Aquaculture 544 (2021) 737052

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Growth performance and fatty acid tissue profile in gilthead seabream juveniles fed with different phospholipid sources supplemented in low-fish meal diets

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ARTICLE INFO

Keywords:
Aquaculture
Plant-based diets
Polar lipids
Neutral lipids
Krill oil
Soy lecithin
Liver histology

ABSTRACT

The aim of the present study was to evaluate the phospholipid requirements for gilthead seabream, fed with low-fish meal diets. A 70-day feeding trial was performed to evaluate the dietary effects of marine versus plant-based phospholipid sources in low-fish meal diets on growth, body composition, liver, muscle and intestinal fatty acid profiles (polar and neutral), as well as liver morphology. Three levels of krill (marine) or soy lecithin (plant) as phospholipid source, at 0.53%, 1% and 2%, were supplemented in diets with 10% fish meal inclusion. The effects were compared to a high-fish meal diet (65%) as a positive control, and a low-fish meal diet without addition of phospholipids as a negative control. Growth, feed and protein efficiency parameters were improved by the addition of phospholipids in the low-fish meal diets, irrespective of the source, but all parameters were lower compared to the positive control. Krill phospholipid supplementation showed optimal results at 0.5% levels and soybean lecithin supplementation at 1% level. Significant differences by the phospholipid supplementation were found in both the neutral and polar lipid profile in the liver and muscle, while only in the neutral lipids in the intestine. Evaluation of liver histology indicated mild improvement of steatosis symptoms. Overall, our results indicate that phospholipid supplementation in low-fish meal diets can improve the growth and liver status in gilthead seabream juveniles.

1. Introduction

Aquaculture is a constantly on-growing food sector, currently providing more than half of the fish supply around the world, since the stagnant fisheries production cannot meet the current demands (FAO, 2020). Such a fast-growing trend leads also to an increasing demand for aquafeeds, translated into a high demand of fisheries-dependent fish meal as the main feed ingredient. To achieve a more sustainable production, the trend over the past decades is to replace fish meal (FM) by alternative and more available sources, such as plant-based ingredients (Tacon and Metian, 2015). Plant-based ingredients such as soybean, sunflower meals and rapeseed meals have been successfully replacing a great part of FM in a number of farmed fish, such as European seabass (*Dicentrarchus labrax*) and Gilthead seabream (*Sparus aurata*) (Kaushik et al., 2004; Kokou et al., 2017; Nengas et al., 1996). However, complete replacement of FM is hindered by the presence of antinutrient factors, or nutritional deficiencies due to unbalanced nutrient profile in amino acids, fatty acids or minerals and vitamins (Francis et al., 2001; Kokou and Fountoulaki, 2018), especially for carnivorous fish. *Re*-evaluating the nutritional requirement of aquaculture species in this new era of alternative aquafeed ingredients is essential in order to be able to create balanced feed formulations.

Dietary phospholipids and their importance in fish nutrition are well documented, with growth improvement, survival, normal skeletal development, and increase in stress resistance being some of their beneficial effects for fish (Tocher et al., 2008). Phospholipids provide

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https://doi.org/10.1016/j.aquaculture.2021.737052
Received 4 May 2021; Received in revised form 8 June 2021; Accepted 12 June 2021
Available online 17 June 2021

choline, phosphorus and essential fatty acids for fish growth (Coutteau et al., 1997), while they also increase the energy flow (transport of fatty acids) from the intestinal enterocytes to the blood (Morais et al., 2007). This leads to a reduced lipid droplet accumulation in the intestine by lipid transport and absorption through chylomicrons (Izquierdo et al., 2000). Generally, levels of phospholipid requirement are around 2–4% in the diets for juvenile fish and higher for larval fish (Tocher et al., 2008). With the current trend of replacing FM by alternative plant ingredients, besides the protein, as much as 10% of lipid which is primarily present in the form of phospholipids, is also replaced. Typically, the dietary lipid content is adjusted by the addition of fish oil or alternative oil sources, maintaining the total lipid content; however, the composition of the phospholipids is also affected (Tocher et al., 2008). Plant meals and oils contain low concentrations of phospholipids in comparison to FM and fish oil-based diets (Sargent et al., 1999). Therefore, substituting marine ingredients with plant-based ones will result in reduced phospholipid concentrations in the diets, and phospholipid requirements may become important. So far, studies on phospholipid requirements focused mostly in larval stages in different fish species such as pikeperch larvae (*Sander lucioperca*) (Hamza et al., 2012), gilthead sea bream larvae (Saleh et al., 2013; Saleh et al., 2015), juvenile cobia (*Rachycentron canadum*) (Trushenski et al., 2013a; Trushenski et al., 2013b), meagre *Argyrosomus*meagre *regius* (Ghaderpour and Estevez, 2020) and Atlantic cod *Gadus morhua* (Li et al., 2015), while especially for juvenile fish, the information is still not widely available.

So far, phospholipid supplementation in juvenile fish have been studied as a means to increase palatability of plant-based diets, due to the low feed intake. Dietary supplementation of soybean lecithin was reported to have a positive effect on feed intake when added in non-FM diets in juvenile amberjack *Seriola dumerilii* and hybrid striped bass *Morone chrysops*, with the attractant effect attributed to the polar lipid fraction of lecithin (phospholipid) (Laporte, 2015; Uyan et al., 2009). Supplementation of marine-origin phospholipids, which are high in the essential fatty acid 22:6ω-3 (Docosahexaenoic acid, DHA) in low-FM diets was less studied so far. Limited studies have shown that supplementation of this phospholipid further promotes growth, other than increasing palatability (Laporte, 2015; Trushenski et al., 2013b). Therefore, the requirements of dietary phospholipid supplementation in low-FM diets, with regard to growth, are not well documented.

Gilthead sea bream (*Sparus aurata* L.) is one of the most important farmed fish species in Mediterranean aquaculture. Studies with regard to phospholipid requirements have focused mainly on the larval stages, showing different levels for marine and plant-origin phospholipids (Saleh et al., 2015), and a better performance for the first one (Betancor et al., 2012; Saleh et al., 2013). For juvenile stages, only very limited information exists with regard to phospholipid requirements (Benedito-Palos et al., 2008), while the information is largely missing when feeding diets with a low-FM content. Therefore, the objectives of the present study were to investigate the effects of two different sources of phospholipids, from marine and plant origin, included at three dietary levels, on growth performance, feed utilization, liver histology, and fatty acid profile of polar and neutral lipids in the liver, muscle and intestine of gilthead sea bream juveniles.

2. Materials and methods

2.1. Fish, experimental diets and sampling

Gilthead seabream juveniles were obtained from a local fish farm. Upon transfer to the Hellenic Centre for Marine Research facilities in Aghios Kosmas (Greece), fish were stocked in 24 tanks of 170 L capacity (30 fish per tank) in an open-circulation system of well-oxygenated sea water and acclimatized for 2 weeks before the start of the feeding experiment. During the experiment, the photoperiod was 12 Light:12-Dark, seawater salinity was 38 g L^{-1} and temperature was at 25.2 ± 1 °C.

Fish of initial weight of 12.05 ± 0.41 g were fed either with a high-FM (65%) as the positive control (FM) or with low-FM diets (10% inclusion), supplemented with two different source of phospholipids: 1) a marine-origin krill oil (K) and 2) a plant-origin soya lecithin (S). Three levels of the phospholipid source were included in the diets: 0.53% (K1 and S1 diets), 1% (K2 and S2 diets) and 2% (K3 and S3 diets). A low-fish meal diet (10% inclusion) without addition of any phospholipid source was used as negative control (KS0). The eight diets were isonitrogenous and isoenergetic (48% crude protein, 18% crude fat), containing a similar composition of raw materials. The diets were formulated and produced by BioMar (BioMar Process Innovation Technology Center, Denmark) by extrusion (Clextral 45 BCE, twin screw, 7-sectons) at a maximal temperature of 115 °C to obtain 2 pellet sizes (1.9 and 3 mm). The detailed diet composition is given in Table 1, while the fatty acid profiles and lipid classes of the diets are given in Table 2 and Table 3. Each diet was supplied by hand *ad libitum* twice per day (09:00 and 15:00 h) to triplicate tanks per diet, for 70 days. Feed conversion, specific growth rate, daily growth index, feed and protein utilization were calculated at the end of the on-growing period, while feed intake and mortalities were determined daily.

At the beginning of the trial and every two weeks, all fish were anaesthetized using diluted clove oil and batch-weighed in groups of five. At the end of the feeding period, all fish were weighed individually. Ten fish from each tank were sampled for gross body composition and kept at -20 °C until analysis. Liver samples from three fish per tank (9

Table 1
Diet composition of the experimental diets.

Ingredients (%)	FM	KS0	K1	K2	K3	S1	S2	S3
Fish meal, SA 68%	64.8	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Soy protein concentrate (Crude protein 60%)		7.50	7.50	7.50	7.50	7.50	7.50	7.50
Corn Gluten (Crude protein 60%)		17.5	17.5	17.5	17.5	17.5	17.5	17.5
Pea Protein (Crude protein 75%)		17.7	17.7	17.7	17.7	17.7	17.7	17.7
Wheat Gluten		10.7	10.7	10.7	10.7	10.7	10.7	10.7
Sunflower cake	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Wheat	16.7	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Fish oil (STD 18%)	5.10	5.40	4.40	3.10	0.60	5.40	5.40	5.40
Rapeseed oil	7.60	8.10	8.10	8.10	8.10	7.10	5.80	3.30
Krill oil[a]			1.00	2.30	4.80			
Soy lecithin[b]						1.00	2.30	4.80
Minerals – Vitamin mix[c]	0.30	0.50	0.50	0.50	0.50	0.50	0.50	0.50
% Gross composition								
Dry matter	92.9	92.7	92.8	93.0	93.0	93.1	93.1	93.0
Protein	46.9	47.1	46.3	46.3	47.1	46.1	46.3	47.5
Fat	18.2	16.1	17.1	17.2	18.2	16.9	17.6	16.5
Ash	11.4	6.6	6.7	6.8	6.8	6.7	6.7	6.8
Nitrogen free extract (NFE)	24.8	29.0	28.7	28.4	29.0	29.5	28.2	28.8
Energy (MJ/Kg)	22.6	23.1	23.0	22.9	22.8	22.9	23.1	23.0

[a] Kril Oil Aker Biomarine, Norway.
[b] Soy lecithin, Cargill, Germany.
[c] Mineral and Vitamin mix contains (mg/kg of premix, except differently stated) vitamins A (1470,96 IU/kg), B1 (thiamine, 356), B2 (riboflavin, 1108), niacin (3310), B5 (pantothenic acid, 2290), B6 (637), biotin (18), folic acid (377), B12 (34), C (ascorbic acid, 10,681), D3 (261,682 IU/kg), E (13677), K3 (1312), iodine (calcium iodate, 89), cobalt (125), copper (cupric sulphate pentahydrate, 433), selenium (sodium selenite, 31), iron (ferrous sulphate monohydrate, 4358), manganese (manganous oxide, 1606), zinc (zinc oxide, 8934), taurine (327.1 g/kg), histidine (181.6 g/kg), and cholesterol (150.2 g/kg). They all added up to 776.9 g/kg and were completed to 1000 with a carrier.

