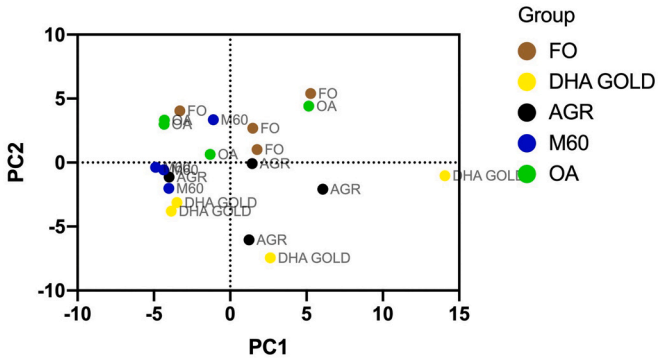

Fig. 1. Principal components of total lipids fatty acid composition of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days. PC1: 40.85%; PC2: 20.70%.

Table 7
Neutral lipids fatty acid composition (% total fatty acids) of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days.

Fatty acids	Diets				
	OA	FO	AGR	DHG	M60
14:0	0.35 ± 0.08^{b}	0.91 ± 0.25^{ab}	0.77 ± 0.07^{a}	2.66 ± 1.17^{ab}	0.68 ± 0.20^{ab}
14:1n-7	0.21 ± 0.20	0.08 ± 0.08	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.00
14:1n-5	0.29 ± 0.28	0.13 ± 0.09	0.03 ± 0.00	0.08 ± 0.04	0.02 ± 0.00
15:0	0.24 ± 0.11	0.43 ± 0.06	0.50 ± 0.02	0.70 ± 0.22	0.33 ± 0.09
15:1n-5	0.13 ± 0.09	0.09 ± 0.06	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
16:0ISO	0.21 ± 0.18	0.12 ± 0.08	0.05 ± 0.04	0.08 ± 0.03	0.02 ± 0.01
16:0	7.04 ± 1.71^{c}	12.29 ± 2.67^{bc}	16.82 ± 1.09^{ab}	22.85 ± 3.30^{a}	11.58 ± 6.76^{bc}
16:1n-7	0.79 ± 0.19^{b}	1.47 ± 0.12^{a}	0.87 ± 0.16^{b}	2.62 ± 1.44^{ab}	0.75 ± 0.15^{b}
16:1n-5	0.27 ± 0.16	0.30 ± 0.11	0.10 ± 0.03	0.20 ± 0.10	0.09 ± 0.02
16:2n-4	0.27 ± 0.11^{ab}	0.30 ± 0.09^{a}	0.29 ± 0.07^{a}	0.08 ± 0.03^{b}	0.23 ± 0.08^{ab}
17:0	0.33 ± 0.23	0.19 ± 0.08	0.10 ± 0.01	0.06 ± 0.04	0.08 ± 0.02
16:3n-4	0.29 ± 0.16	0.29 ± 0.07	0.22 ± 0.07	0.20 ± 0.14	0.14 ± 0.04
16:3n-3	0.18 ± 0.15	0.19 ± 0.12	0.06 ± 0.02	0.17 ± 0.05	0.05 ± 0.01
16:3n-1	0.25 ± 0.13	0.17 ± 0.05	0.17 ± 0.06	0.13 ± 0.02	0.12 ± 0.01
16:4n-3	0.34 ± 0.15	0.19 ± 0.06	0.15 ± 0.06	0.09 ± 0.01	0.09 ± 0.03
18:0	6.10 ± 0.82^{b}	6.72 ± 0.53^{ab}	8.07 ± 0.25^{a}	8.35 ± 0.56^{a}	8.51 ± 1.28^{a}
18:1n-9	24.42 ± 3.89^{a}	18.55 ± 2.69^{ab}	10.29 ± 0.31^{c}	13.65 ± 1.22^{abc}	12.36 ± 1.32^{bc}
18:1n-7	1.52 ± 0.05^{b}	2.48 ± 0.06^{a}	1.76 ± 0.12^{b}	2.48 ± 0.42^{ab}	1.78 ± 0.22^{b}
18.1n-5	0.16 ± 0.20	0.17 ± 0.06	0.09 ± 0.02	0.09 ± 0.02	0.07 ± 0.02
18:2n-9	5.00 ± 0.72^{a}	3.42 ± 0.35^{b}	1.11 ± 0.32^{cd}	0.43 ± 0.10^{d}	1.90 ± 0.18^{c}
18:2n-6	16.93 ± 3.18	11.92 ± 4.03	14.80 ± 1.09	11.01 ± 3.11	18.18 ± 2.15
18:2n-4	0.18 ± 0.14	0.18 ± 0.10	0.04 ± 0.01	0.09 ± 0.04	0.02 ± 0.01
18:3n-6	3.24 ± 0.35^{a}	4.97 ± 3.93^{ab}	2.43 ± 0.41^{ab}	1.67 ± 0.32^{b}	3.40 ± 0.42^{a}
18:3n-4	0.19 ± 0.15	4.17 ± 6.84	0.09 ± 0.04	0.09 ± 0.04	0.05 ± 0.01
18:3n-3	0.73 ± 0.07^{b}	1.34 ± 0.32^{a}	0.87 ± 0.07^{b}	0.91 ± 0.13^{ab}	1.04 ± 0.16^{ab}
18:4n-3	0.38 ± 0.26^{ab}	0.95 ± 0.56^{ab}	0.24 ± 0.03^{b}	0.42 ± 0.04^{a}	0.31 ± 0.05^{ab}
18:4n-1	0.26 ± 0.20	0.20 ± 0.13	0.04 ± 0.01	0.07 ± 0.03	0.04 ± 0.02
20:0	0.48 ± 0.11	0.57 ± 0.19	0.49 ± 0.09	0.43 ± 0.07	0.46 ± 0.07
20:1n-9	0.24 ± 0.12	0.60 ± 0.30	0.16 ± 0.04	0.19 ± 0.03	0.13 ± 0.02
20:1n-7	1.24 ± 0.15	1.83 ± 0.31	1.40 ± 0.12	1.24 ± 0.12	1.51 ± 0.17
20:1n-5	0.29 ± 0.24	0.30 ± 0.14	0.24 ± 0.16	0.17 ± 0.03	0.16 ± 0.06
20:2n-9	1.12 ± 0.10^{a}	0.60 ± 0.19^{b}	0.32 ± 0.08^{c}	0.12 ± 0.04^{c}	0.65 ± 0.05^{b}
20:2n-6	0.65 ± 0.14^{ab}	0.66 ± 0.16^{a}	0.48 ± 0.13^{ab}	0.36 ± 0.06^{b}	0.41 ± 0.07^{ab}
20:3n-9	1.26 ± 0.68^{abc}	0.43 ± 0.48^{abc}	0.76 ± 0.07^{b}	0.04 ± 0.02^{c}	1.07 ± 0.03^{a}
20:3n-6	0.76 ± 0.50	0.59 ± 0.41	0.23 ± 0.06	0.38 ± 0.10	0.23 ± 0.07
20:4n-6	2.05 ± 0.15^{a}	2.34 ± 1.76^{ab}	2.18 ± 0.14^{a}	2.02 ± 0.38^{ab}	1.41 ± 0.19^{b}

