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Regulation of glucose and lipid metabolism by dietary carbohydrate levels and lipid sources in gilthead sea bream juveniles

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Abstract

The long-term effects on growth performance, body composition, plasma metabolites, liver and intestine glucose and lipid metabolism were assessed in gilthead sea bream juveniles fed diets without carbohydrates (CH–) or carbohydrate-enriched (20 % gelatinised starch, CH+) combined with two lipid sources (fish oil; or vegetable oil (VO)). No differences in growth performance among treatments were observed. Carbohydrate intake was associated with increased hepatic transcripts of glucokinase but not of 6-phosphofructokinase. Expression of phosphoenolpyruvate carboxykinase was down-regulated by carbohydrate intake, whereas, unexpectedly, glucose 6-phosphatase was up-regulated. Lipogenic enzyme activities (glucose-6-phosphate dehydrogenase, malic enzyme, fatty acid synthase) and Δ6 fatty acyl desaturase (*FADS2*) transcripts were increased in liver of fish fed CH+ diets, supporting an enhanced potential for lipogenesis and long-chain PUFA (LC-PUFA) biosynthesis. Despite the lower hepatic cholesterol content in CH+ groups, no influence on the expression of genes related to cholesterol efflux (ATP-binding cassette G5) and biosynthesis (lanosterol 14 α-demethylase, cytochrome P450 51 cytochrome P450 51 (*CYP51A1*); 7-dehydrocholesterol reductase) was recorded at the hepatic level. At the intestinal level, however, induction of CYP51A1 transcripts by carbohydrate intake was recorded. Dietary VO led to decreased plasma phospholipid and cholesterol concentrations but not on the transcripts of proteins involved in phospholipid biosynthesis (glycerol-3-phosphate acyltransferase) and cholesterol metabolism at intestinal and hepatic levels. Hepatic and muscular fatty acid profiles reflected that of diets, despite the up-regulation of *FADS2* transcripts. Overall, this study demonstrated that dietary carbohydrates mainly affected carbohydrate metabolism, lipogenesis and LC-PUFA biosynthesis, whereas effects of dietary lipid source were mostly related with tissue fatty acid composition, plasma phospholipid and cholesterol concentrations, and LC-PUFA biosynthesis regulation. Interactions between dietary macronutrients induced modifications in tissue lipid and glycogen content.

Key words: Carbohydrate content: Cholesterol: Fatty acid bioconversion: Lipid sources: Nutrient metabolism

Besides being a source of high-quality protein and essential micronutrients for humans, fish are unique sources of *n*-3 long-chain PUFA (LC-PUFA), namely EPA (20 : 5*n*-3) and DHA (22 : 6*n*-3), which were proven to be beneficial for human health$^{(1)}$. Driven by the crescent awareness of the health beneficial effects of *n*-3 LC-PUFA in a range of human pathologies (including CVD, inflammatory and neurological diseases) the global fish consumption is rising$^{(1)}$ and an increasing proportion of this fish is now being supplied by aquaculture$^{(2)}$.

Fishmeal (FM) and fish oil (FO) have been widely used as main ingredients in aquafeeds for carnivorous fish species. However, environmental sustainability and economical issues, related to the limited availability of fisheries resources and escalating costs, forced the aquafeed industry to search for alternative and eco-friendly ingredients such as plant feedstuffs and vegetable oils (VO). However, the unbalanced amino acid and fatty acid (FA) profiles, relatively high amounts of carbohydrates and the presence of antinutritional factors may limit their use in aquafeeds, especially for carnivorous fish species that are metabolically adapted to diets rich in LC-PUFA and almost devoid of carbohydrates$^{(1,3–5)}$.

It is well known that modifications of dietary macronutrients (e.g. lipid source, carbohydrates, etc.) may have marked effects on tissue FA composition and lipid deposition of fish$^{(6–9)}$.

Abbreviations: CYP51A1, lanosterol 14 α-demethylase, cytochrome P450 51; elovl5, elongase 5; FA, fatty acid; FAS, fatty acid synthase; FO, fish oil; G6Pase, glucose 6-phosphatase; G6PD, glucose-6-phosphate dehydrogenase; LC-PUFA, long-chain PUFA; ME, malic enzyme; PL, phospholipids; VO, vegetable oil.

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For instance, replacement of FO by VO generally leads to a decrease of *n*-3 LC-PUFA and an increase of 18C PUFA precursors, linoleic acid (18 : 2*n*-6) and α-linolenic acid (18 : 3*n*-3), in the fillet(7,9–11). Increased tissue lipid deposition with dietary incorporation of VO(6,8,9) or carbohydrates(7,12) was also reported. Such effects may occur because of different mechanisms that are recognised to regulate the quantity and quality of the fish lipid depots, such as modification of lipogenesis, β-oxidation, tissue lipid uptake and transport or FA desaturation and elongation processes(13,14).

Although not always consistent, several studies in fish reported that dietary lipid source and carbohydrates regulate gene expression, key transcription factors and/or activity of enzymes involved in lipogenesis, β-oxidation and lipid uptake. Accordingly, it was observed that replacing dietary FO by VO decreased(15,16) or increased(17–19) gene expression or activity of lipogenic enzymes (such as glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), fatty acid synthase (FAS)). Similarly, dietary VO was reported to regulate gene expression or activities of lipolytic enzymes either positively(20,21) or negatively(19,20,22,23) (including carnitine palmitoyltransferase (CPT) I and II, lipoprotein lipase) in a tissue-specific manner. Dietary carbohydrate or glucose administration was also reported to enhance lipogenesis(24). However, regarding β-oxidation, data are contradictory, as either stimulation(25) or inhibition(26) effects were reported. Regarding LC-PUFA biosynthesis, strong induction of desaturase (fatty acyl desaturase (*FAD*) Δ5 and Δ6 desaturases, *FADS1* and *FADS2*, respectively) and elongase expression by VO administration was reported in freshwater fish and salmonids(10,27), but in marine fish such induction of gene expression is not so clear(11,28–30). Desaturases and elongases were also shown to be up-regulated by dietary carbohydrates in salmonids(25,27), but in marine fish such an effect has never been demonstrated(7).