Table 2
Fatty acids composition (% of total fatty acids) of the experimental diets fed to gilthead seabream juveniles.

	FM	KS0	K1	K2	K3	S1	S2	S3
Saturated	20.6	17	16.5	16.24	16.8	16.8	19.5	21.9
Mono-unsaturated	47.3	50.9	51.2	51	50.3	49.5	45.5	39.5
ω-9	43.9	45.2	46.5	47.3	46.5	44.2	38.8	34.7
ω-6	13	17.2	17	17.3	17.6	19.2	19.8	24.1
ω-3	18.8	15	15.2	15.5	15.4	14.5	15.3	14.4
ArA	0.43	0.3	0.31	0.25	0.27	0.29	0.28	0.33
EPA	6.74	4.22	4.26	4.5	4.63	4.23	4.51	4.56
DHA	5.49	3.27	3.43	3.34	2.81	3.16	3.88	3.55
ω-3/ω-6	1.44	0.87	0.89	0.9	0.87	0.76	0.77	0.6

ArA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

Table 3
Lipid classes (%) of the experimental diets fed to gilthead seabream juveniles.

	FM	KS0	K1	K2	K3	S1	S2	S3
SM					3.3			
PC	8.4	2.6	3.8	9.3	14.5	4.7	5.2	7.0
PS + PI	1.5					1.8	2.1	4.0
PE	2.91	0.8	1.3	2.4	3.4	4.1	5.1	7.4
∑ Polar lipids	**12.8**	**3.4**	**5.1**	**11.7**	**21.1**	**10.7**	**12.4**	**18.4**
MAG	2.17	3.04	3.30	4.00	3.23	3.69	3.85	3.50
C	13.0	12.3	11.6	10.5	11.1	13.2	11.0	11.3
TAG	67.4	75.1	74.5	69.0	60.8	68.4	68.8	63.5
CE	4.65	6.13	5.54	4.87	3.67	4.05	3.89	3.32
∑ Neutral Lipids	**87.2**	**96.6**	**94.9**	**88.3**	**78.9**	**89.3**	**87.6**	**81.6**

Abbreviations: Polar lipids – SM, sphingomyelin, PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; SL, sphingolipids.
Neutral lipids – MAG, monoacylglycerol; C, cholesterol; TAG, triacyglycerol; CE, cholesterol ester.

samples/ treatment) were excised for fat determination. Liver, muscle and intestine samples from three fish per tank (9 samples/treatment) were pooled and immediately stored at -70 °C for fatty acid profile analysis. Samples of liver were removed from 3 fish of each replicate tank (9 samples/treatment), fixed in 10% buffered formalin and processed for histological observations.

2.2. Biochemical analysis

Proximate composition analysis of the experimental diets and whole fish were determined according to AOAC (2005). The crude fat content of the whole body was determined using the Soxhlet extraction method. Crude fat in the extruded diets was determined as the ether extract after acid hydrolysis. Acid hydrolysis was used to facilitate fat extraction from the extruded diets by breaking of covalent and ionic bonds of lipids to proteins and carbohydrates. Ash content was measured by ignition of samples at 500 °C overnight. Liver fat was determined gravimetrically according to Folch et al. (1957).

For the tissue and experimental diets fatty acid profiles, lipid extraction and separation into neutral and polar lipid fractions were performed as previously described by Fountoulaki et al. (2003). Briefly lipid extraction was made according to Folch et al. (1957). Extracts were dried under vacuum and weighed for tissue fat determination, then were dissolved in chloroform and separated into polar and neutral fractions using amino cartridges (Biotage CatNo 470–0200-C ISOLUTE NH_2g/6 mL) according to Kim and Salem Jr (1990). Fatty acids methyl esters were prepared by trans-esterification with anhydrous methanol containing 2% sulphuric acid, for 16 h at 50 °C under nitrogen (Christie, 1989; Kok Leong, 1983). Fatty acids methyl esters were separated by gas chromatography (GC), on a Varian Model 3300 gas chromatograph equipped with flame ionisation detector, as described by Fountoulaki et al. (2009). Lipid classes (one replicate per sample, three per diet) were separated by double-development, high performance thin-layer chromatography (HPTLC) using 10 × 10 cm plates (VWR, Lutterworth, UK), according to Henderson and Tocher (1987). Total lipid samples (1–2 μg) were applied as 3 mm origins and the plates developed in methyl acetate/isopropanol/chloroform/ methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) to 5.2 cm for the determinations of polar lipids. Excess solvent was evaporated via air drying and vacuum desiccation; plates were developed to 9.5 cm using a solvent mixture containing isohexane/diethyl ether/acetic acid (80:20:1, by vol.), before termination and drying as described above, for the determination of neutral lipids. Lipid classes were visualized by spraying with 3% (*w/v*) aqueous cupric acetate containing 8% (*v/v*) phosphoric acid and charring plates at 160 °C for 20 min. Lipid classes were quantified by densitometry using a CAMAG-3 TLC Scanner (version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with win CATS software (Planar Chromatography Manager, version 1.2.3).

2.3. Histology

Standard histological methods were used. Briefly, tissues were transferred to ethanol 70%, dehydrated in ascending ethanol concentrations to 100%, embedded in paraffin and sections (5 mm) (microtome Leica RM 2255, Nussloch, Germany) were stained with haematoxylin and eosin (Leica Auto Stainer XL, Nussloch, Germany). The sections were examined under light microscopy (Olympus VANOX-T, NJ, USA) equipped with a digital camera (Infinity, Lumenera, Ontario, Canada). Images were processed by Image analysis software (Digital Image Systems, Athens, Greece). Liver sections were evaluated on lipid degeneration level and the integrity of hepatocytes as described by Kokou et al. (2017).

2.4. Statistical analysis

All values are presented as means ± standard error of the mean (SEM) and differences present at 5% level were considered significant. Normal distribution and homogeneity of variance were checked using Kolmogorov-Smirnov and Levene tests, respectively. One-Way ANOVA and Tukey's post hoc test were performed. When data were not normally distributed, Kruskal-Wallis and Mann-Whitney *U* test were applied. A Principal component analysis was applied to show the profiles of fatty acids between the different tissues and treatments; the significance of clustering was verified with One-way Permanova. Pearson correlation (r) was applied to understand the relationship between the phospholipid levels and the lipid profiles in the different tissues.

3. Results

3.1. Growth performance and feed utilization

At the start of the experiment, there were no significant differences between means and range of gilthead seabream initial body weights (IBW) for the eight diets (Table 4). No mortalities were observed during the whole experimental period.

All growth parameters were affected by the inclusion of phospholipids in the low-FM diets (Table 4). The lowest growth was observed by the KS0 diet (44.9 g final individual body weight, FBW) where no phospholipids were added, while the highest growth was observed in the FM diet (51.3 g FBW). Addition of both marine and plant origin phospholipids resulted in a growth improvement, already from the lowest inclusion level for the marine phospholipids (0.5%), while for the plant phospholipid from the second highest level (1%). The same was reflected also for the specific growth rate (SGR) and the daily growth index (DGI). There was a tendency for better growth in the fish fed on the krill supplemented diets compared to soya lecithin diets, however this was

Table 4
Growth and feed utilization of gilthead seabream juveniles fed with the experimental diets.

	FM	KS0	K1	K2	K3	S1	S2	S3
IBW (g)	12.2 ± 0.5	11.8 ± 0.5	12.4 ± 0.4	12.1 ± 0.1	11.7 ± 0.6	12.0 ± 0.3	12.4 ± 0.3	11.8 ± 0.1
FBW (g)	51.3 ± 1.5^{a}	44.9 ± 1.0^{b}	49.6 ± 1.7^{a}	48.5 ± 1.5^{ab}	48.1 ± 2.7^{ab}	47.1 ± 0.7^{ab}	49.5 ± 1.7^{a}	48.2 ± 1.3^{ab}
WI	44.9 ± 1.0^{a}	33.0 ± 1.2^{c}	37.1 ± 1.3^{ab}	36.4 ± 1.5^{abc}	36.3 ± 2.2^{abc}	35.1 ± 0.6^{ab}	37.2 ± 1.4^{bc}	36.4 ± 1.2^{abc}
[1]SGR	2.2 ± 0.04^{b}	2.0 ± 0.08^{a}	2.1 ± 0.02^{ab}	2.1 ± 0.06^{ab}	2.2 ± 0.04^{ab}	2.1 ± 0.03^{b}	2.1 ± 0.03^{b}	2.2 ± 0.0^{ab}
[2]DGI	2.2 ± 0.04^{b}	2.0 ± 0.07^{b}	2.1 ± 0.04^{a}	2.1 ± 0.06^{a}	2.1 ± 0.06^{ab}	2.0 ± 0.02^{ab}	2.1 ± 0.0^{ab}	2.1 ± 0.04^{ab}
[3]FE	89.2 ± 2.3	82.6 ± 4.3	87.4 ± 1.2	86.7 ± 1.5	87.6 ± 2.2	86.1 ± 2.4	86.9 ± 1.1	87.2 ± 2.3
[4]FCR	1.12 ± 0.03	1.21 ± 0.06	1.14 ± 0.02	1.15 ± 0.02	1.14 ± 0.03	1.16 ± 0.03	1.15 ± 0.01	1.15 ± 0.03
[5]PER	1.9 ± 0.05	1.7 ± 0.09	1.9 ± 0.03	1.9 ± 0.03	1.8 ± 0.05	1.9 ± 0.05	1.9 ± 0.02	1.8 ± 0.05
[6]ANPU	33.9 ± 0.6	30.4 ± 1.3	33.5 ± 0.7	33.5 ± 1.2	32.0 ± 2.9	33.7 ± 0.6	33.9 ± 0.8	32.9 ± 2.2
[7]DFC (%)	2.12 ± 0.04	2.17 ± 0.06	2.11 ± 0.04	2.14 ± 0.01	2.13 ± 0.08	2.14 ± 0.06	2.16 ± 0.02	2.14 ± 0.04