(*continued on next page*)

Table 7 (*continued*)

Fatty acids	Diets				
	OA	FO	AGR	DHG	M60
20:3n-3	0.43 ± 0.32	0.31 ± 0.09	0.22 ± 0.04	0.20 ± 0.04	0.14 ± 0.03
20:4n-3	0.42 ± 0.32	0.56 ± 0.23	0.35 ± 0.07	0.38 ± 0.06	0.26 ± 0.03
20:5n-3	1.63 ± 0.37	2.81 ± 1.10	1.76 ± 0.41	2.83 ± 1.38	1.25 ± 0.08
22:1n-1	0.42 ± 0.30	0.85 ± 0.33	0.12 ± 0.02	0.13 ± 0.06	0.08 ± 0.03
22:1n-9	3.15 ± 1.10	1.26 ± 0.39	1.03 ± 0.33	0.57 ± 0.15	1.37 ± 0.48
22:4n-6	0.78 ± 0.65	0.96 ± 0.79	0.15 ± 0.03	0.23 ± 0.09	0.15 ± 0.04
22:5n-6	1.76 ± 0.47^{b}	0.86 ± 0.53^{b}	3.70 ± 0.08^{a}	4.13 ± 1.32^{ab}	5.24 ± 0.15^{a}
22:5n-3	1.00 ± 0.63	1.75 ± 1.02	0.49 ± 0.11	0.90 ± 0.42	0.30 ± 0.05
22:6n-3	12.03 ± 2.13^{bc}	10.42 ± 2.67^{c}	25.86 ± 0.80^{a}	16.39 ± 2.38^{b}	23.25 ± 0.80^{a}
SFA	14.74 ± 1.92^{c}	21.23 ± 3.44^{bc}	26.79 ± 1.32^{ab}	35.13 ± 4.27^{a}	21.67 ± 5.16^{bc}
MUFA	33.12 ± 1.11^{a}	28.12 ± 2.83^{a}	16.15 ± 0.60^{c}	21.48 ± 2.71^{b}	18.37 ± 1.82^{bc}
n-3	17.14 ± 4.34^{b}	18.52 ± 4.55^{ab}	30.00 ± 1.24^{a}	22.30 ± 3.25^{ab}	26.69 ± 1.00^{ab}
n-6	26.17 ± 2.29^{ab}	22.30 ± 4.39^{ab}	23.96 ± 1.13^{ab}	19.81 ± 4.88^{b}	29.02 ± 2.67^{a}
n-9	35.19 ± 2.82^{a}	24.87 ± 2.88^{b}	13.66 ± 0.66^{c}	15.01 ± 0.98^{c}	17.47 ± 1.47^{bc}
n-3/n-6	0.67 ± 0.22^{b}	0.90 ± 0.37^{ab}	1.26 ± 0.10^{a}	1.17 ± 0.23^{ab}	0.93 ± 0.08^{ab}
n-3 LC-PUFA	15.51 ± 3.74^{b}	15.85 ± 4.54^{ab}	28. 68 ± 1.22^{a}	20.71 ± 3.18^{ab}	25.19 ± 0.92^{a}
n-6 LC-PUFA	6 ± 0.86	5.41 ± 1.63	6.73 ± 0.13	7.12 ± 0.89	7.44 ± 0.24
EPA/ARA	0.79 ± 0.13	1.47 ± 0.36	0.80 ± 0.14	1.36 ± 0.51	0.90 ± 0.09
EPA/DHA	0.13 ± 0.01^{a}	0.27 ± 0.07^{ab}	0.07 ± 0.02^{b}	0.17 ± 0.08^{ab}	0.05 ± 0.00^{b}

SFA: saturated fatty acids; MUFA: Monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids.
Values (mean ± SE; n = 4) with the different superscripts are significantly different.