Besides the reported effects on tissue FA composition and lipid deposition of fish, the use of plant feedstuffs and VO can also compromise fish physiological functions and, ultimately, fish health(31,32). For example, there is an increased awareness of potential effects due to reduced dietary phospholipids (PL) or cholesterol in plant feedstuff-based diets. Besides their important roles in membrane structure, cholesterol and PL also have important functional roles. Cholesterol is a precursor of physiologically active compounds such as bile acids, vitamin D, adrenal corticoids and sex hormones, and PL are precursors of eicosanoids, diacylglycerol, inositol phosphates and platelet-activating factors(33,34). In a number of fish species, dietary VO was reported to decrease plasma PL(7,35,36), cholesterol and LDL-cholesterol(8,35,37), and to modulate the activity or expression of genes involved in PL synthesis(38) and cholesterol synthesis and absorption(7,10,39). Recently, it was also demonstrated that dietary carbohydrates regulate plasma cholesterol and PL concentrations and the transcription of proteins involved in cholesterol metabolism(7).

Therefore, to provide adequate background for successful use of plant feedstuffs in aquafeeds, all aspects related to fish physiological functions and the nutritional quality of the final product must be better understood. For that purpose, we assessed the long-term effect of dietary lipid source, carbohydrate content and interactions between both on growth performance, tissue composition, liver and intestine enzymatic activity and expression of genes related with lipid metabolism (lipogenesis, β-oxidation, FA bioconversion, cholesterol and PL metabolism) in gilthead sea bream juveniles. In this study, the selection of the relevant genes involved in lipid metabolism was performed taking advantage of the recent advances in the molecular and functional characterisation of a number of new gilthead sea bream sequences related to FA, PL and cholesterol metabolism(40–47). In addition, as an increasing number of studies have reported that glucose metabolism was distinctly regulated by different dietary lipid sources(7,17,48–50), we also investigated the effects of these dietary manipulations on mechanisms involved in glucose utilisation/metabolism (such as glycolysis and gluconeogenesis pathways).

Methods

Experimental diets

Four diets differing in carbohydrate content (0 and 20 % gelatinised starch, diets CH– and CH+, respectively) and lipid source (diets FO or VO) were formulated (Table 1). The increase in carbohydrate content in CH+ diets was achieved by decreasing protein, which was kept well above the requirements of the species(51). The major lipid source of FO diets was cod liver oil. In VO diets, 100 % of the cod liver oil was replaced by a VO blend composed of 20 % rapeseed, 50 % linseed and 30 % palm oils. FM was added as a major dietary protein source to isolate the impacts of dietary VO and to avoid the interference of dietary plant protein on lipid metabolism, especially on cholesterol metabolism.

Animals, experimental conditions and sampling

The experiment was directed by trained scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was performed at the Marine Zoological Station, University of Porto, Portugal, in a thermoregulated recirculation water system equipped with twelve fibreglass cylindrical tanks of 300 litres water capacity, and supplied with continuous flow of filtered seawater. After 2 weeks of adaptation to the experimental conditions, twelve groups of twenty-one gilthead sea bream (*Sparus aurata*) juveniles with an initial body weight of 70·8 (SD 0·03) g were established and the experimental diets were randomly assigned to triplicate groups of these fish.

The growth trial lasted for 81 d, and during this period fish were hand-fed twice a day, 6 d/week, to apparent visual satiety. At the end of the trial, fish were unfed for 1 d to empty the gut content and then bulk-weighed after mild anesthesia with 0·3 ml/l methylethanol. At the beginning and at the end of the growth trial, fifteen fish from the stock population and three fish from each tank were, respectively, sampled, pooled and frozen until whole-body composition analysis. During the trial, salinity averaged 34·7 (SD 0·8) g/l, dissolved O_2 was kept near saturation and water temperature was regulated to 24·0 (SD 0·5)°C.

Table 1. Ingredient and chemical composition of the experimental diets

	Experimental diets			
Lipid source…	FO		VO	
CH…	CH–	CH+	CH–	CH+
Ingredients (% dry weight)				
Fishmeal*	87·3	65·1	87·3	65·1
Starch†	0	20	0	20
Cod liver oil‡	9·2	11·4	0	0
VO§	0	0	9·2	11·4
Vitamins‖	1·5	1·5	1·5	1·5
Minerals¶	1·0	1·0	1·0	1·0
Binder**	1·0	1·0	1·0	1·0
Proximate analyses (% DM)				
DM	87·0	86·8	87·2	87·6
Crude protein (CP)	66·3	50·3	66·3	50·4
Crude fat (CF)	18·4	18·4	18·2	18·3
Starch	–	16·8	–	18·0
Energy (kJ/g DM)	22·7	23·3	23·3	22·7
Ash	14·1	11·2	14·3	11·1
Cholesterol	0·59	0·39	0·49	0·39
Protein/energy (g/MJ)	29·2	21·6	28·5	22·2

FO, fish oil; VO, blend of vegetable oils; CH, carbohydrate; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch.

* Steam-dried low-temperature fishmeal (Superprime; Inproquisa) (CP: 74·6 % DM; crude lipid: 10·1 % DM).

† C-Gel Instant-12018; Cerestar.

‡ Labchem; Laborspirit Lda.

§ 30 % palm oil (Colmi), 50 % linseed oil (Sociedade Portuense de Drogas) and 20 % rapeseed oil (Huilerie Emile Noël S.A.S).

‖ Vitamins (mg/kg diet): retinyl acetate, 18 000 IU/kg diet (6·19 mg); cholecalciferol, 2000 IU/kg diet (0·04 mg); *α*-tocopheryl acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid, 10; cyanocobalamin, 0·02; biotin, 1·5; ascorbic acid, 50; inositol, 400 (premix).

¶ Minerals (mg/kg diet): cobalt sulphate, 1·91; copper sulphate, 19·6; iron sulphate, 200; sodium fluoride, 2·21; potassium iodide, 0·78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0·66; zinc oxide, 37·5; dibasic calcium phosphate, 5·93 (g/kg diet); potassium chloride, 1·15 (g/kg diet); sodium chloride, 0·40 (g/kg diet) (premix).

** Aquacube (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate) (Agil).

To eliminate handling stress, after the growth trial fish, continued to be fed for one more week, and then 18 h after the last meal (the previous day afternoon meal) nine fish from each tank were randomly sampled for blood, liver, intestine and muscle collection. Blood was collected from the caudal vein using heparinised syringes and centrifuged at 2500 ***g*** for 10 min and the recovered plasma was kept at –20°C until analysis. Thereafter, fish were killed with a sharp blow to the head, and whole body, viscera and liver were weighed for determination of hepatosomatic (HSI) and viscerosomatic (VSI) indexes. Liver, intestine and muscle sections were frozen in liquid N_2 and then stored at –80°C until biochemical, enzymatic and molecular analyses.