Data are presented as means of three replicates ± S.D. Different letters at the same column indicate significant difference between diets ($P < 0.05$).
[1]SGR (Specific growth rate (% day^{-1}) = 100× (ln final weight - ln initial weight)/days; [2] DGI (Daily growth index) = (Final BW^{3})-(Initial BW^{3})*100/days of rearing; [3] FE (Feed efficiency) = Weight increase*100/feed consumed per fish (g);[4] FCR (Feed conversion ratio) = consumed dry feed (g) / weight gain (g);[5]PER (Protein efficiency ratio) = weight increase g/ protein consumed g; [6]ANPU (Apparent net protein utilization = 100*(final body protein/fish - initial body protein/fish)/total protein consumed/fish; [7]Daily feed consumption = {(feed intake g/fish*100) / (Initial body weight/fish * final body weight/fish)$^{0.5}$)}/days of feeding.

not significant ($P > 0.05$). Feed efficiency (FE) was higher in the FM diet (89.2%) and lower in the KS0 diet (82.6%). The addition of both source of phospholipids in low-FM diets improved feed utilization (86–87.5%) and FCR, although differences were not significant because of high variations between replicate tanks. A similar effect was observed also for protein utilization (ANPU), with both phospholipid sources resulting in an improvement and values closer to the FM diet.

3.2. Whole body composition

Body protein and fat content were increased compared to the initial population, as expected, but no differences were observed between the different dietary treatments (Table 5). Liver fat content was lower in the FM diet and higher in the rest of the diets. Although there was not significant differences between the phospholipid supplemented diets and the KS0 diet, there was a tendency of a reduction in fat content at the higher inclusion phospholipid level for both sources of phospholipids.

3.3. Fatty acid profile and lipid classes of the experimental diets

Fatty acid composition of the experimental diets is shown in Table 2. FM-diet differed from the rest of the diets in the content of ω-6, being lower from all phospholipid-supplemented diets, but mostly from the plant-origin phospholipid diets. Levels of ω-3, EPA and DHA were also higher in the FM-diet compared to the phospholipid supplemented diets, while KS0 diet was formulated to have the same levels of ω-3, EPA and DHA with all the phospholipid-supplemented diets, in order to cover dietary requirements of essential fatty acids. This was in order not to attribute possible differences between those diets to ω-3 deficiencies. Some differences were observed in saturated, monounsaturated and ω-9, levels between the FM and the rest of the diets, but this was expected due to the raw materials used in the formulation of the diets.

Analysis on the lipid classes of the experimental diets (Table 3) showed a high content of neutral lipids, ranging from 78.9% (K3 diet) to 96.6% (KS0 diet), and a lower content of polar lipids, ranging from 3.4% (KS0 diet) to 18.4% (S3 diet). The addition of phospholipids increased the content of polar lipid up to 21.1% by the marine source, and up to 18.4% by the plant source. Phosphatidyl choline (PC) was the predominant phospholipid in all diets, followed by phosphatidyl ethanolamine, while phosphatidyl serine and phosphatidyl inositol (PS + PI) were present only in the FM and the plant phospholipid diets (Table 3).

With regard to the neutral lipids, opposite to the polar lipids, the addition of phospholipids decreased their content from 96.6% (KS0 diet) to 78.9% and 81.6% for the marine and plant origin, respectively. Concerning the neutral lipids triacylglycerol (TAG) was the most abundant lipid in all diets, ranging from 60.8% (K3 diet) to 75.1% (KS0 diet), followed by cholesterol (C), ranging from 10.5% (K2 diet) to 13.0% (FM diet). Monoacylglycerol (MAG) and cholesterol ester (CE) were found to have a lower concentration (below 6%).

3.4. Fatty acid profile and lipid classes in the liver

Analysis of the polar and neutral fatty acids in the liver showed significant differences between high and low-FM diets ($P < 0.05$; Fig. 1A and B; Supplementary Table 1 and 2).

PCA analysis showed that all low-FM treatments clustered close to each other based on the polar lipid (PL) profiles, except the S3 diet, which clustered separately, with the differences driven by the saturates and the ω-9 fatty acid profiles (Fig. 1A). Overall, PL were characterized by high levels of saturates, ranging from 41% to 48% and low levels of arachidonic acid, ranging from 0.8% to 1.7% (Table S1). A significant increase with the addition of marine phospholipid in the diets was found for monounsaturates coming from ω-9, while the opposite trend was observed for the plant phospholipid inclusion ($P < 0.05$). The ω-9 levels were significant low in the FM diet and higher in the phospholipids supplemented diets except from diet S3, where level was similar to FM diet. ArA, EPA and DHA levels were significantly higher in the FM diet and lower in the rest of the diets reflecting the dietary levels and the same was observed for the ω-3/ω-6 ratio ($P < 0.001$).

PCA analysis showed that all low-FM treatments clustered further from the high-FM diet based on the neutral lipid (NL) profiles (Fig. 1B). However, plant and marine phospholipid-supplemented diets clustered separately, with the differences driven by the saturates and the ω-6 profiles (Fig. 1B). Overall, neutral lipids were characterized by high levels of monounsaturates, ranging from 45.6% to 52.3% expressed mostly as ω-9, which ranged from 42.6% to 48.8% (Table S2). ArA,

Table 5
Whole body composition of gilthead seabream juveniles fed with the experimental diets.

%	Initial population	FM	KS0	K1	K2	K3	S1	S2	S3
Moisture	73.3	64.8 ± 0.7	65.9 ± 0.7	64.6 ± 1.1	64.8 ± 1.5	65.9 ± 1.0	64.4 ± 0.7	64.4 ± 0.2	64.8 ± 1.0
Fat	8.0	14.2 ± 0.5	13.3 ± 0.3	14.4 ± 0.6	14.2 ± 0.8	13.8 ± 0.1	14.4 ± 0.3	14.4 ± 0.2	14.1 ± 0.3
Protein	14.3	17.0 ± 0.5	16.6 ± 0.1	16.8 ± 0.5	17.0 ± 0.5	16.6 ± 0.8	17.1 ± 0.4	17.1 ± 0.4	16.9 ± 0.5
Ash	3.9	3.8 ± 0.1	3.6 ± 0.1	3.9 ± 0.1	3.9 ± 0.4	3.7 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.9 ± 0.1

Data are presented as means of three replicates ± S.D. Different letters at the same column indicate significant difference between diets.

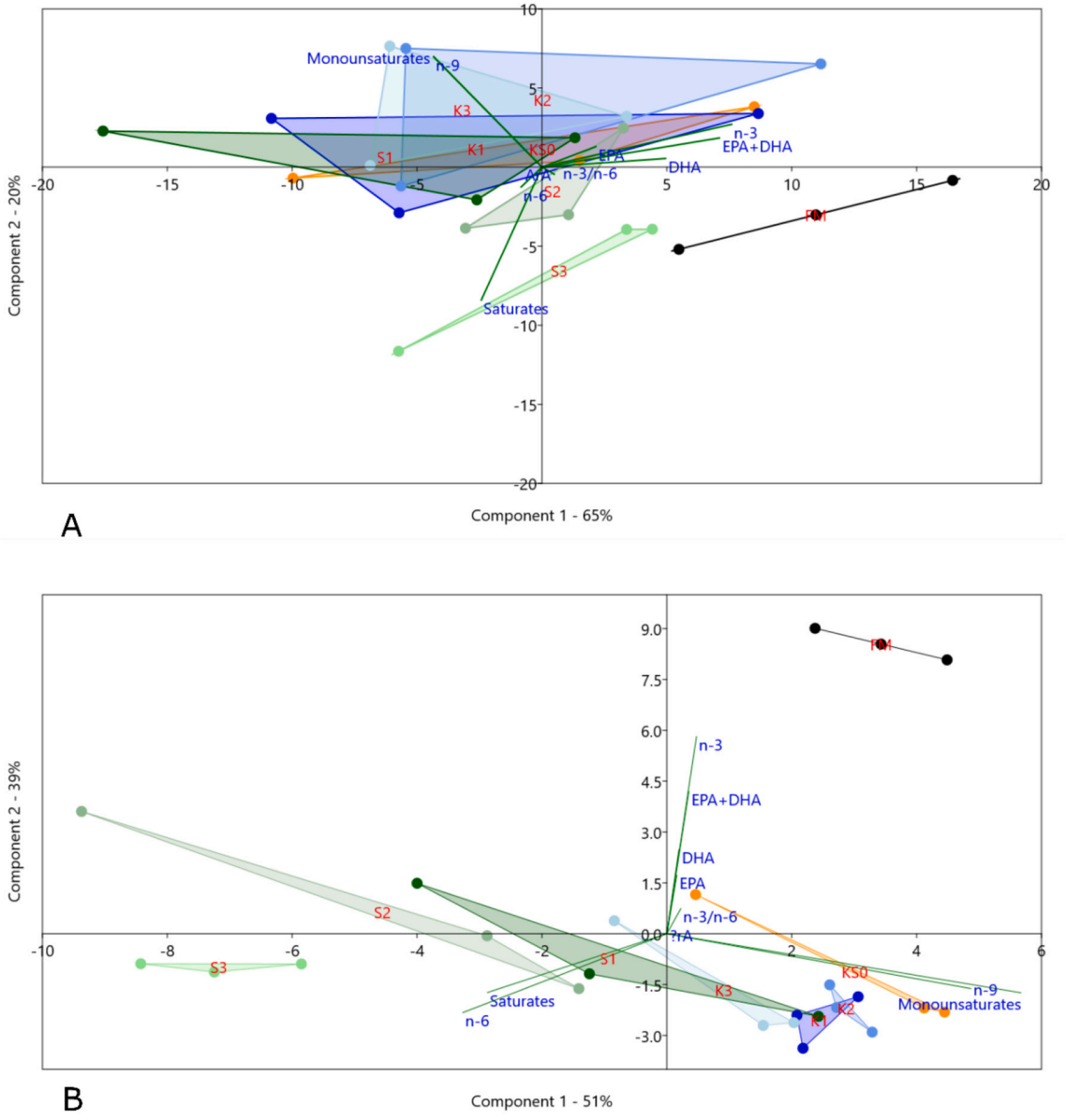

Fig. 1. Principal component analysis showing A. the polar and B. the neutral lipids in the liver of gilthead seabream fed with different phospholipids sources. A significant diet effect was found in the neutral lipid composition (Kruskal-Wallis test, *P*-value <0.05), as also indicated by the different clustering between the plant (green) and marine phospholipids (blue). All low-FM diets clustered further closer, compared to the high-FM diet (black). The S3 diet clustered further from the rest of the diets based on the polar lipid profiles, with the differences driven mainly by the saturates profiles, as indicated by the triplots (green lines) in the PCA. For the neutral lipids, saturates and ω-6 profiles differentiated the plant-phospholipid diets from the rest, as indicated by the triplot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Liver lipid classes in sea bream juveniles fed on different phospholipid sources.