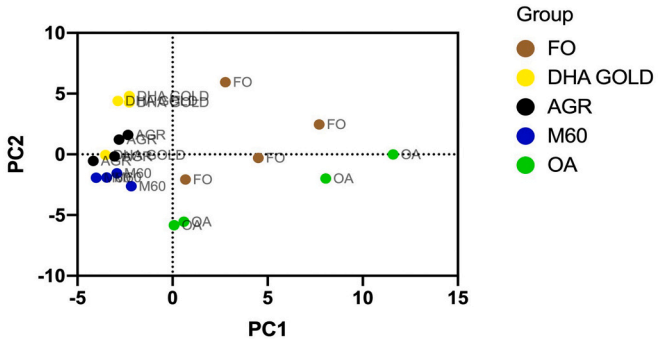

Fig. 2. Principal components of neutral lipids fatty acid composition of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days. PC1: 36.31%; PC2: 17.80%.

elongation and desaturation from 18:1n-9 or 18:6n-6, were higher in OA larvae, followed by FO larvae (Table 8). The contents on 18:2n-6 in PL were lower than in diet and similar that their respective values in NL, and despite the values of this fatty acid for OA larvae were the highest in agreement with the dietary levels, they were also high in PL of M60 larvae, regardless the low content in M60 larvae. Finally, ARA, EPA and DHA contents in PL (Table 8) were higher than their respective values in the diet. The PCA results showed that PC1 explained 36.24% of the total

Table 8
Polar lipids fatty acid composition (% total fatty acids) of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days.

Fatty acids	Diets				
	OA	FO	AGR	DHG	M60
14:0	0.19 ± 0.06^{b}	0.31 ± 0.13^{b}	0.39 ± 0.10^{ab}	0.69 ± 0.13^{a}	0.30 ± 0.17^{b}
14:1n-7	0.06 ± 0.05	0.06 ± 0.04	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
14:1n-5	0.13 ± 0.15	0.06 ± 0.03	0.03 ± 0.02	0.03 ± 0.00	0.01 ± 0.01
15:0	0.20 ± 0.08	0.22 ± 0.05	0.35 ± 0.08	0.27 ± 0.03	0.18 ± 0.07
15:1n-5	0.08 ± 0.07	0.06 ± 0.06	0.03 ± 0.03	0.02 ± 0.00	0.04 ± 0.04
16:0ISO	0.08 ± 0.08	0.07 ± 0.05	0.04 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
16:0	14.11 ± 2.56^{b}	19.28 ± 7.23^{ab}	22.58 ± 3.83^{ab}	24.69 ± 2.69^{a}	21.29 ± 3.53^{ab}
16:1n-7	0.56 ± 0.05^{b}	0.75 ± 0.18^{ab}	1.02 ± 0.22^{a}	1.10 ± 0.16^{a}	0.83 ± 0.18^{ab}
16:1n-5	0.19 ± 0.07	0.20 ± 0.05	0.13 ± 0.06	0.10 ± 0.01	0.09 ± 0.01
16:2n-4	0.33 ± 0.08^{a}	0.40 ± 0.06^{a}	0.42 ± 0.08^{a}	0.05 ± 0.01^{b}	0.30 ± 0.07^{a}
17:0	0.15 ± 0.11	0.16 ± 0.04	0.12 ± 0.07	0.06 ± 0.00	0.11 ± 0.06
16:3n-4	0.24 ± 0.06	0.21 ± 0.01	0.30 ± 0.09	0.21 ± 0.00	0.17 ± 0.02
16:3n-3	0.12 ± 0.12	0.18 ± 0.06	0.07 ± 0.03	0.10 ± 0.03	0.04 ± 0.01
16:3n-1	1.04 ± 0.11	1.03 ± 0.03	1.37 ± 0.14	0.99 ± 0.11	1.27 ± 0.14
16:4n-3	0.86 ± 0.05^{a}	0.59 ± 0.12^{b}	0.38 ± 0.05^{c}	0.35 ± 0.03^{c}	0.46 ± 0.07^{bc}
16:4n-1	0.15 ± 0.06	0.17 ± 0.08	0.10 ± 0.07	0.09 ± 0.01	0.11 ± 0.01
18:0	9.14 ± 0.45	11.42 ± 2.94	11.24 ± 2.01	10.00 ± 0.22	10.45 ± 1.79
18:1n-9	21.17 ± 1.45^{a}	17.20 ± 2.95^{ab}	11.99 ± 2.18^{bc}	10.94 ± 0.30^{bc}	8.46 ± 5.03^{c}
18:1n-7	1.66 ± 0.01	2.42 ± 0.38	1.83 ± 0.24	1.73 ± 0.04	1.72 ± 0.18
18:1n-5	0.11 ± 0.06	0.12 ± 0.01	0.09 ± 0.02	0.10 ± 0.04	0.06 ± 0.01
18:2n-9	2.26 ± 0.11^{a}	1.49 ± 0.11^{b}	0.62 ± 0.20^{cd}	0.33 ± 0.01^{d}	0.95 ± 0.13^{c}
18:2n-6	17.32 ± 1.18^{a}	13.81 ± 0.10^{ab}	12.01 ± 2.83^{b}	11.53 ± 0.73^{b}	15.72 ± 1.41^{ab}
18:3n-6	1.49 ± 0.07^{a}	1.23 ± 0.20^{ab}	0.89 ± 0.06^{b}	2.18 ± 1.11^{ab}	1.15 ± 0.15^{ab}
18:3n-4	0.08 ± 0.07	0.11 ± 0.07	0.05 ± 0.01	0.06 ± 0.02	0.07 ± 0.03
18:3n-3	0.44 ± 0.08	0.69 ± 0.16	0.43 ± 0.05	0.45 ± 0.02	0.58 ± 0.14
18:4n-3	0.17 ± 0.10	0.18 ± 0.07	0.08 ± 0.01	0.11 ± 0.04	0.09 ± 0.02
18:4n-1	0.16 ± 0.12	0.08 ± 0.02	0.04 ± 0.02	0.06 ± 0.03	0.03 ± 0.01
20:0	0.53 ± 0.07^{a}	0.46 ± 0.05^{ab}	0.40 ± 0.16^{ab}	0.29 ± 0.05^{b}	0.34 ± 0.06^{ab}
20:1n-9	0.18 ± 0.12^{ab}	0.26 ± 0.00^{a}	0.17 ± 0.16^{ab}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}
20:1n-7	1.32 ± 0.07^{a}	1.48 ± 0.14^{a}	0.85 ± 0.22^{b}	0.67 ± 0.04^{b}	0.87 ± 0.14^{b}
20:1n-5	0.17 ± 0.09	0.18 ± 0.05	0.15 ± 0.09	0.07 ± 0.02	0.11 ± 0.04
20:2n-9	0.78 ± 0.10^{a}	0.56 ± 0.07^{b}	0.19 ± 0.08^{cd}	0.10 ± 0.02^{d}	0.35 ± 0.04^{c}
20:2n-6	0.84 ± 0.13^{a}	0.85 ± 0.04^{a}	0.52 ± 0.12^{b}	0.42 ± 0.03^{b}	0.53 ± 0.06^{b}
20:3n-9	1.22 ± 0.16^{a}	0.91 ± 0.66^{abc}	0.63 ± 0.09^{b}	0.02 ± 0.01^{c}	0.95 ± 0.04^{a}
20:3n-6	0.18 ± 0.09^{b}	0.31 ± 0.28^{ab}	0.05 ± 0.02^{b}	0.49 ± 0.01^{a}	0.04 ± 0.01^{b}
20:4n-6	1.87 ± 0.21^{ab}	1.71 ± 0.87^{ab}	1.98 ± 0.43^{ab}	1.97 ± 0.09^{a}	1.34 ± 0.09^{b}