Diets, whole fish, liver, muscle and plasma analysis

Chemical analysis of the diets, whole fish, liver and muscle was conducted according to AOAC(52) and by following the procedures given below: DM after drying at 105°C until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein content (N × 6·25) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit (models 1015 and 1026, Tecator Systems; Höganäs); and lipid by petroleum diethyl ether extraction (Soxtec HT System; Höganäs). Starch was determined according to Beutler(53) and gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 6200; Parr Instruments).

Hepatic and muscular glycogen contents were determined as described by Roehrig & Allred(54), and lipids were determined according to the method of Folch *et al.*(55). FA methyl esters were prepared by acid transmethylation of total lipids using boron trifluoride in methanol (14 %), as described by Shantha & Ackman(56), and analysed by GC (Varian 3900; Varian; for details see Castro *et al.*(48)). Total cholesterol in diets, liver and muscle was assayed on total lipid extract by the Liebermann–Burchard method(57). Plasma metabolites were analysed using commercial kits from Spinreact: glucose (ref: 1001191), TAG (ref: 1001312), total cholesterol (ref: 1001090) and PL (ref: 1001140).

Enzymatic activity assays

The activity of key lipogenesis enzymes was determined in the liver (three fish per tank). For that purpose, liver was homogenised (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0·1 mM-EDTA and 0·1 % triton X-100 (v/v), pH 7·8). All procedures were performed on ice. Homogenates were centrifuged at 30 000 ***g*** for 30 min at 4°C. After centrifugation, the resultant supernatant was collected and aliquots were stored at –80°C until analysis. All enzyme activities were measured at 37°C, by monitoring the changes in absorbance of NADPH at 340 nm in a Multiskan GO microplate reader (model 5111 9200; Thermo Scientific), using 6·22 mM/cm as the millimolar extinction coefficient for NADPH. The optimal substrate and protein concentrations for measurement of each enzyme activity were established by preliminary assays. G6PD (*EC* 1.1.1.49), ME (*EC* 1.1.1.40) and FAS (*EC* 2.3.1.38) activities were determined as previously described by Castro *et al.*(7).

Gene expression

Analyses of mRNA levels were performed on liver and intestine samples (two fish per tank). Tissues for RNA analyses were homogenised in 2-ml tubes containing Trizol reagent (Invitrogen) using rapid vibration (liver: 2 × 10 s, with an interval of 10 s, at 5000 rpm; intestine: 3 × 10 s, with 10-s intervals, at 6500 rpm) in Precellys®24 (Bertin Technologies). Extraction of total RNA was then performed according to the manufacturer's recommendations. RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000; NanoDrop Labtech). Complementary DNA (cDNA) synthesis was performed with 1 µg of the resulting total RNA using SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen) and random primers (Promega). Real-time quantitative PCR (q-PCR) analyses were performed in a total volume of 6 µl (detailed information of the reaction mix in Castro *et al.*(7)) using LightCycler® 480 II apparatus (Roche Diagnostics) to assess the gene expression levels. Primers were either obtained in the literature (Mininni *et al.*(44); Pérez-Sánchez *et al.*(45); Sánchez-Gurmaches *et al.*(46); Enes *et al.*(59); Diez *et al.*(74)) or designed from gilthead sea bream-expressed sequence tag sequences available on the SIGENAE database (http://www.sigenae.org) using the Primer3 software(58) (online Supplementary Table S1). For gene targets that had not been previously validated, primers were tested on a pool of cDNA and amplified products were systematically sequenced. The PCR protocol followed the conditions described previously by Castro *et al.*(7). Each PCR run included duplicates of reverse transcription for each sample and negative controls (RT-free samples, RNA-free samples). PCR run for reference gene included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and negative controls. Quantification of the target gene transcripts in the liver and intestine was done using β-actin gene expression as reference, as previously used in gilthead sea bream by Pérez-Sánchez *et al.*(45), and that was stably expressed in the present study (data not shown). Relative quantification of the target gene transcript with the β-actin reference gene transcript was performed using the mathematical model described by Pfaffl(60). The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and the threshold cycle (C_t) deviation (ΔCT) of the unknown sample compared with a control sample and expressed in comparison with the β-actin reference gene:

$$R = [(\mathrm{Etarget\ gene})\,\Delta CT\ \mathrm{target\ gene}\,(\mathrm{mean\ control} - \mathrm{mean\ sample})]$$

$$/[(\mathrm{E}\beta\,\mathrm{actin})\,\Delta CT\,\beta\,\mathrm{actin}(\mathrm{mean\ control} - \mathrm{mean\ sample})].$$

Efficiency of q-PCR was measured by the slope of a standard curve using serial dilutions of cDNA. The fish fed the FOCH – diet was used as the control group.

Statistical analysis

Data were checked for normality and homogeneity of variances, and they were normalised when appropriate. Statistical evaluation of data was carried out by a 2 × 2 factorial arrangement of treatments in a completely randomised experimental design (two-way ANOVA) with carbohydrate content and lipid source as fixed factors. The significance level of 0·05 was used for rejection of the null hypothesis. In cases in which interaction was significant, one-way ANOVA was performed for each factor. All statistical analyses were conducted using the SPSS 22.0 software package (IBM Corp.) for Windows.

Results

Dietary fatty acid composition

The four diets presented small differences in the proportions of total SFA, whereas MUFA were higher in FO diets, and *n*-3 an *n*-6 PUFA were higher in VO diets (Table 2). Within MUFA, high levels of oleic acid (18 : 1*n*-9) were recorded in VO diets, whereas the opposite occurred for palmitoleic acid (16 : 1*n*-7), eicosenoic acid (20 : 1*n*-9) and erucic acid (22 : 1*n*-9). Among *n*-3 PUFA, VO diets were particularly rich in linolenic acid (18 : 3*n*-3) and poor in EPA and DHA. The proportion of total *n*-6 PUFA was strongly higher in VO diets mainly because of linoleic acid (18 : 2*n*-6) levels.

Growth performance and feed utilisation

Fish promptly accepted the experimental diets, and no mortality was recorded during the trial. Dietary treatments had no effects on fish growth performance or feed utilisation (Table 3). Feed intake (g/kg average body weight per d) was similar among diets. N retention, expressed per unit weight gain, was not affected by diet composition. However, protein efficiency ratio was higher with the CH+ diets, which had a lower protein content. Dietary carbohydrate intake increased lipid retention per unit weight gain only when fish were fed the VO diet (Table 3). In fish fed CH– diets, lipid retention per unit weight gain was lower in the VO group. In addition, within the VO group, lipid retention per unit weight gain was lower in fish fed the CH– diet (carbohydrate and lipid source interaction).