	FM	KS0	K1	K2	K3	S1	S2	S3	P-value	SEM
PC	18.3^{b}	13.2^{a}	12.2^{a}	11.5^{a}	10.8^{a}	11.8^{a}	10.6^{a}	10.3^{a}	***	0.96
PS + PI	4.06^{b}	2.43^{a}	2.57^{a}	2.39^{a}	2.54^{a}	2.76^{a}	2.39^{a}	2.32^{a}	**	0.29
PE	9.06	7.17	7.40	6.98	7.28	7.23	6.74	5.98	ns	0.62
∑Polar	**31.4^{b}**	**22.8^{a}**	**22.2^{a}**	**20.9^{a}**	**20.6^{a}**	**21.8^{a}**	**19.8^{a}**	**18.6^{a}**	**	1.78
MAG	7.2^{ab}	6.6^{a}	11.0^{abc}	12.2^{c}	11.6^{bc}	13.1^{c}	11.5^{bc}	11.3^{abc}	*	0.97
C	6.92^{c}	5.27^{bc}	3.85^{ab}	5.19^{bc}	3.87^{ab}	4.03^{ab}	3.26^{ab}	2.72^{a}	**	0.48
TAG	49.3^{a}	60.2^{b}	59.0^{ab}	58.8^{ab}	57.5^{ab}	56.6^{ab}	60.2^{b}	61.3^{b}	*	2.21
CE	5.10^{bc}	5.09^{bc}	3.92^{ab}	2.88^{a}	6.39^{c}	4.42^{b}	5.22^{bc}	6.08^{c}	***	0.28
∑Neutral	**68.6^{a}**	**77.2^{ab}**	**77.8^{b}**	**79.1^{b}**	**79.4^{b}**	**78.2^{b}**	**80.2^{b}**	**81.4^{b}**	**	1.78

Different letters at the same raw means that there is significant differences between diets, ns = not significant $P > 0.1$; *P < 0.05; **$P < 0.01$; ***$P < 0.001$.
Abbreviations: Polar lipids – PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine.
Neutral lipids – MAG, monoacylglycerol; C, cholesterol; TAG, triacylglycerol; CE, cholesterol ester.

levels were low ranging from 0.1% to 0.4%. A significant increase in saturates was observed with the inclusion of plant phospholipids, while the opposite trend was observed in the levels of monounsaturates with the inclusion of plant phospholipid source. ω-9 levels, decreased with the inclusion of plant phospholipids, while levels of ω-6, increased at the highest levels of inclusion for both phospholipids' sources ($P < 0.001$). Levels of ω-3, ArA, EPA and DHA, were low compared to that in the polar lipids.

Lipid classes in the liver (Table 6) showed significant differences between the FM diet and the rest of the diets, as this was also reflected by the PCA analysis. Total polar lipids were significantly higher in the FM diet (31.4%) and lower in the rest of the diets, ranging from 18.6% to 22.8%. The predominant polar lipid was PC, followed by PE and PS + PI; their content was higher in the FM diet, but significant differences were evident only for PC and PS + PI. No correlations were observed with dietary polar lipid concentrations. In the neutral lipid classes, FM diet showed the lowest values (68.6%), while the un-supplemented and supplemented diets showed higher values ranging from 81.4% to 77.2%. Differences were significant between the FM and the rest of the diets for all neutral lipid classes. The predominant neutral lipid was TAG, followed by the rest of the neutral lipid classes in the following order: TAG>MAG > CE > C. TAG was significantly lower in the FM diet and higher in the rest of diets. A decreasing tendency was observed in the marine phospholipid supplemented diets, compared to the plant ones. C was higher in the FM and the KS0 diet, whilst the supplementation of both source of phospholipids at the highest inclusion level decrease significantly the % of C.

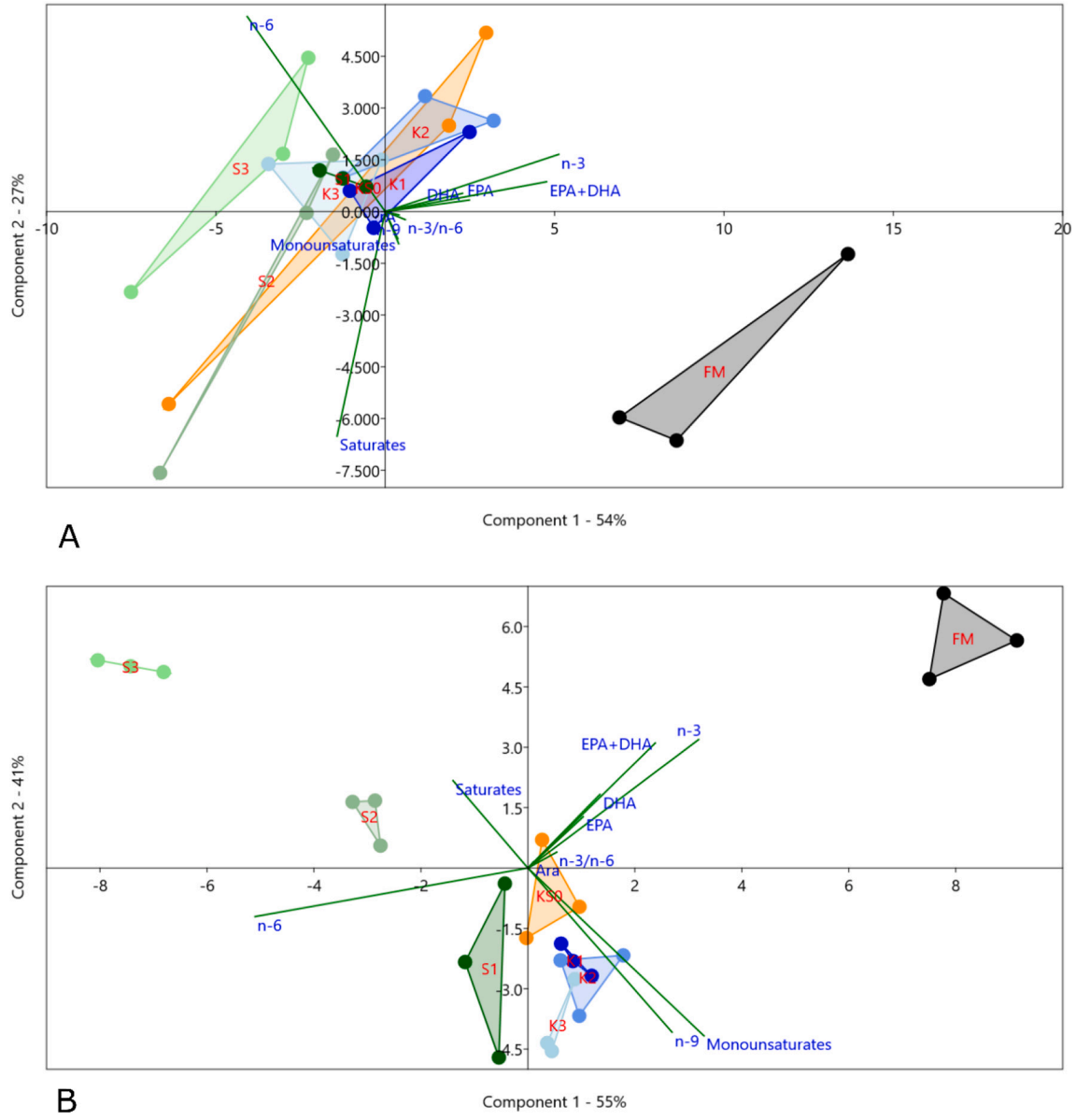

Fig. 2. Principal component analysis showing A. the polar and B. the neutral lipids in the muscle of gilthead seabream fed with different phospholipids sources. A significant diet effect was found in both the polar and neutral lipid composition (Kruskal-Wallis test, P-value <0.05), as also indicated by the different clustering between the plant (green) and marine phospholipids (blue), compared to the FM diets (black). Moreover, for the neutral lipids there was also a clustering according to the type of phospholipids in the diets – plant (green) vs marine (blue). The differences were driven mainly by the ω-6 and ω-9 profiles in the diets (Kruskal-Wallis test, P-value <0.01), as also indicated by the triplots (green lines) in the PCA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Fatty acid profile and lipid classes in the muscle

Analysis of the polar and neutral fatty acids in the muscle showed significant differences between the diets (Fig. 2; Table S3 and S4).

PCA analysis showed that all low-FM treatments clustered close to each other based on the polar lipid profiles (Fig. 2A). Polar lipids (PL) were characterized by high levels of saturates, ranging from 36.7% to 42% and low levels of ArA, ranging from 0.4% to 1.2% (Table S3). Monounsaturates mostly as ω-9, tended to decrease in the plant-based diets in both phospholipid sources at the highest inclusion level. ω-6, was significantly low in the FM diet while EPA and DHA was higher. Significant difference was found between the FM and the rest of the diets reflecting dietary levels.

PCA analysis showed that all low-FM treatments clustered further from the high-FM diet based on the neutral lipid (NL) profiles (Fig. 2B). However, plant and marine phospholipid-supplemented diets clustered separately, with the differences driven by the saturates and the ω-6 profiles (Fig. 2B). Neutral lipids were characterized by high levels of monounsaturates, ranging from 39.9% to 48.0% and ω-9, ranging from 37.7% to 44.6%, and low levels of ArA, ranging from 0.1% to 0.4% (Table S4). A significant increase in saturates and ω-6 was observed with the inclusion of plant phospholipids. Inclusion of marine phospholipid increased the levels of monounsaturates at the highest level of krill inclusion, while the opposite was observed in the soy lecithin inclusion. ω-3, EPA and DHA, were significantly low in the KS0 diets as well as in all phospholipid-supplemented diets.