(*continued on next page*)

Table 8 (*continued*)

Fatty acids	Diets				
	OA	FO	AGR	DHG	M60
20:3n-3	0.10 ± 0.06	0.27 ± 0.06	0.12 ± 0.01	0.14 ± 0.02	0.10 ± 0.01
20:4n-3	0.20 ± 0.17	0.40 ± 0.15	0.20 ± 0.05	0.21 ± 0.02	0.18 ± 0.03
20:5n-3	1.29 ± 0.05	2.26 ± 1.31	1.55 ± 0.94	1.23 ± 0.18	1.14 ± 0.26
22:1n-1	0.23 ± 0.19^{ab}	0.41 ± 0.19^{a}	0.12 ± 0.06^{ab}	0.15 ± 0.10^{ab}	0.07 ± 0.04^{b}
22:1n-9	0.44 ± 0.17^{a}	0.35 ± 0.09^{ab}	0.20 ± 0.08^{ab}	0.18 ± 0.12^{ab}	0.16 ± 0.02^{b}
22:4n-6	0.36 ± 0.12	0.62 ± 0.42	0.56 ± 0.66	0.17 ± 0.04	0.11 ± 0.02
22:5n-6	2.39 ± 0.38^{bc}	1.51 ± 0.85^{c}	3.60 ± 0.84^{b}	6.28 ± 0.57^{a}	5.58 ± 0.80^{a}
22:5n-3	0.78 ± 0.20	1.57 ± 0.93	0.71 ± 0.66	0.38 ± 0.08	0.27 ± 0.06
22:6n-3	14.57 ± 2.00	13.25 ± 8.15	19.56 ± 7.30	20.89 ± 2.27	23.20 ± 6.31
SFA	24.40 ± 2.64^{b}	31.92 ± 10.32^{ab}	35.11 ± 5.70^{ab}	36.03 ± 2.94^{a}	32.69 ± 5.32^{ab}
MUFA	26.28 ± 0.49^{a}	23.55 ± 3.41^{ab}	16.61 ± 2.77^{bc}	15.16 ± 0.46^{c}	12.52 ± 4.85^{c}
n-3	18.53 ± 2.48	19.39 ± 10.89	23.09 ± 7.75	23.86 ± 2.49	26.07 ± 6.64
n-6	24.45 ± 0.85^{a}	20.04 ± 1.89^{b}	21.42 ± 1.55^{ab}	23.03 ± 0.97^{ab}	24.50 ± 1.24^{a}
n-9	26.04 ± 0.96^{a}	20.75 ± 2.14^{ab}	13.80 ± 2.45^{bc}	11.62 ± 0.33^{c}	10.93 ± 4.82^{c}
n-3/n-6	0.76 ± 0.11	0.92 ± 0.49	1.07 ± 0.35	1.03 ± 0.07	1.07 ± 0.28
n-3 LC-PUFA	16.93 ± 2.27	17.75 ± 10.58	22.13 ± 7.78	22.84 ± 2.52	24.90 ± 6.63
n-6 LC-PUFA	5.64 ± 0.88^{b}	5.00 ± 1.74^{ab}	8.51 ± 4.36^{ab}	9.32 ± 0.74^{a}	7.60 ± 0.83^{ab}
EPA/ARA	0.70 ± 0.08^{ab}	1.22 ± 0.46^{a}	0.50 ± 0.13^{b}	0.62 ± 0.06^{b}	0.84 ± 0.15^{ab}
EPA/DHA	0.09 ± 0.01^{b}	0.19 ± 0.02^{a}	0.08 ± 0.05^{b}	0.06 ± 0.01^{b}	0.05 ± 0.01^{b}