Whole-body, liver and muscle composition

At the end of the trial, only whole-body lipid and DM contents were affected by dietary treatments. Under a VO-based diet regimen, whole-body lipid content was higher in CH+ than in CH– groups. In addition, whole-body lipid was lower in VO than in FO groups only when fish were fed no-carbohydrate diets (CH– diets) (carbohydrate and lipid source interaction). Whole-body DM content was higher in fish fed the CH+ diets (Table 4).

Higher HSI and VSI were observed in the CH+ groups, but no effect of dietary lipid source was noticed (Table 4).

Table 2. Fatty acid composition (% of total fatty acids) of the experimental diets

	Experimental diets			
Lipid source…	FO		VO	
CH…	CH–	CH+	CH–	CH+
14 : 0	5·8	5·9	2·5	2·0
15 : 0	0·7	0·6	0·4	0·3
16 : 0	18·7	17·2	21·3	21·0
17 : 0	0·5	0·4	0·4	0·3
18 : 0	4·0	3·5	4·9	4·5
20 : 0	0·2	0·1	0·3	0·2
∑SFA	29·9	27·8	29·9	28·6
16 : 1*n*-7	7·1	7·5	2·3	1·8
18 : 1*n*-9	18·3	19·1	25·4	27·6
20 : 1*n*-9	4·9	5·8	0·9	0·7
22 : 1*n*-9	3·9	4·3	0·5	0·4
∑MUFA	34·4	36·8	29·2	30·5
18 : 2*n*-6	2·2	2·2	9·0	10·6
18 : 3*n*-6	0·1	0·1	0·0	0·1
20 : 2*n*-6	0·3	0·3	0·1	0·1
20 : 3*n*-6	0·13	0·12	0·08	0·08
20 : 4*n*-6	1·2	1·0	0·9	0·7
∑*n*-6 PUFA	4·0	3·8	10·4	11·6
18 : 3*n*-3	1·1	1·2	15·7	19·0
18 : 4*n*-3	1·9	2·1	0·5	0·4
20 : 3*n*-3	0·14	0·15	0·06	0·04
20 : 4*n*-3	0·6	0·6	0·2	0·1
20 : 5*n*-3	7·8	8·0	3·5	2·5
21 : 5*n*-3	0·3	0·3	0·1	0·1
22 : 5*n*-3	1·2	1·2	0·7	0·5
22 : 6*n*-3	11·7	10·8	7·2	4·9
∑*n*-3 LC-PUFA	24·8	24·3	28·0	27·5
Ratios				
SFA:PUFA	1·0	0·9	0·8	0·7
n-3:*n*-6	6·2	6·4	2·7	2·4
Unsat. index	181·4	179·5	169·2	160·2

FO, fish oil; VO, blend of vegetable oils; CH, carbohydrate; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; unsat. index, unsaturation index = sum (fatty acid percentage) × (number of double bonds).

Lipid content in muscle was higher in the CH+ group, whereas in the liver an increase in lipid content with carbohydrate intake was only evident when fish were fed VO-based diets (carbohydrate and lipid source interaction). Carbohydrate intake also increased the glycogen content in liver, but in muscle a similar effect was only recorded when fish were fed FO-based diets (carbohydrate and lipid source interaction). Dietary lipid source induced no changes on hepatic and muscular lipid content, and glycogen content in both tissues increased in the VO group when fish were fed CH– diets (carbohydrate and lipid source interaction). Hepatic cholesterol content was lower in CH+ groups, but no differences were recorded in muscle cholesterol content. Dietary lipid source did not affect hepatic and muscular cholesterol content.

Liver and muscle fatty acid profiles

Muscle and liver FA composition were affected by diet composition and resembled the FA composition of the dietary lipid sources (Tables 5 and 6).

Except for muscle SFA content that was similar among CH+ and CH– diets, liver and muscle FA profiles of fish fed the CH+ diets were characterised by a higher proportion of SFA (particularly 16 : 0) and MUFA (particularly 18 : 1*n*-9) and a lower proportion of *n*-3 PUFA and *n*-6 PUFA. In the liver, replacing FO by VO resulted in higher proportions of SFA (mainly 16 : 0) and *n*-6 PUFA (mainly 18 : 2*n*-6) and a lower proportion of *n*-3 PUFA (mainly 22 : 6*n*-3, 20 : 5*n*-3, 22 : 5*n*-3) and MUFA (mainly 16 : 1*n*-7, 20 : 1*n*-9, 22 : 1*n*-9).

The muscle of fish fed the VO diets presented a lower proportion of SFA (mainly 14 : 0) and MUFA (mainly 16 : 1*n*-7, 20 : 1*n*-9, 22 : 1*n*-9) and an increased proportion of *n*-6 (mainly 18 : 2*n*-6) and *n*-3 (mainly 18 : 3*n*-3) PUFA.

Plasma metabolites and enzyme activity

After 18 h of feeding diets, CH+ plasma glucose concentration was lower than in fish fed CH– diets (Table 7).

Plasma cholesterol was also lower in fish fed the CH+ diet when combined with VO (carbohydrate and lipid source interaction). On the contrary, plasma TAG was higher in fish fed the CH+ diet, but only in fish fed FO (carbohydrate and lipid source interaction). Plasma PL and cholesterol were lower in fish fed the VO diets. Among fish fed the CH+ diets, plasma TAG was lower in the VO group than in the FO group (carbohydrate and lipid source interaction).

The CH+ diet promoted an increase in FAS, G6PD and ME activities. G6PD activity was also responsive to dietary lipid source, being higher in VO diets (Table 7).

Gene expression

Hepatic transcript levels of glucokinase (*GK*), the first glycolytic enzyme, was higher in CH+ than in CH– groups, whereas 6-phosphofructokinase (*PFK*) transcript levels were not affected by dietary carbohydrate (Fig. 1). Hepatic transcript levels of phosphoenolpyruvate carboxykinase (PEPCK), the first key enzyme involved in gluconeogenesis, were lower in CH+ than in CH– groups, whereas the opposite occurred for hepatic glucose 6-phosphatase (*G6Pase*) mRNA levels, the enzyme involved in the last step of gluconeogenesis. Among glycolytic and gluconeogenic enzymes, only hepatic *G6Pase* transcript levels were up-regulated by dietary VO. At the intestinal level, no transcriptional regulation by diet composition of *PFK* and of *PEPCK* was observed (Fig. 1).