Lipid classes in the muscle tissue (Table 7) showed significant differences between diets. Total polar lipids were lower in the FM (27.3%) and the KS0 diets (27.1%), and higher in the phospholipid-supplemented diets, ranging from 28.7% to 36.7%. The predominant polar lipid was PC, followed by PE and PS + PI. Significant differences were observed in the PC and PE; the highest krill supplementation level resulted in significant higher muscle concentration in both PC and PE, while in the soy lecithin supplementation, values were higher in all inclusion levels. No correlations were observed between dietary polar lipid concentrations and muscle polar lipid concentrations. In the neutral lipid classes, the two control diets FM (72.7%) and KS0 (72.9%), showed the highest values, while the supplementation of both sources of phospholipids resulted in decreasing the levels in all supplemented diets, ranging from 63.3% to 71.2%. Differences were significant between diets K3, S1, S2 S3 and the two control diets FM and KS0. The predominant neutral lipid was TAG, followed by the rest of the neutral lipid classes in the following order TAG>C > MAG. TAG was higher in the FM and KS0 diets and lower in the rest of diets. The levels were significantly decreasing at the higher marine phospholipid level (K3), while the addition of plant phospholipids resulted in further decrease already from the lowest inclusion level (S1 diet).

3.6. Fatty acid profile and lipid classes in the intestine

Analysis of the polar and neutral fatty acids in the intestine indicated significant differences between the diets (Fig. 3; Table S5 and S6), mainly in the neutral lipids.

PCA analysis showed that all low-FM treatments clustered close to each other based on the polar lipid profiles (Fig. 3A). Polar lipids (PL) were characterized by high levels of saturates, ranging from 40.3% (K2 diet) to 48% (FM diet) and low levels of ArA, ranging from 0.6% to 0.9% (Table S5). A significant increase for ω-6 ($P < 0.01$) was found with phospholipid inclusion, while no differences were found in ω-3, EPA and DHA.

PCA analysis showed that all low-FM treatments clustered further from the high-FM diet based on the neutral lipid (NL) profiles (Fig. 3B). However, plant and marine phospholipid-supplemented diets clustered separately, with the differences driven by the ω-6 profiles (Fig. 3B). Neutral lipids in the intestine were characterized by significant differences between different dietary groups (Table S6). High levels of monounsaturates ranging from 41.9% (S3 diet) to 52.8% (K1 diet) and ω-9, ranging from 39.1% (S3 diet) to 49.5% (K1 diet), and low levels of ArA, ranging from 0.1% to 0.3%. In the low FM based diets levels of ω-6 increased, while ω-3 decreased. Values for EPA and DHA decreased, but at highest inclusion levels for both source of phospholipids values increased.

Lipid classes in the intestine (Table 8) showed the presence of sphingomyelin (SM), while this phospholipid was absent in the rest of the tissues examined. Levels of SM ranged from 0.7 (S1 diet) to 1.4% (K3 and S3 diets). PS + PI showed significant differences between diets, with a decrease in the highest levels of plant phospholipid inclusion (S2 and S3 diets), while for PE, the opposite trend was observed where a significant increase was observed by the increase of the plant phospholipids. No correlations were observed between dietary polar lipid concentrations and intestine polar lipid concentrations. In the total neutral lipid classes, there were no significant differences between diets and values ranged from 75.6 (S3 diet) to 80.7% (S2 diet). The predominant neutral lipid was TAG, followed by the rest of the neutral lipid classes in the following order: TAG>C > MAG. CE was present only in the two control diets and the marine phospholipid supplemented diet, while no CE was found in the plant phospholipid supplemented diet. Differences were significant in the C and TAG contents, with C being higher in the higher inclusion level for both phospholipids' sources (12.3% and 11.6%, respectively), while TAG was lower in higher inclusion level for both phospholipids' sources.

3.7. Fatty acid profile comparison between tissues

Polar lipids, in general, were characterized by higher levels of saturates and ω-3, and lower levels of monounsaturates and ω-9, compared to neutral lipids. Polar lipids from examined tissues were more conservative and correlations between dietary fatty acids and tissue fatty acids were either weak (r 0.7–0.9) or non-existing (Table 9). Intestine polar lipids were not affected by dietary treatments, and differences between diets were present only in the ω-6 levels and consequently in the ratio ω-3/ω-6, indicating differences between the metabolic functions of the

Table 7
Muscle lipid classes in gilthead seabream juveniles fed on different phospholipid sources.

	FM	KS0	K1	K2	K3	S1	S2	S3	Pvalue	SEM
PC	14.4^{abc}	11.9^{a}	13.0^{ab}	14.0^{abc}	16.5^{bcd}	17.7^{cd}	18.0^{d}	16.8^{bcd}	***	0.50
PS + PI	4.08	5.62	6.25	6.14	6.67	6.62	5.69	6.28	ns	0.22
PE	8.90^{a}	9.56^{ab}	9.51^{ab}	9.90^{abc}	12.3^{d}	12.3^{d}	11.0^{bcd}	11.6^{cd}	***	0.28
∑Polar	**27.3^{a}**	**27.1^{a}**	**28.7^{ab}**	**30.1^{abc}**	**35.6^{cd}**	**36.7^{d}**	**34.7^{bcd}**	**34.6^{bcd}**	***	0.86
MAG	3.41^{c}	3.45^{c}	5.04^{de}	4.21^{cd}	3.08^{bc}	5.58^{e}	1.50^{a}	1.64^{ab}	***	0.29
C	6.30^{ab}	5.83^{a}	7.39^{abc}	8.24^{c}	7.69^{bc}	8.90^{c}	8.47^{c}	7.68^{bc}	***	0.23
TAG	62.9^{cd}	63.6^{d}	58.8^{bcd}	57.5^{bcd}	53.7^{ab}	48.8^{a}	55.3^{abc}	55.9^{abc}	***	1.05
∑Neutral	**72.7^{d}**	**72.9^{d}**	**71.2^{cd}**	**69.9^{bcd}**	**64.4^{ab}**	**63.3^{a}**	**65.3^{abc}**	**65.3^{abc}**	***	0.86

Neutral lipids – MAG, monoacylglycerol; C, cholesterol; TAG, triacyglycerol.
Different letters at the same raw means that there is significant differences between diets, ns = not significant P > 0.1; *P < 0.05; **P < 0.01; ***P < 0.001.
Abbreviations: Polar lipids – SM, sphingomyelin, PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine.

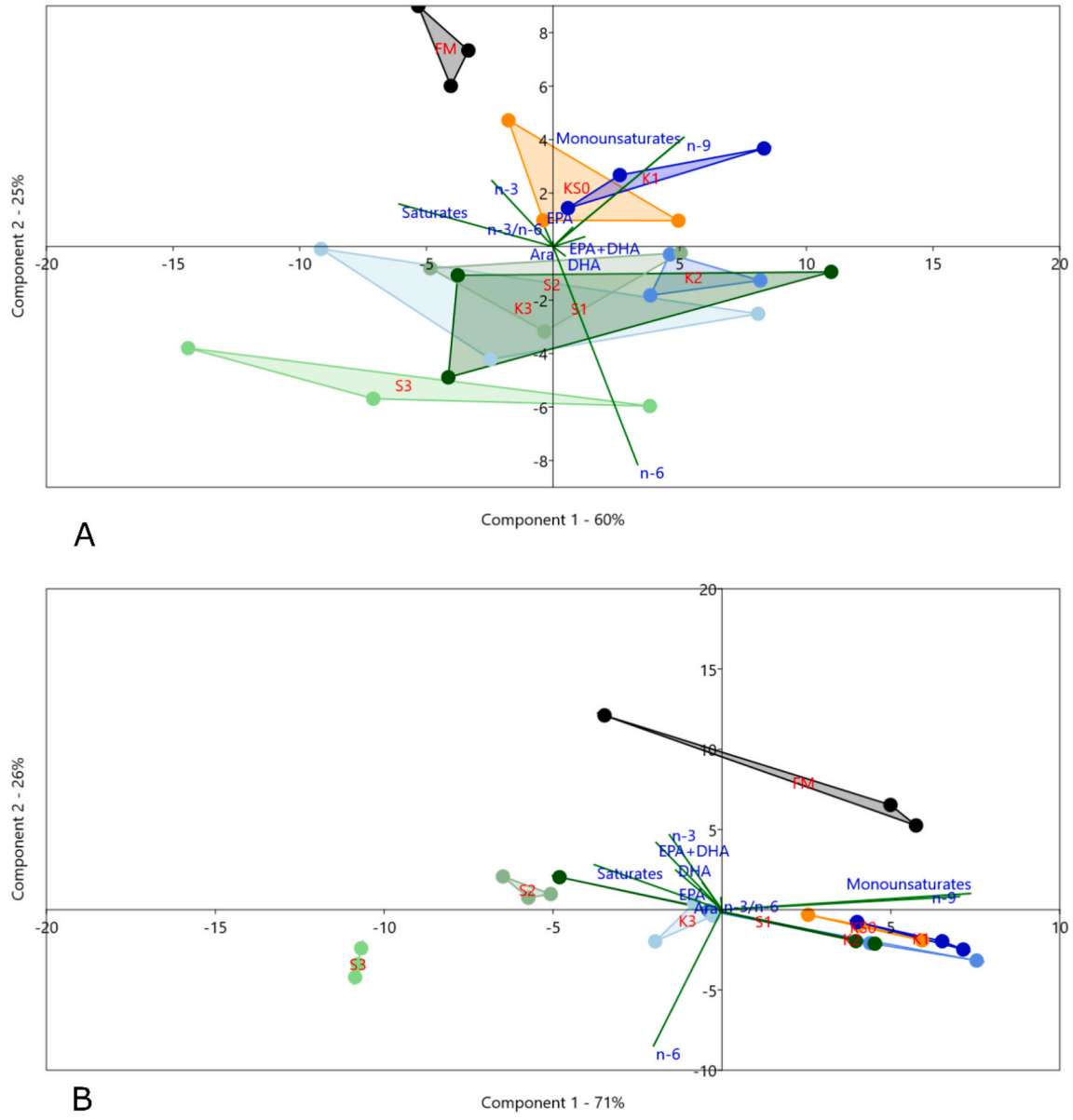

Fig. 3. Principal component analysis showing A. the polar and B. the neutral lipids in the intestine of gilthead seabream fed with different phospholipids sources. A significant diet effect was found in both the polar and neutral lipid composition (Kruskal-Wallis test, P-value <0.05), as also indicated by the different clustering between the plant (green) and marine phospholipids (blue), compared to the FM diets (black). Moreover, for the neutral lipids there was also a clustering according to the type of phospholipids in the diets – plant (green) vs marine (blue). The differences were driven mainly by the ω-6 profiles in the diets (Kruskal-Wallis test, P-value <0.01), as also indicated by the triplots (green lines) in the PCA, which separated S2 and S3 diets from the rest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 8
Intestine lipid classes in gilthead seabream juveniles fed on different phospholipid sources.