SFA: saturated fatty acids; MUFA: Monounsaturated fatty acids; LC-PUFA: long-chain polyunsaturated fatty acids.
Values (mean ± SE; $n = 4$) with the different superscripts are significantly different.

variation in fatty acid composition of PL in sea bream larvae, whereas PC2 explained 18.96% (Fig. 3). PC1 was positively correlated with MUFA, n-9 and, particularly, with 20:2n-6, 18:2n-9, 20:2n-9, 22:1n-9, as well as 18:4n-3 contents and negatively correlated with SFA, 16:0, n-3, n-3 LC-PUFA, 22:5n-6 and 22:6n-3, clearly differentiating FO and OA diets from those containing algal products. PC2 was positively correlated with 18:0 and negatively correlated with n-3 LC-PUFA.

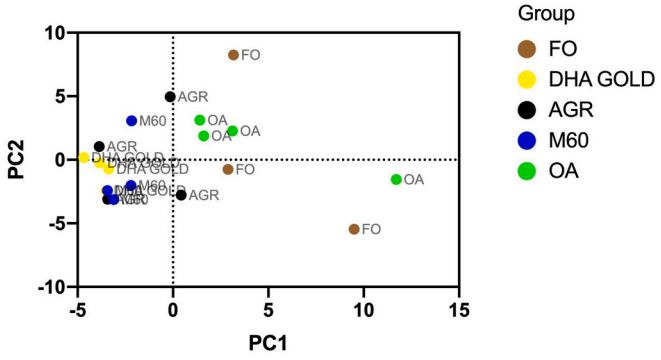

Fig. 3. Principal components of polar lipids fatty acid composition of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days. PC1: 36.24%; PC2: 18.96%.

3.3. Larval hepatic histomorphology

In general, larvae were well developed irrespectively of the diets. Morphological studies focused liver as a main organ for lipid metabolism. Hepatocytes presented a round and proeminent nucleus, sometimes displaced from the central position of the cell by lipid vacuoles. Irrespective of the experimental group, a moderate lipid infiltration within the hepatocytes was observed (Table 9; Fig. 4).

3.4. Gene expression

The expression of *fads2* and *g6p* was the highest in larvae fed the OA diet, followed by those fed FO and was downregulated in sea bream larvae fed the diets containing microalgae ($P < 0.05$; Fig. 5). Indeed, expressions of both genes were negatively correlated with the dietary levels of DHA ($r^2 = 0.99$, $P = 0.001$ and $r^2 = 0.87$, $P = 0.02$, respectively) and total n-3 LC-PUFA ($r^2 = 0.98$, $P = 0.001$ and $r^2 = 0.90$ $P = 0.01$, respectively). Furthermore, *fads2* expression was also correlated with DHA content in larval phospholipids ($r^2 = 0.83$, $P = 0.04$). In contrast, no significant differences were found in the relative expression of *5-lox*, *cox-2* and *ppar-α* among larvae fed the different microdiets. However, there was a moderate negative correlation ($R^2 = 0.67$; $P = 0.03$) between the EPA/ARA ratio in larval PL and *cox-2* expression, as well as a positive, despite not significant, relation between DHA contents in PL and *5-lox* expression ($R^2 = 0.59$).

4. Discussion

In marine fish, an insufficient supply of n-3 LC-PUFA, especially DHA and EPA, can lead to low survival, reduced stress resistance, growth depletion, altered feeding and swimming activities, and immune-suppression (Izquierdo, 2005Hamre et al., 2013). This is particularly important in larvae, which have higher essential fatty acids (EFA) requirements than juveniles (Izquierdo and Koven, 2011). In the present study, FO replacement by OA in microdiets for seabream, reduced final larval survival by 30% and survival after stress exposure by 70%, in agreement with the increase demand in EFA to deal with stress and recover homeostasis (Montero et al., 2019). Moreover, FO replacement by OA lead to the lowest growth in terms of body weight and total length, confirming the symptoms of EFA deficiency (Tocher, 2010; Hamre et al., 2013). In fact, the OA diet contained only 0.3% n-3 LC-PUFA on a dry matter basis (dw), well below the minimum requirement estimated for gilthead sea bream (1.5% dw; Izquierdo and Koven, 2010), in agreement with the poor survival and growth of the larvae fed this diet.