Hepatic and intestinal transcript levels of key enzymes involved in lipogenesis (FAS), *β*-oxidation (carnitine palmitoyltransferase 1A (*CPT1A*)) and PL synthesis (glycerol-3-phosphate acyltransferase (*GPAT*)) were not affected by diet composition (Fig. 2).

With the exception of lanosterol 14 *α*-demethylase, cytochrome P450 51 (*CYP51A1*) transcript levels in the intestine, which were higher in CH+ groups, no variation in hepatic or intestine transcript levels of proteins involved in cholesterol efflux (ATP-binding cassette G5 (*ABCG5*)), and cholesterol synthesis (7-dehydrocholesterol reductase (*DHCR7*); *CYP51A1*) and catabolism (liver X receptor *α* (*LXRα*)) were observed (Fig. 3).

The expression of genes encoding key proteins involved in the LC-PUFA-biosynthesis pathway (Δ6 fatty acyl desaturase-liver isoform (*FADS2*); elongase 5 (*elovl5*)) were nutritionally

Table 3. Growth performance and feed utilisation of gilthead sea bream fed the experimental diets
(Mean values and standard deviations; n 3)

	Experimental diets										
LS…	FO				VO						
CH…	CH–		CH+		CH–		CH+		P*		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH× LS
IBW (g)	70·8	0·03	70·7	0·03	70·8	0·03	70·7	0·02	1·000	0·370	0·094
FBW (g)	222·3	8·5	221·8	8·8	203·9	18·8	212·1	4·62	0·577	0·066	0·524
DGI†	2·37	0·10	2·37	0·10	2·16	0·22	2·26	0·05	0·553	0·073	0·503
Feed intake(g/kg ABW‡ per d)	18·4	0·6	18·4	1·5	16·7	0·8	17·3	1·3	0·633	0·059	0·716
FE§	0·70	0·03	0·69	0·06	0·71	0·04	0·72	0·04	1·000	0·453	0·899
PER‖	1·05	0·05	1·38	0·12	1·08	0·06	1·42	0·09	<0·001	0·514	0·895
N intake (g/kg ABW per d)	1·95	0·08	1·49	0·14	1·77	0·18	1·39	0·13	0·001	0·122	0·637
N retention (g/kg ABW per d)¶	0·34	0·03	0·34	0·01	0·35	0·01	0·34	0·02	0·648	0·878	0·648
Lipid intake (g/kg ABW per d)	3·37	0·14	3·39	0·31	3·04	0·30	3·15	0·29	0·681	0·104	0·757
Lipid retention (g/kg ABW per d)**	2·21^{B}	0·05	2·31	0·12	1·84^{a,A}	0·13	2·36^{b}	0·07	0·001	0·023	0·006

LS, lipid source; FO, fish oil; VO, blend of vegetable oils; CH, carbohydrates; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch; IBW, initial body weight; FBW, final body weight; ABW, average body weight; FE, feed efficiency; PER, protein efficiency ratio.

^{a,b,A,B} If interaction was significant, one-way ANOVA was performed for each factor, and means in the same line with different capital and small letters indicate significant differences ($P<0{\cdot}05$) between the two tested LP and two CH levels, respectively; means with no letters are not significantly different ($P>0{\cdot}05$).

* Significant differences at $P<0{\cdot}05$ two-way ANOVA.

† DGI: ((FBW$^{1/3}$ – IBW$^{1/3}$)/time (d)) × 100.

‡ ABW: (IBW + FBW)/2.

§ FE: wet weight gain/dry feed intake.

‖ PER: wet weight gain/crude protein intake.

¶ N retention*n* = ((FBW × carcass N content) – (IBW × carcass N content))/(ABW × the number of days).

** Lipid retention = ((FBW × carcass lipid content) – (IBW × carcass lipid content))/(ABW × the number of days).

Table 4. Whole-body, liver and muscle composition (wet-weight basis), hepatosomatic (HSI) and viscerosomatic (VSI) indexes of gilthead sea bream fed the experimental diets*
(Mean values and standard deviations)

	Experimental diets										
LS…	FO				VO						
CH…	CH–		CH+		CH–		CH+		P†		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
Whole-body composition‡											
Protein (%)	16·54	0·73	16·64	0·18	17·48	0·46	16·87	0·82	0·484	0·134	0·341
Lipids (%)	14·27^{B}	0·25	14·83	0·64	12·75^{a,A}	0·68	15·35^{b}	0·38	0·001	0·123	0·009
DM (%)	35·00	0·23	36·13	0·62	34·01	0·72	35·94	0·28	0·001	0·078	0·208
Ash	4·01	0·24	4·31	0·29	4·50	0·19	4·39	0·31	0·095	0·562	0·212
HSI§	1·23	0·17	1·87	0·20	1·23	0·22	1·96	0·31	<0·001	0·478	0·343
VSI‖	6·18	0·68	6·82	0·89	6·35	0·94	6·77	1·05	0·003	0·755	0·528
Liver composition											
Lipids (%)	13·88	3·25	12·01	2·79	10·39^{a}	3·15	13·95^{b}	2·98	0·413	0·461	0·021
Cholesterol (%)	0·34	0·068	0·26	0·034	0·35	0·058	0·257	0·025	<0·001	0·811	0·809
Glycogen (mg/g liver)	73·49^{a,A}	11·75	134·89^{b}	7·77	89·80^{a,B}	11·27	125·46^{b}	11·21	<0·001	0·338	0·001
Muscle composition											
Lipids (%)	8·23	1·06	9·85	2·10	8·08	1·17	9·43	1·05	0·020	0·661	0·877
Cholesterol (%)	0·097	0·008	0·088	0·008	0·09	0·009	0·097	0·010	0·811	0·831	0·051
Glycogen (µg/g muscle)	12·28^{a,A}	2·47	29·84^{b}	7·89	23·66^{B}	3·40	31·35	8·57	<0·001	<0·001	0·001

LS, lipid source; FO, fish oil; VO, blend of vegetable oils; CH, carbohydrates; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch.

^{a,b,A,B} If interaction was significant, one-way ANOVA was performed for each factor, and means in the same line with different capital and small letters indicate significant differences ($P<0{\cdot}05$) between the two tested LS and two CH levels, respectively; means with no letters are not significantly different ($P>0{\cdot}05$).

* n 3 For whole-body composition; n 6 for lipids and cholesterol; n 9 for glycogen; n 18 for HSI and VSI.

† Significant differences at $P<0{\cdot}05$ two-way ANOVA.

‡ Initial body composition on the fish: DM 28·96 %; protein 16·15 %; lipid 7·87 %; ash 5·84 %.