	FM	KS0	K1	K2	K3	S1	S2	S3	Pvalue	SEM
SM	1.40^{bc}	1.10^{abc}	0.80^{ab}	1.00^{abc}	1.40^{c}	0.70^{a}	1.30^{abc}	1.40^{c}	**	0.06
PC	9.20	9.42	8.84	8.27	9.57	8.22	7.74	10.10	ns	0.27
PS + PI	3.69^{c}	4.15^{c}	3.61^{bc}	3.22^{abc}	3.33^{abc}	3.78^{c}	1.80^{a}	2.05^{ab}	**	0.19
PE	6.30^{a}	6.93^{ab}	6.77^{ab}	7.58^{ab}	5.99^{a}	7.48^{ab}	8.51^{b}	10.92^{c}	***	0.33
∑Polar	**20.5**	**21.6**	**20.0**	**20.1**	**20.3**	**20.2**	**19.3**	**24.4**	ns	0.47
MAG	2.71	3.69	4.53	3.72	4.04	3.56	2.52	3.96	ns	0.54
C	9.12^{a}	9.78^{ab}	8.69^{a}	9.13^{a}	12.3^{c}	9.60^{ab}	9.82^{ab}	11.6^{bc}	***	0.28
TAG	62.7^{abc}	60.9^{ab}	64.6^{abc}	62.4^{abc}	58.7^{a}	66.6^{bc}	68.3^{c}	60.0^{ab}	**	0.77
CE	4.91^{a}	4.05^{a}	2.11^{b}	4.69^{a}	4.64^{a}	0^{c}	0^{c}	0^{c}	***	0.28
∑Neutral	**79.5**	**78.4**	**80.0**	**79.9**	**79.7**	**79.8**	**80.7**	**75.6**	ns	0.47

Different letters at the same raw means that there is significant differences between diets, ns = not significant P > 0.1; *P < 0.05; **P < 0.01; ***P < 0.001.
Abbreviations: Polar lipids – SM, sphingomyelin, PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine. Neutral lipids – MAG, monoacylglycerol; C, cholesterol; TAG, triacyglycerol; CE, cholesterol ester.

Table 9
Correlation between dietary fatty acids and tissue composition in polar lipids.

	Liver	Muscle	Intestine
Saturated	ns	0.76*	0.87***
Monounsated	0.86**	0.79*	0.82*
∑ω-9	0.81*	0.74*	0.77*
∑ω-6	0.93**	0.85**	0.74*
∑ω-3	0.86**	0.92**	ns
EPA + DHA	0.86**	0.88**	ns
ω-3/ω-6	0.97***	0.97***	0.85**

ns = not significant $P > 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

different tissues, with the intestine being more conserved than the liver. Moreover, the content of arachidonic acid, EPA and DHA were higher in liver tissue, compared to muscle and intestine. Overall, neutral lipids in all tissues were characterized by higher levels of monounsaturates and ω-9, and lower levels of saturates and ω-3, compared to the polar lipids. A strong positive correlation between dietary fatty acids and tissue fatty acids ($r = 0.72$–0.98) was evident for most fatty acids, with the exception of saturates in the liver (Table 10).

3.8. Liver histology

Histological examination of liver did not indicate differences between the dietary treatments (Fig. 4). All sections examined had a similar morphology, with centrally located nuclei and regular size of hepatocytes. In some of the specimens examined coming from KS0, K1 and S1 diets, mild lipid accumulation with a simultaneous peripheral nuclei translocation was observed (Fig. 4B, C and F, asterisk).

4. Discussion

Phospholipids are known to facilitate digestion and absorption of lipids, form the structure of cellular membranes, serve as carriers of bioactive long-chain polyunsaturated fatty acids and precursors to other physiological active molecules (Tocher et al., 2008). Dietary phospholipids are well-known to improve survival, development and growth of larval stages of many freshwater and marine species, including gilthead seabream (Betancor et al., 2012; Saleh et al., 2013; Saleh et al., 2015; Seiliez et al., 2006), meagre (Ghaderpour and Estevez, 2020) and Atlantic cod (Li et al., 2015). Data on juvenile fish, like yellowtail (*Seriola quinqueradiata*)(La et al., 2018), European sea bass and turbot (*Psetta maximus*)(Geurden et al., 1997), rainbow trout (*Oncorhynchus mykiss*)(Azarm et al., 2013), amberjack (*Seriola dumerilii*)(Uyan et al., 2009) and cobia (Trushenski et al., 2013a; Trushenski et al., 2013b) confirm the same result. Optimal requirements levels for juvenile fish as referred in literature are ranging from 1.5% to 14% inclusion levels in the diet (Tocher et al., 2008). Such a great range in the dietary phospholipid levels may originate from differences in target species, fish size (most studies examined the requirements of smaller fish than the present study) and duration of the trials. For juvenile gilthead seabream, no studies for phospholipids requirements have been reported, when fish are fed with low-FM diets. In the present study, a range of 0.5 to 2% was used based on studies from Atlantic salmon (National Research Council, 2011). To our knowledge, this is the first study to report such effects for juvenile gilthead seabream, using different sources of phospholipids (marine versus plant) on low-FM diets. In addition to the effects on growth, the current study provided data on the fatty acid profile and lipid classes distribution in the liver, muscle and intestine, while we also evaluated the effects of dietary phospholipids on liver steatosis.

Table 10
Correlation between dietary fatty acids and tissue composition in neutral lipids.

	Liver	Muscle	Intestine
Saturated	ns	0.91**	0.84**
Monounsated	0.85**	0.95***	0.92**
∑ω-9	0.94***	0.98***	0.87**
∑ω-6	0.93**	0.93***	0.91**
∑ω-3	0.89**	0.92**	0.72*
EPA + DHA	0.98***	0.97***	0.79*
ω-3/ω-6	0.94***	0.96***	0.89**

ns = not significant $P > 0.1$; *P < 0.05; **P < 0.01; ***P < 0.001.
Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

Dietary alterations such as fish meal replacement or addition of different sources of phospholipids may alter the dietary phospholipids content and compositions, leading also to alterations in growth performance. There are few studies available that compare the efficacy of different phospholipids sources, like soya lecithin, egg lecithin and occasionally marine phospholipids for juvenile fish. In rainbow trout, addition of 2% of egg lecithin gave a better growth performance compared to soybean lecithin, and this was attributed to the different fatty acid composition, and specifically the content of HUFA and ω-3/ω-6 ratio (Azarm et al., 2013). In two studies with juvenile cobia and hybrid stiped bass fed with low-fish meal diets supplemented with soybean lecithin and marine-origin lecithin, similar to our experimental setup, it was observed that marine phospholipids had a better growth performance, attributing these results to the quality of the dietary polar lipids (Laporte, 2015; Trushenski et al., 2013b). Similar results were also obtained from a similar study in gilthead sea bream larvae, comparing soybean versus krill phospholipid sources, showing a better growth for the latter, attributing such effects on the higher level of phosphatidylcholine (PC) and DHA and EPA levels in the diets (Saleh et al., 2015). In our study, both phospholipid types led to a growth performance improvement (weight increase, specific and daily growth rates), while this was more evident for the lowest marine phospholipid levels (0.5%) and the second lowest plant phospholipid levels (1%), thus supporting previous findings.

Concerning the fatty acid profile of the experimental diets used in the current study, the levels of EPA and DHA of the supplemented diets were carefully formulated to contain similar levels with the un-supplemented diet (KS0) and to be adequate for gilthead seabream requirements, in order to be able to discriminate a phospholipid effect from a fatty acid effect. Therefore, the growth promoting effect for both sources of phospholipid is a good indication that this is not due to EPA and DHA, but due to dietary phospholipids. Moreover, we observed a higher level of ω-6 in the phospholipid-supplemented diets compared to the KS0, which was more prominent in the soybean lecithin-supplemented diets. As a result, the ω-3/ω-6 ratio was decreasing in these diets with the highest inclusion levels of the phospholipid supplementation. This was also reflected in the fatty acid profile of the tissues examined, where we observed a higher level of ω-6 for the higher dietary levels of soybean lecithin. The content of polar lipids in the experimental diets increased with the addition of both phospholipid sources, and mainly of the marine-origin, which could explain the results for a better performance at lower inclusion levels of this supplement compared to the plant-origin. More specifically, we observed an increase in PC and the presence of sphingomyelin (SM) at the higher inclusion levels of the marine phospholipid. Higher levels of polar lipids and more specifically PC, have been associated with an increase in feed intake through the stimulation of gustatory responses (Izquierdo et al., 2001). In the present trial, no differences in feed intake were observed, but diets supplemented with marine phospholipids showed higher levels of PC, while diets supplemented with plant phospholipids showed higher levels of PE.