Larvae fed OA diet also showed the lowest n-3 LC-PUFA, particularly DHA, levels in total and neutral lipids, denoting the marked influence of dietary fatty acids on body composition (Cowey and Sargent, 1972). Particularly, the n-3 LC-PUFA contents in fish are markedly dependent on dietary levels, since marine fish larvae have a very limited capacity for their synthesis (Izquierdo et al., 2001). Interestingly, OA larvae also showed the highest contents on 18:2n-9, 20:2n-9, 18:3n-6, 20:3n-6 and ARA, products from the elongation and desaturation pathways of 18:1n-9 and 18:2n-6, respectively, in the total and, particularly, the

Table 9
Hepatic histological evaluation of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days.

	Diets				
	OA	FO	AGR	DHG	M60
Lipid infiltration*	2.00 ± 0.76	2.83 ± 0.79	2.83 ± 0.62	2.50 ± 0.83	2.23 ± 0.42

Values are presented as mean ± SE.
* 0, no lipid infiltration; 1, few lipid vacuolization; 2, medium lipid vacuolization; 3, severe lipid vacuolization.

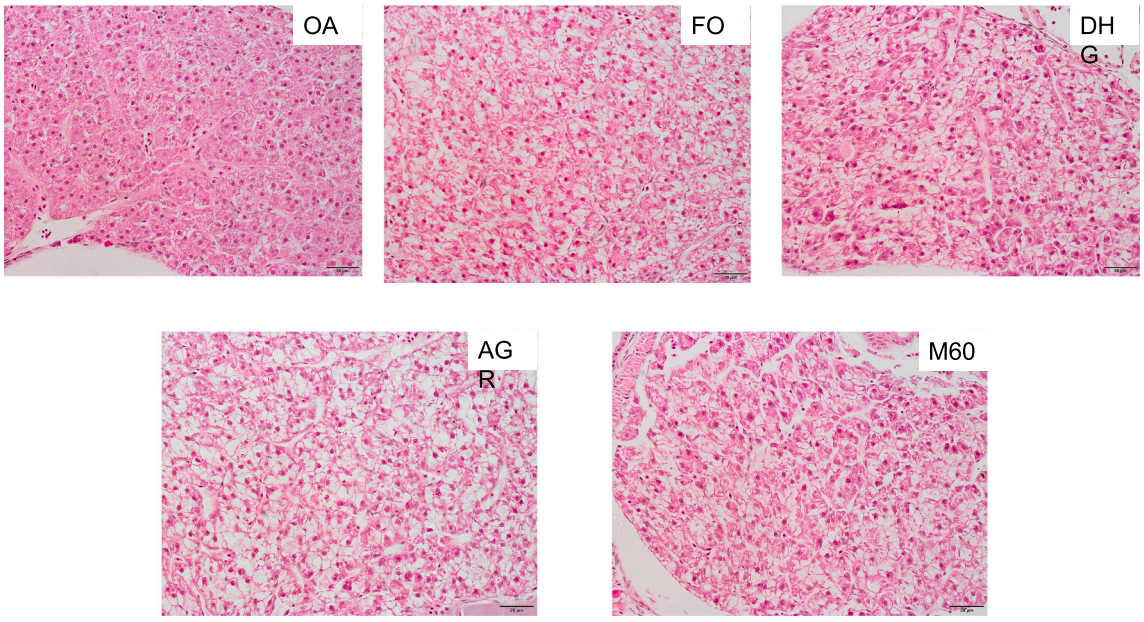

Fig. 4. Hepatic histomorphology of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days.

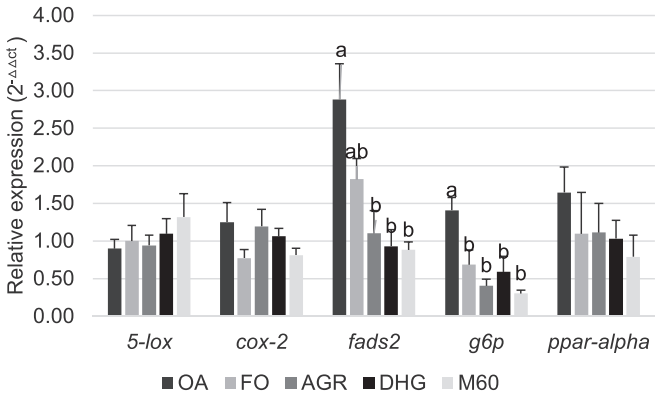

Fig. 5. Relative expression of fatty acid metabolism-related genes of gilthead sea bream larvae fed the experimental microdiets with different oils for 21 days. Values (mean ± SE; $n = 4$) with the different letters above SE bars are significantly ($P < 0.05$) different.