§ HSI: (liver weight/body weight) × 100.

‖ VSI: (viscera weight/body weight) × 100.

regulated both in the liver and in the intestine (Fig. 4). In the liver, *FADS2* transcript levels were upregulated by dietary carbohydrate and VO. *Elovl5* expression in the liver was down-regulated in CH+ groups, but in the intestine down-regulation was only evident in fish fed FO-based diets (carbohydrate and lipid source interaction). In addition,

Table 5. Liver fatty acid profile (% of total fatty acids) of gilthead sea bream fed the experimental diets*
(Mean values and standard deviations; *n* 6)

	Experimental diets										
LS…	FO				VO						
CH…	CH–		CH+		CH–		CH+		*P*†		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
14 : 0	4·3^{b,B}	0·29	3·5^{a,B}	0·46	2·4^{A}	0·28	2·8^{A}	0·32	0·185	<0·001	<0·001
15 : 0	0·51	0·04	0·32	0·03	0·36	0·06	0·25	0·03	<0·001	<0·001	0·073
16 : 0	19·40	0·87	22·29	0·69	22·03	2·13	25·60	1·48	<0·001	<0·001	0·643
17 : 0	0·45	0·04	0·31	0·01	0·43	0·04	0·27	0·01	<0·001	0·011	0·305
18 : 0	4·6^{a,A}	0·38	6·4^{b}	0·42	5·6^{B}	0·50	5·99	0·30	<0·001	0·074	<0·001
20 : 0	0·13	0·02	0·14	0·01	0·13	0·04	0·10	0·01	0·215	0·061	0·079
∑SFA	29·48	0·87	33·03	0·76	31·00	2·26	35·02	1·73	<0·001	0·011	0·737
16 : 1*n*-7	6·5^{B}	0·32	6·3^{B}	0·34	3·5^{a,A}	0·18	4·4^{b,A}	0·21	0·003	<0·001	<0·001
18 : 1*n*-9	22·14	1·62	27·92	1·24	27·07	1·87	32·20	1·51	<0·001	<0·001	0·475
20 : 1*n*-9	3·64	0·59	3·53	0·31	0·77	0·20	0·48	0·03	0·034	<0·001	0·108
22 : 1*n*-9	2·16	0·35	2·01	0·36	0·30	0·17	0·08	0·03	0·008	<0·001	0·068
∑MUFA	34·68	1·73	39·91	1·34	31·73	2·17	37·24	1·61	<0·001	0·001	0·795
18 : 2*n*-6	2·41	0·16	1·97	0·22	8·21	0·45	7·90	0·34	0·002	<0·001	0·109
18 : 3*n*-6	0·18^{B}	0·06	0·19^{B}	0·01	0·10^{a,A}	0·05	0·30^{b,A}	0·05	<0·001	0·864	<0·001
20 : 2*n*-6	0·26^{b}	0·02	0·21^{a,B}	0·01	0·25^{b}	0·05	0·15^{a,A}	0·01	<0·001	0·003	0·029
20 : 3*n*-6	0·14	0·02	0·11	0·01	0·08	0·04	0·11	0·02	0·515	0·031	0·071
20 : 4*n*-6	1·4^{b}	0·22	1·0^{a,B}	0·11	1·5^{b}	0·35	0·64^{a,A}	0·06	<0·001	0·003	0·003
∑*n*-6 PUFA	4·61	0·31	3·56	0·21	10·08	0·70	9·10	0·30	<0·001	<0·001	0·141
18 : 3*n*-3	0·91^{b,A}	0·09	0·69^{a,A}	0·08	11·1^{B}	0·82	10·5^{B}	0·84	<0·001	<0·001	0·012
18 : 4*n*-3	1·0^{b,B}	0·12	0·84^{a,B}	0·10	0·30^{a,A}	0·04	0·69^{b,A}	0·12	0·002	<0·001	<0·001
20 : 3*n*-3	0·18^{b,A}	0·03	0·13^{a,A}	0·02	0·65^{b,B}	0·16	0·37^{a,B}	0·04	<0·001	<0·001	0·020
20 : 4*n*-3	0·85	0·11	0·76	0·06	0·36	0·06	0·34	0·06	0·131	<0·001	0·412
20 : 5*n*-3	5·3^{b,B}	0·36	4·2^{a,B}	0·30	2·5^{b,A}	0·42	1·2^{a,A}	0·05	<0·001	<0·001	0·003
21 : 5*n*-3	0·27	0·03	0·21	0·05	0·04	0·05	0·00	0·01	0·023	<0·001	0·439
22 : 5*n*-3	2·6^{b,B}	0·31	2·0^{a,B}	0·24	1·3^{b,A}	0·36	0·47^{a,A}	0·05	<0·001	<0·001	0·017
22 : 6*n*-3	14·7^{b,B}	1·44	9·6^{a,B}	0·68	8·6^{b,A}	1·55	3·2^{a,A}	0·11	<0·001	<0·001	0·016
∑*n*-3PUFA	25·82	1·63	18·44	1·02	24·89	1·80	16·74	0·87	<0·001	0·024	0·398
Ratios											
SFA:PUFA	0·93	0·07	1·42	0·08	0·87	0·10	1·33	0·11	<0·001	0·060	0·846
n-3:*n*-6	5·6^{b,B}	0·18	5·2^{a,B}	0·28	2·5^{b,A}	0·20	1·8^{a,A}	0·05	<0·001	<0·001	0·007
Unsat. index	191·02	8·95	151·51	5·08	159·30	16·39	122·88	3·41	<0·001	<0·001	0·705

LS, lipid source; FO, fish oil; VO, blend of vegetable oils; CH, carbohydrates; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; unsat. index, unsaturation index = sum (fatty acid percentage) × (number of double bonds).

^{a,b,A,B} If interaction was significant, one-way ANOVA was performed for each factor, and means in the same line with different capital and small letters indicate significant differences ($P < 0·05$) between the two tested LS and two CH levels, respectively; means with no letters are not significantly different ($P > 0·05$).

* FA ≥ 0·02 %, <0·02 % was not considered in the table.

† Significant differences at $P < 0·05$ two-way ANOVA.

intestinal *elovl5* mRNA levels increased in VO groups when fed a carbohydrate-rich diet (carbohydrate and lipid source interaction).