The promoting effect of phospholipids can be associated with their different lipid classes. There are not so many data available on the efficacy of different phospholipid classes when administrated within requirement levels. Studies focusing on soybean lecithin, egg lecithin, and some marine phospholipids, reported different abundances in phospholipid classes, mainly PC, PE, PI and PS. Soya lecithin contains a range of 50 to 86% phospholipids, from which 13–18% is PC, 10–15% is

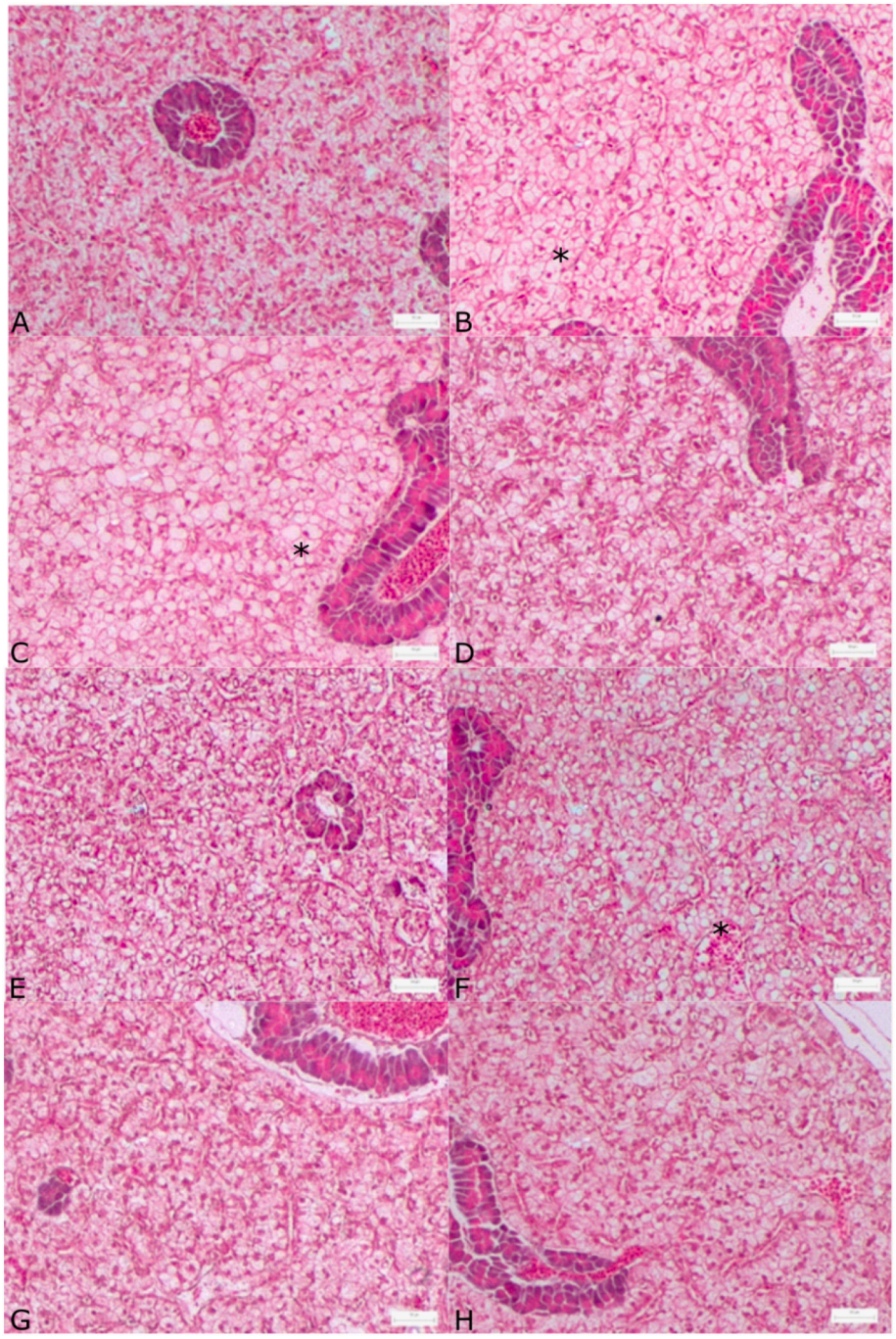

Fig. 4. Histological sections under light microscopy from liver of gilthead sea bream fed the different diets: FM-A, KS0-B, K1—C, K2—D, K3-E, S1—F, S2-G, S3—H. Hepatocytes in FM groups had central located nuclei, while in some fish in KS0, K1 and S1 groups, there was very mild steatosis with hepatocytes with some lipid vacuoles and nuclei displacement (star). Staining with Haematoxylin and Eosin. Bars 50 μm.

PE, 10–15% is PI and 5–12% is phosphatases (PA)(Daniel and PhD, 2004). Both PC and PI are effective to meet phospholipid requirements in larval ayu, whereas PE was shown to be less effective (Kanazawa et al., 1985; Kanazawa et al., 1983). In Japanese flounder larvae, PC, but not PI and PE resulted in growth improvement (Kanazawa, 1993). In carp, PC was more important for growth, while PI was more important for normal development (Geurden et al., 1998). In the present trial, dietary PC levels were increasing when source of phospholipids were supplemented in the diet. Growth improvement started to be evident already from the lowest inclusion level for krill (0.5%) resulting in 3.8% of PC, while for soybean lecithin, an evident increase was present from 1% inclusion levels resulting in 5.2% of PC. Although FM diet contained lower levels of PC compared to the highest level of krill and soya lecithin, growth improvement by the addition of phospholipids was not equivalent to that observed in the FM diet, but only better with regard to the non-supplemented diets (KS0). Overall, we observed a tendency for the krill-supplemented diets to perform better than the soya lecithin-supplemented diets in agreement with Trushenski et al. (2013b) in juvenile cobia. Similar results were previously reported for Atlantic salmon, where results were significant at the stages below 2.5 g but not when the fish grew to smolt stage from 2.5 g onwards (Taylor et al., 2015). Moreover, similar to the study from Tacon and Metian (2015), we did not observed an improvement of the growth performance at higher levels of phospholipids (higher than 0.5% and 1% for krill and soybean

respectively). The authors hypothesized that addition of phospholipids does not lead to an increase in energy when compared to triacylglycerols, which are more important for juvenile or adult stages compared to early life stages (Bell and Koppe, 2011; Taylor et al., 2015). During these later stages where enterocyte metabolism is normally functioning, phospholipids have a less important role in exporting dietary lipids from the intestine and thus higher levels in the diet at the expense of triacylglycerols (Mansbach, 2001; Taylor et al., 2015) may have a negative impact on growth performance.

The fatty acid composition of liver PL largely reflected that of the diet, which is consistent with other studies on Atlantic salmon (Bell et al., 2003; Bransden et al., 2003; Brodtkorb et al., 1997). The relative DHA content of liver PL was always considerably higher than that of the diet, indicating the important role of this fatty acid in cell membranes (Sargent et al., 2002). The results from the different fatty acid composition of the neutral lipid fraction compared to polar lipid fraction is in agreement with previous studies with different fish species (Henderson and Tocher, 1987), and gilthead seabream specifically (Fountoulaki et al., 2003; Ibeas et al., 1997; Kalogeropoulos et al., 1993). In the current study, it was observed that fatty acids deposition was positively correlated with dietary fatty acids in the neutral lipids of all the tissues examined, which comes in agreement with previous findings (Fountoulaki et al., 2003; Fountoulaki et al., 2009; Sargent et al., 1995). In the polar lipids of the intestine tissue, it was shown that ω-3, mostly as EPA + DHA, was not correlated to the dietary fatty acids, indicating the specificity of this tissue. Moreover, we observed the existence of sphingomyelin (SM) in the lipid classes of the intestine. Previous studies have demonstrated the importance of SM in epithelial barriers of fish and other vertebrates, despite the structural differences between marine and terrestrial epithelia (Cheng et al., 2018; Feingold, 2007; Pullmannová et al., 2014). In fact, this polar lipid, disposed in the outer layer of the cell membrane with another choline-container lipid as PC (Tocher et al., 2008), is more abundant in membranes of temperate-water fish suggesting its role in the membrane fluidity (Palmerini et al., 2009).

Histological examination of the liver revealed that addition of krill from 0.5% and for soybean lecithin from 1% on improved symptoms of steatosis. It was previously reported in Atlantic salmon that supplementation of both soybean and krill phospholipids prevented symptoms of intestinal steatosis, and this was associated with the levels of PC in the diets; diets with PC levels above 10% did not show steatosis symptoms (Taylor et al., 2015). In our study, such results were more evident at lower PC levels, starting from 3.8% for krill and 5.2% for soybean, although overall we observed that a low number of individuals showed severe symptoms of steatosis.

In conclusion, the current study showed that addition of phospholipids to low fish meal diets can improve the growth and liver status in gilthead seabream juveniles. Krill phospholipids showed optimal results at 0.5% levels and soybean lecithin at 1% level. The fact that both sources of phospholipids gave similar results, argues also with the fish meal sparing effect, thus supporting sustainability in aquafeeds. Further research is required to elucidate the functions of phospholipids and their associated fatty acids as well as their requirements with regard to growth performance in juvenile fish fed with low fish meal diets.

Author statement

Fotini Kokou: Laboratory and experimental work, Formal analysis, Manuscript original draft and review; **Antigoni Vasilaki:** Laboratory and experimental work, Formal analysis, Manuscript review; **Chrysanthi Nikoloudaki**: Laboratory and experimental work; **Ataman Bilge Sari**: Laboratory work; **Vasileios Karalazos**: Conceptualization, Funding acquisition; **Eleni Fountoulaki**; Conceptualization, Supervision, Funding acquisition, Formal analysis, Manuscript Original Draft and review.

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.

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

Acknowledgements

This study has received funding from the EU 7th Framework Programme, under the ARRAINA project (Grant agreement 288925): “Advanced Research Initiatives for Nutrition & Aquaculture”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737052.