neutral lipids, despite the lowest dietary contents in these fatty acids. These results suggested the activation of the biosynthetic pathway of PUFA trough FADS2 in OA larvae, in agreement with the highest mRNA relative levels for *fads2*. This gene was first isolated in a marine fish in gilthead seabream (Seiliez et al., 2003) and its expression is modulated by dietary lipids (Izquierdo et al., 2008), although the ability to synthesize DHA was insufficient to satisfy its requirement (Hamre et al., 2013). Posteriorly, other studies have reported the up-regulation of *fads2* in tissues of fish fed low n-3 LC-PUFA levels (Carvalho et al., 2018, 2020). This up-regulation could be modulated by deficient contents of the products (DHA, EPA or ARA, essential fatty acids for marine species) or/and by high fatty acids substrates (often LA or LNA). In the present study, desaturation products in OA larvae appeared in n-6 and n-9 FA rather than in n-3 series, according to the higher levels of 18:1n-9 and 18:2n-6 in this diet. Both FA are substrates for FADS2 which have typically demonstrated a Δ6 activity in gilthead sea bream (Houston et al., 2017). Thus, the poor survival and growth performance of OA larvae confirms the low efficiency of seabream to synthesize more elongated and desaturated FA, such as DHA, possibly in relation to the low activity of Δ4 desaturase, which may also produce this EFA. In addition, an activation of the elongation pathways through ELOVL5 and/or 6 is also often observed in marine fish fed OA-based diets (Houston et al., 2017; Carvalho et al., 2018).

In contrast, despite the lowest DHA contents in OA diet, as well as in total and neutral lipids of OA larvae, the levels of DHA in phospholipids of these larvae were similar to those of fish fed the FO diet. The relatively high content of DHA in polar lipids of OA larvae in comparison to FO larvae, could be related to the selective incorporation of DHA by some of the enzymes involved in phospholipid synthesis and its low catabolic rate. Thus, this fatty acid is preferentially retained in PL, even when they are deficient in diet, to preserve cell membrane fluidity and functionality (Izquierdo, 2005). This selective retention was reported in previous studies in gilthead sea bream (Rodriguez et al., 1994; Fountoulaki et al., 2009), as well in other fish species (Bell et al., 2001; Borges et al., 2014; Carvalho et al., 2018), in concordance with the important biological functions of DHA in the early stages of development for plastic purposes (Izquierdo, 2005).

Gluconeogenesis is also an important energy supply metabolic pathway during starvation, nutrient or energy-restricted periods, which leads to endogenous glucose production (Enes et al., 2008; Wang et al., 2019). Studies in mammals have reported the inducible effect of the dietary lipids in gluconeogenesis (Massillon et al., 1997) and this effect have been described in fish as well (Panserat et al., 2002). Indeed, a previous study with rainbow trout reported a higher expression and activity of G6P with the increase of the dietary lipids and free fatty acids. For instance, the activation of PPAR, involved in FA oxidation, stimulates the expression of G6P and other gluconeogenic enzymes (Caseras et al., 2002). In agreement, in the present study sea bream larvae fed the OA diet also showed the highest expression in *g6p*, which is consistent with the highest absolute numeric value in *ppar* expression. The expression of *g6p* alone is not sufficient to estimate an effect in glucose metabolism and further analysis of glucose or other related parameters would be necessary. Furthermore, whether this possible effect is associated to an energy restriction in these EFA-deficient fish or due to a possible higher FA oxidation needs to be further studied. For instance, in mammals, high levels of free FA impaired glucose metabolism, leading to impaired glucose tolerance (Randle, 1998). A similar interaction was described in rainbow trout, which led to high fat-induced persistent hyperglycaemia and reduced insulin sensitivity (Figueiredo-Silva et al., 2012). Furthermore, linoleic acid and oleic acid increase glucose

production in chicken hepatocytes (Suh et al., 2008), which is agreement with the highest levels of these FA in OA diet. Further studies would be needed to confirm if this condition is impairing fish health and metabolism with metabolic consequences for fish production.

Indeed, replacement of dietary FO by any of the microalgal products did not negatively affected survival, stress resistance or growth of larval gilthead seabream. For instance, survival of gilthead sea bream larvae ranged around 46–53%, in agreement with the survival rates observed in previous studies with this species reared with microalgae supplemented-microdiets during a similar period (Eryalçın et al., 2015). Also, survival after air exposure of larvae fed the microalgae-based diets, was very high and similar to that of larvae fed the FO-based diet. This, along with the highest growth performance found in these fish, indicates that these microalgae products were very efficient in meeting the n-3 LC-PUFA requirements for gilthead sea bream larvae. The three microdiets tested containing microalgal products, AGR, DHG and M60, had very high dietary n-3 LC-PUFA levels (2.8, 3.1 and 2.6% dw, respectively). However, a slight improvement in growth performance and survival after stress of larvae fed DHG and M60 diets compared to those fed FO or AGR was observed and might be related with the higher n-6 DPA 22:5n-6 in the former diets, in agreement with recent studies in fish larvae (Garcia et al., 2008; Basford et al., 2020). This result suggests the need to further study the role of n-6 DPA, not commonly found on the traditional lipid sources used in aquafeeds such as FO or VO but present in high quantities in some microalgal products. Thus, feeding AGR, DHG or M60 diets led to the highest contents of n-3 LC-PUFA, particularly DHA, as well as n-6 DPA (22:5n-6) in the larval lipid fractions. Whereas FA profiles of neutral lipids clearly reflect dietary profiles, phospholipid composition is more stable, in relation to a substrate specificity for certain fatty acids during phospholipids synthesis and a limited catabolism, supporting cell membrane structure and fluidity functions (Sargent et al., 1993).