In the liver transcription of *PPARα* and *PPARγ* was down-regulated in CH+ groups while in the intestine down-regulation of *PPARβ* transcript levels in CH+ groups was only observed when fish were fed the VO diet (carbohydrate and lipid source interaction) (Fig. 4). Additionally, intestine from the CH– groups exhibited an up-regulation of *PPARβ* transcripts when fish were fed the VO diet (carbohydrate and lipid source interaction).

Discussion

Effects of dietary carbohydrates

Although gilthead sea bream is a carnivorous species (trophic level 3·3–3·5 according to Fish Base), it tolerates up to 20 % dietary starch without detrimental effects in growth performance and feed efficiency. These results support previous evidences(61,62) and indicate the possibility to reduce aquafeed costs and alleviate the overexploitation of fisheries marine resources through the use of the carbohydrate component in gilthead sea bream diets.

The low glycaemia values at 18 h after feeding are in accordance with results of Peres *et al.*(63) that observed in a glucose tolerance test that seabream was able to restore glucose levels within 12 h after receiving an overdose of glucose.

Hepatic GK is a key player in blood glucose homoeostasis by catalysing the phosphorylation of glucose and providing the first substrate for glycolysis, glycogenesis and the pentose phosphate pathway(64). An induction of hepatic *GK* transcripts by dietary carbohydrates was recorded in the present study, as in a previous study in this species(65). On the contrary, dietary carbohydrates did not affect transcription levels of hepatic *PFK*, another key glycolytic enzyme.

Table 6. Muscle fatty acid profile (expressed as % of total fatty acids) of gilthead sea bream fed the experimental diets*
(Mean values and standard deviations; *n* 6)

	Experimental diets										
LS…	FO				VO						
CH…	CH–		CH+		CH–		CH+		*P*†		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
14 : 0	4·76	0·17	4·59	0·19	2·72	0·17	2·39	0·14	0·001	<0·001	0·108
15 : 0	0·52	0·02	0·43	0·01	0·34	0·01	0·24	0·01	<0·001	<0·001	0·002
16 : 0	19·24	0·54	19·99	0·71	19·06	0·82	19·63	0·33	0·018	0·307	0·754
17 : 0	0·39	0·02	0·30	0·01	0·32	0·02	0·23	0·01	<0·001	<0·001	0·179
18 : 0	3·30	0·22	3·72	0·17	3·59	0·15	4·01	0·10	<0·001	<0·001	0·875
20 : 0	0·15	0·01	0·14	0·00	0·20	0·01	0·18	0·01	0·013	<0·001	0·238
∑SFA	28·41	0·58	29·22	0·80	26·32	1·04	26·77	0·56	0·058	<0·001	0·585
16 : 1*n*-7	7·92	0·16	7·80	0·12	4·69	0·44	4·18	0·20	0·009	<0·001	0·058
18 : 1*n*-9	$24·42^{a,A}$	0·85	$25·76^{b,A}$	0·32	$28·76^{a,B}$	0·39	$32·23^{b,B}$	0·35	<0·001	<0·001	<0·001
20 : 1*n*-9	$3·83^{B}$	0·29	$4·06^{B}$	0·11	$1·14^{b,A}$	0·03	$0·95^{a,A}$	0·08	0·020	<0·001	<0·001
22 : 1*n*-9	$2·13^{a,B}$	0·18	$2·40^{b,B}$	0·12	$0·55^{b,A}$	0·04	$0·41^{a,A}$	0·04	0·778	<0·001	<0·001
∑MUFA	$38·50^{a,B}$	0·48	$40·20^{b,B}$	0·28	$35·28^{a,A}$	0·79	$37·93^{b,A}$	0·30	<0·001	<0·001	0·030
18 : 2*n*-6	4·19	0·32	3·66	0·47	9·13	0·55	9·25	0·35	0·140	<0·001	0·051
18 : 3*n*-6	0·28	0·04	0·26	0·06	0·20	0·07	0·25	0·04	0·429	0·059	0·081
20 : 2*n*-6	0·26	0·01	0·23	0·01	0·25	0·01	0·21	0·01	<0·001	0·004	0·241
20 : 3*n*-6	$0·14^{b,B}$	0·02	$0·11^{a,A}$	0·01	$0·11^{A}$	0·01	$0·12^{B}$	0·02	0·026	0·180	0·007
20 : 4*n*-6	$0·98^{b,B}$	0·03	$0·79^{a,B}$	0·04	$0·87^{b,A}$	0·07	$0·59^{a,A}$	0·04	<0·001	<0·001	0·007
∑*n*-6 PUFA	$5·85^{b,A}$	0·30	$5·04^{a,A}$	0·52	$10·56^{B}$	0·59	$10·41^{B}$	0·42	0·013	<0·001	0·047
18 : 3*n*-3	$1·10^{b,A}$	0·03	$1·03^{a,A}$	0·06	$11·19^{a,B}$	0·94	$12·60^{b,B}$	0·58	0·301	<0·001	0·001
18 : 4*n*-3	1·21	0·09	1·27	0·08	0·45	0·03	0·45	0·04	0·410	<0·001	0·206
20 : 3*n*-3	0·15	0·02	0·14	0·02	0·42	0·04	0·40	0·03	0·071	<0·001	0·884
20 : 4*n*-3	0·75	0·03	0·69	0·03	0·38	0·02	0·35	0·03	0·001	<0·001	0·723
20 : 5*n*-3	$5·10^{B}$	0·13	$5·01^{B}$	0·07	$2·75^{b,A}$	0·16	$1·87^{a,A}$	0·11	<0·001	<0·001	<0·001
21 : 5*n*-3	$0·25^{B}$	0·01	$0·23^{B}$	0·02	$0·11^{b,A}$	0·02	$0·03^{a,A}$	0·04	0·002	<0·001	0·006
22 : 5*n*-3	$2·15^{b,B}$	0·17	$1·86^{a,B}$	0·12	$1·44^{b,A}$	0·08	$0·99^{a,A}$	0·04	<0·001	<0·001	0·007
22 : 6*n*-3	$11·32^{b,B}$	0·86	$10·17^{a,B}$	0·09	$8·37^{b,A}$	0·54	$5·82^{a,A}$	0·38	<0·001	<0·001	0·001
∑*n*-3 LC PUFA	22·02	1·05	20·39	0·24	25·12	1·26	22·50	0·52	<0·001	<0·001	0·212
Ratios											
SFA:PUFA	0·97	0·05	1·10	0·05	0·72	0·06	0·80	0·03	<0·001	<0·001	0·423
n-3:*n*-6	$3·78^{B}$	0·29	$4·08^{B}$	0·46	$2·38^{b,A}$	0·15	$2·16^{a,A}$	0·11	0·943	<0·001	0·023
Unsat. index	$173·26^{b}$	5·55	$163·51^{a,B}$	1·57	$170·48^{b}$	5·27	$153·53^{a,A}$	2·59	<0·001	0·001	0·029

LS, lipid source; FO, fish oil; VO, blend of vegetable oils; CH, carbohydrates; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; unsat. index, unsaturation index = sum (fatty acid percentage) × (number of double bonds).