References

AOAC, 2005. Official Methods of Analysis of the Association of the Official Analytical Chemists.
Azarm, H.M., Kenari, A.A., Hedayati, M., 2013. Effect of dietary phospholipid sources and levels on growth performance, enzymes activity, cholecystokinin and lipoprotein fractions of rainbow trout (Oncorhynchus mykiss) fry. Aquac. Res. 44, 634–644.
Bell, J., Koppe, W., 2011. Welfare and Health of Fish Fed Vegetable Oils as Alternative Lipid Sources to Fish Oil. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. Taylor & Francis. CRC Press, Boca Raton, pp. 21–59.
Bell, J.G., McGhee, F., Campbell, P.J., Sargent, J.R., 2003. Rapeseed oil as an alternative to marine fish oil in diets of post-smolt Atlantic salmon (Salmo salar): changes in flesh fatty acid composition and effectiveness of subsequent fish oil “wash out”. Aquaculture. 218, 515–528.
Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Bell, J.G., 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.
Betancor, M.B., Nordrum, S., Atalah, E., Caballero, M.J., Benítez-Santana, T., Roo, J., Robaina, L., Izquierdo, M., 2012. Potential of three new krill products for seabream larval production. Aquac. Res. 43, 395–406.
Bransden, M.P., Carter, C.G., Nichols, P.D., 2003. Replacement of fish oil with sunflower oil in feeds for Atlantic salmon (Salmo salar L.): effect on growth performance, tissue fatty acid composition and disease resistance. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 135, 611–625.
Brodtkorb, T., Rosenlund, G., Lie, Ø., 1997. Effects of dietary levels of 20: 5n-3 and 22: 6n-3 on tissue lipid composition in juvenile Atlantic salmon, Salmo salar, with emphasis on brain and eye. Aquac. Nutr. 3, 175–187.
Cheng, K., Bou, M., Ruyter, B., Pickova, J., Ehtesham, E., Du, L., Venegas, C., Moazzami, A.A., 2018. Impact of reduced dietary levels of eicosapentaenoic acid and docosahexaenoic acid on the composition of skin membrane lipids in Atlantic Salmon (Salmo salar L.). J. Agric. Food Chem. 66, 8876–8884.
Christie, W.W., 1989. Gas Chromatography and Lipids (Oily).
Coutteau, P., Geurden, I., Camara, M., Bergot, P., Sorgeloos, P., 1997. Review on the dietary effects of phospholipids in fish and crustacean larviculture. Aquaculture. 155, 149–164.
Daniel, K.T., PhD, C., 2004. Soy Lecithin: From Sludge to Profit.
FAO, 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in Action, FAO Rome, Italy.
Feingold, K.R., 2007. Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. J. Lipid Res. 48, 2531–2546.
Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226.
Fountoulaki, E., Alexis, M., 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.
Fountoulaki, E., Vasilaki, A., Hurtado, R., Grigorakis, K., Karacostas, I., Nengas, I., Rigos, G., Kotzamanis, Y., Venou, B., Alexis, M., 2009. Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (Sparus aurata L.); effects on growth performance, flesh quality and fillet fatty acid profile: recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. Aquaculture. 289, 317–326.
Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199, 197–227.
Geurden, I., Coutteau, P., Sorgeloos, P., 1997. Effect of a dietary phospholipid supplementation on growth and fatty acid composition of European sea bass (Dicentrarchus labrax L.) and turbot (Scophthalmus maximus L.) juveniles from weaning onwards. Fish Physiol. Biochem. 16, 259–272.

Geurden, I., Marion, D., Charlon, N., Coutteau, P., Bergot, P., 1998. Comparison of different soybean phospholipidic fractions as dietary supplements for common carp, Cyprinus carpio, larvae. Aquaculture. 161, 225–235.
Ghaderpour, S., Estevez, A., 2020. Effect of short-term rotifer enrichment with marine phospholipids on growth, survival, and composition of meager (Argyrosomus regius) larvae. Front. Mar. Sci. 7, 1082.
Hamza, N., Kestemont, P., Khemis, I.B., Mhetli, M., Cahu, C., 2012. Effect of different sources and levels of dietary phospholipids on performances and fatty acid composition of pikeperch (Sander lucioperca) larvae. Aquac. Nutr. 18, 249–257.
Henderson, R.J., Tocher, D.R., 1987. The lipid composition and biochemistry of freshwater fish. Prog. Lipid Res. 26, 281–347.
Ibeas, C., Cejas, J., Fores, R., Badia, P., Gomez, 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.
Izquierdo, M., Socorro, J., Arantzamendi, L., Hernández-Cruz, C., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem. 22, 97–107.
Izquierdo, M., Tandler, A., Salhi, M., Kolkovski, S., 2001. Influence of dietary polar lipids' quantity and quality of ingestion and assimilation of labelled fatty acids by larval gilthead seabream. Aquac. Nutr. 7, 153–160.
Kalogeropoulos, N., Alexis, M., Henderson, R., 1993. Effect of dietary lipids on tissue fatty acid composition of gilthead bream (Sparus aurata). Colloques de l'INRA (France). 61, 257–267.
Kanazawa, A., 1993. Essential phospholipids of fish and crustaceans. Fish Nutrition in Practice. 519–530.
Kanazawa, A., Teshima, S.-I., Kobayashi, T., Takae, M., Iwashita, T., Uehara, R., 1983. Necessity of dietary phospholipids for growth of the larval ayu. 鹿児島大学水産学部紀要= Memoirs of Faculty of Fisheries Kagoshima University, pp. 115–120.
Kanazawa, A., Teshima, S.-I., Sakamoto, M., 1985. Effects of dietary bonito-egg phospholipids and some phospholipids on growth and survival of the larval ayu, *Plecoglossus altivelis*. Zeitschrift für angewandte Ichthyologie. 1, 165–170.
Kaushik, S.J., Covès, D., Dutto, G., Blanc, D., 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture. 230, 391–404.
Kim, H.-Y., Salem Jr., N., 1990. Separation of lipid classes by solid phase extraction. J. Lipid Res. 31, 2285–2289.
Kok Leong, W., 1983. Studies on intensive snakehead (Channa sp.) culture with special reference to their nutrition. PhD Thesis. Stirling University.
Kokou, F., Fountoulaki, E., 2018. Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients. Aquaculture. 495, 295–310.
Kokou, F., Sarropoulou, E., Cotou, E., Kentouri, M., Alexis, M., Rigos, G., 2017. Effects of graded dietary levels of soy protein concentrate supplemented with methionine and phosphate on the immune and antioxidant responses of gilthead sea bream (*Sparus aurata* L.). Fish & Shellfish Immunol. 64, 111–121.
La, T.X., Ishikawa, M., Tola, S., Fukada, H., Masumoto, T., 2018. Effects of dietary phospholipid level and fraction on the feed intake of non-fish meal diet in yellowtail, Seriola quinqueradiata Temminck & Schlegel, 1845. Aquac. Res. 49, 569–575.
Laporte, J., 2015. Amending reduced fish meal feeds with phospholipids to improve performance of hybrid striped bass. J. Anim. Res. 1, 7.
Li, K., Østensen, M.-A., Attramadal, K., Winge, P., Sparstad, T., Bones, A.M., Vadstein, O., Kjørsvik, E., Olsen, Y., 2015. Gene regulation of lipid and phospholipid metabolism in Atlantic cod (Gadus morhua) larvae. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 190, 16–26.
Mansbach, C.M., 2001. Triacylglycerol movement in enterocytes, intestinal lipid metabolism. Springer, pp. 215–233.
Morais, S., Conceição, L., Rønnestad, I., Koven, W., Cahu, C., Infante, J.Z., Dinis, M., 2007. Dietary neutral lipid level and source in marine fish larvae: effects on digestive physiology and food intake. Aquaculture. 268, 106–122.
National Research Council, 2011. *Nutrient requirements of fish and shrimp*. National academies press, Vancouver.
Nengas, I., Alexis, M.N., Davies, S.J., 1996. Partial substitution of fishmeal with soybean meal products and derivatives in diets for the gilthead sea bream *Sparus aurata* (L.). Aquac. Res. 27, 147–156.
Palmerini, C.A., Mazzoni, M., Giovinazzo, G., Arienti, G., 2009. Blood lipids in Antarctic and in temperate-water fish species. J. Membr. Biol. 230, 125–131.
Pullmannová, P., Staňková, K., Pospíšilová, M., Školová, B., Zbytovská, J., Vávrová, K., 2014. Effects of sphingomyelin/ceramide ratio on the permeability and microstructure of model stratum corneum lipid membranes. Biochimica et Biophysica Acta (BBA)-Biomembranes 1838, 2115–2126.
Saleh, R., Betancor, M., Roo, J., Benítez-Santana, T., Hernández-Cruz, C.M., Moyano, F. J., Izquierdo, M., 2013. Optimum krill phospholipids content in microdiets for gilthead seabream (S parus aurata) larvae. Aquac. Nutr. 19, 449–460.
Saleh, R., Betancor, M., Roo, J., Benítez-Dorta, V., Zamorano, M.J., Bell, J.G., Izquierdo, M., 2015. Effect of krill phospholipids versus soybean lecithin in microdiets for gilthead seabream (S parus aurata) larvae on molecular markers of antioxidative metabolism and bone development. Aquac. Nutr. 21, 474–488.
Sargent, J., Bell, J., Bell, M., Henderson, R., Tocher, D., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol. 11, 183–198.
Sargent, J., Bell, G., McEvoy, L., Tocher, D., Estevez, A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture. 177, 191–199.
Sargent, J., Tocher, D., Bell, J., 2002. The lipids. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego.
Seiliez, I., Bruant, J., Zambonino Infante, J.-L., Kaushik, S., Bergot, P., 2006. Effect of dietary phospholipid level on the development of gilthead sea bream (Sparus aurata) larvae fed a compound diet. Aquac. Nutr. 12, 372–378.
Tacon, A.G.J., Metian, M., 2015. Feed matters: satisfying the feed demand of aquaculture. Rev. Fish. Sci. & Aquacul. 23, 1–10.
Taylor, J.F., Martinez-Rubio, L., del Pozo, J., Walton, J.M., Tinch, A.E., Migaud, H., Tocher, D.R., 2015. Influence of dietary phospholipid on early development and performance of Atlantic salmon (Salmo salar). Aquaculture. 448, 262–272.
Tocher, D., Bendiksen, E., Campbell, P., Bell, J.G., 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture. 280, 21–34.
Trushenski, J., Woitel, F., Schwarz, M., Yamamoto, F., 2013a. Saturated fatty acids limit the effects of replacing fish oil with soybean oil with or without phospholipid supplementation in feeds for juvenile cobia. N. Am. J. Aquac. 75, 316–328.
Trushenski, J., Schwarz, M., Pessoa, W.V.N., Mulligan, B., Crouse, C., Gause, B., Yamamoto, F., Delbos, B., 2013b. Amending reduced fish-meal feeds with marine lecithin, but not soy lecithin, improves the growth of juvenile cobia and may attenuate heightened responses to stress challenge. J. Anim. Physiol. Anim. Nutr. 97, 170–180.
Uyan, O., Koshio, S., Ishikawa, M., Yokoyama, S., Uyan, S., Ren, T., Hernandez, L., 2009. The influence of dietary phospholipid level on the performances of juvenile amberjack, Seriola dumerili, fed non-fishmeal diets. Aquac. Nutr. 15, 550–557.