Interestingly, despite the high DHA content in AGR diet, reflected in the high DHA contents in larval total and neutral lipids, the larval polar lipids did not show a so high DHA content. Moreover, the DHA content in larval neutral lipids fed this diet was the highest among all larvae, suggesting a good absorption and incorporation into the neutral lipids, but a poorer incorporation of DHA into polar lipids and, hence, into biomembranes. These results suggested the lower bioavailability of DHA in All G Rich than in DHA Gold or M060. Indeed, although not significant, feeding the AGR diet led to a slight lower growth or survival after activity test than DHG and M60 diets. The incorporation of dietary DHA into larval polar lipids may be related to the total PL content of the diet, which was higher in DHG and M60 diets compared to AGR, or the DHA content in the PL. Thus, enzymatic, histological and biochemical evidence demonstrate that feeding with PL, particularly if they are rich in n-3 LC-PUFA, enhances lipid transport and the incorporation n-3 LC-PUFA into larval membrane lipids promoting fish growth (Izquierdo et al., 2000). In agreement, in the present study, there was a correlation between final total length and n-3 LC-PUFA contents in larval PL (R^2 = 0.63), whereas such relation was not found for larval NL (R^2 = 0.17). All G Rich as well as M60 are obtained from *Schizochytrium limacinum,* whose lipids contain mainly triacylglycerol, sterol esters, and phosphatidylcholine (Morita et al., 2006). However, PL content and the DHA content in the PL fraction may decrease along the culture in *Schizochytrium limacinum* (Morita et al., 2006), what could explain the lower incorporation into larval polar lipids according to previous studies in this species (Izquierdo et al., 2000). DHA Gold is obtained from an improved strain of *Schizochytrium* sp. with a higher content of EPA, as reflected in the DHG diet.

Alternative lipid sources can affect fish production by altering EPA/ARA ratios in fish, closely associated with an alteration in fish health metabolic functions, namely eicosanoids production. Indeed, it is recognized that ARA-derived eicosanoids (pro-inflammatory) are more active than EPA-derived eicosanoids (anti-inflammatory). In mammals, LOX and COX have a higher affinity for ARA than for EPA (Calder, 2012), although in marine fish the production of EPA-derived eicosanoids has an important role in metabolism (Ganga et al., 2006). Consequently, the imbalance in EPA/ARA ratios may lead to an imbalance in eicosanoids metabolism, resulting in excessive production of pro-inflammatory eicosanoids and affecting fish health and growth. Indeed, EPA has a down-regulating effect on the production of prostaglandin 2 (PGE2), an ARA-derived eicosanoid in fish (Ghioni et al., 2002). Moreover, *cox-2* and *5-lox* gene expressions are significantly down-regulated in sea bream larvae with EPA/ARA ratios in whole-body lower than 1 (Martins et al., 2012). In agreement, in the present study there was a moderate negative relation (R^2 = 0.67) between the EPA/ARA ratio in larval PL and *cox-2* expression, suggesting a down-regulation of *cox-2* by the increase in EPA/ARA ratios in fish membranes. On the contrary, no relation was found in the present study between EPA/ARA ratios in diets or larval lipids and *5-lox* expression, but a positive relation was found between DHA contents in PL and *5-lox* expression (R^2 = 0.59). DHA has been found to be a good substrate for a number of lipoxigenases (LOX). For instance, in sea bream, DHA regulates cortisol production through LOX-derived products in adrenocorticotropic hormone stimulated interrenal cells (Ganga et al., 2006). Nevertheless, FO replacement by the microalgal products tested in the present study did not significantly affect the expression of these genes, suggesting that the eicosanoid cascade metabolism was not compromised. Further studies on the expression of these genes, under stressed conditions for instance, would be of interest.

Overall, the results of the present study agree with the successful replacement of fish oil by other microalgae in larvae as well as in juveniles of the same species (Eryalçın et al., 2015; Carvalho et al., 2020), corroborating the potential of microalgae as DHA sources for fish at different developmental stages.

5. Conclusions

In conclusion, all microalgae products used in the present study (All G RICH, DHA Gold and MO060) were effective total replacers of FO in the diets of gilthead sea bream larvae, providing the necessary dietary n-3 LC-PUFA for optimal growth and survival. Even though, DHA GOLD and MO060 further improved growth and survival under stress conditions, possibly related with a higher dietary n-6 DPA or PL contents. Worth noting is that microalgae diets raised the DHA content in the whole body of larvae compared to OA or FO. The relative gene expression of lipid catabolism (*ppar-α*), eicosanoid metabolism (*cox-2* and *5-lox*) were unaffected, but FA synthesis though *fads2* was down-regulated by microalgae when compared to a diet based in oleic acid as lipid source, possibly due to the high DHA level in microalgae diets.

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CRediT authorship contribution statement

Marta Carvalho: Investigation, Formal analysis, Writing – original draft. **Bruno Marotta:** Investigation, Formal analysis, Writing – review & editing. **Hanlin Xu:** Formal analysis. **Pierre-André Geraert:** Resources, Funding acquisition, Writing – review & editing. **Sachi Kaushik:** Writing – review & editing. **Daniel Montero:** Resources, Funding acquisition, Writing – review & editing, Supervision. **Marisol Izquierdo:** Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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