[a,b,A,B] If interaction was significant, one-way ANOVA was performed for each factor, and means in the same line with different capital and small letters indicate significant differences ($P < 0·05$) between the two tested LS and two CH levels, respectively; means with no letters are not significantly different ($P > 0·05$).

* FA ≥ 0·02 %, <0·02 % was not considered in the table.

† Significant differences at $P < 0·05$ two-way ANOVA.

Hepatic transcript levels of *PEPCK*, the first key enzyme of gluconeogenesis, were down-regulated by dietary carbohydrate, but the transcript levels of *G6Pase*, another key enzyme of gluconeogenesis, was up-regulated by dietary carbohydrates. Although a similar response of hepatic G6Pase by dietary carbohydrate at the transcriptional level was previously reported in carnivorous rainbow trout(24), present data apparently contradict results of Panserat *et al.*(66) in this species, who observed that *G6Pase* and *FBPase* were down-regulated by dietary carbohydrates at the transcriptional level, whereas *PEPCK* was not affected. At the enzymic activity level, carbohydrates in gilthead sea bream diets induced minor effects(62,67) or even an increase(61) in the hepatic activities of gluconeogenesis enzymes. Recent studies pointed out that the intestine has important functions in glucose homoeostasis(68,69). However, present results do not support that assumption, as expression of glycolytic (*PFK*) and gluconeogenesis (*PEPCK*) enzymes in this tissue did not respond to dietary carbohydrate.

In parallel with the hepatic up-regulation of *GK* transcripts, increased HSI and VSI were recorded in fish fed carbohydrate-rich diets, which may indicate that the hepatic glucose pool was directed towards glycogen and/or FA synthesis. Indeed, in liver, an increased deposition of glycogen and a higher lipogenic potential, indicated by FAS, G6PD and ME activities, were recorded in CH+ groups. Increased HSI may have unwanted physiological effects; therefore, histomorphological analysis of liver should be considered in future studies to discard any histopathological damage induced by diet.

Despite the fact that lipogenesis was nutritionally regulated by dietary carbohydrates, the contribution to the overall lipid deposition in liver or whole body of fish fed FO-based diet seemed to be minor, as lipids in liver and whole body increased with carbohydrate intake only under a VO-based diet regimen.

Recently, it was suggested that dietary carbohydrates have a role in cholesterol biosynthesis by inducing (at least at a molecular level) the capacity to produce it(7). In the present

Table 7. Plasma metabolite concentrations (n 18) (mmol/l) and enzymatic activity (μ/mg protein) of selected enzymes involved in lipogenesis(n 9) in gilthead sea bream fed the experimental diets
(Mean values and standard deviations)

	Experimental diets										
LS…	FO				VO						
CH…	CH–		CH+		CH–		CH+		*P**		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
Plasma metabolites											
GLU	3·73	0·60	3·16	0·49	3·47	0·34	3·19	0·46	<0·001	0·312	0·203
CHOL	7·37^{B}	0·53	7·25^{B}	0·95	6·80^{b,A}	0·99	5·82^{a,A}	0·65	0·005	<0·001	0·027
TAG	2·87^{a}	0·63	3·73^{b,B}	0·73	3·15	0·86	3·15^{A}	0·67	0·015	0·383	0·016
PL	15·37	1·47	15·45	1·44	14·46	2·54	13·42	1·26	0·246	0·001	0·178
Enzyme activity											
G6PD	140·1	36·3	203·7	49·1	198·6	27·6	240·0	68·6	0·004	0·009	0·511
ME	6·97	3·10	9·58	3·49	5·51	1·96	11·63	4·37	0·001	0·791	0·130
FAS	4·28	2·76	7·38	1·75	5·46	2·91	8·60	3·61	0·003	0·225	0·981

LS, lipid source; FO, fish oil; VO, blend of vegetable oils; CH, carbohydrates; CH content, 0 % (CH–) or 20 % (CH+) gelatinised maize starch; GLU, glucose; CHOL, total cholesterol; PL, phospholipids; G6PD, glucose-6-phosphate dehydrogenase; ME, malic enzyme; FAS, fatty acid synthase.

[a,b,A,B] If interaction was significant, one-way ANOVA was performed for each factor, and means in the same line with different capital and small letters indicate significant differences ($P < 0.05$) between the two tested LS and two CH levels, respectively; means with no letters are not significantly different ($P > 0.05$).

* Significant differences at $P < 0.05$ two-way ANOVA.

study, it was observed that gilthead sea bream fed carbohydrate-rich diets exhibited reduced liver and plasma (only in VOCH+ group) cholesterol content. Furthermore, an increased expression at the intestinal level of one gene encoding for an enzyme involved in the last steps of cholesterol biosynthesis, *CYP51A1*, was also observed. We hypothesise that the depletion of the cholesterol pool was promoted by a lower dietary input of cholesterol, and the up-regulation of CYP51A1 may reflect an increased synthesis of sterols by the intestinal cells in response to the low dietary supply. Metabolic adjustments in *de novo* cholesterol biosynthesis in response to the dietary load of cholesterol were previously described in Atlantic salmon(70).

In this work, we demonstrated a transcriptional up-regulation by dietary carbohydrate of liver *FADS2*, a key desaturase involved in LC-PUFA synthesis. With the up-regulation of *FADS2* gene, it was expected to observe increased transcript levels of *elovl5*, a fatty acyl elongase involved in LC-PUFA biosynthesis. However, and somehow surprisingly, the reverse pattern was observed both in the intestine (only in FOCH+ group) and in the liver of gilthead sea bream fed CH+ diets. In the present state of knowledge, we have no clear explanation for these conflicting results, and therefore further studies are needed to better understand the apparent atypical molecular regulation of some enzymes of the LC-PUFA synthesis pathway by dietary carbohydrate in gilthead sea bream. Irrespective of the regulation of *FADS2* expression, increased liver or muscle n-3 LC-PUFA content of gilthead sea bream fed CH+ diets were not observed. On the contrary, reduced n-3 LC-PUFA content in CH+ groups was recorded, which could be related to an increase in SFA derived by lipogenesis from carbohydrates, as previously reported in other species such as rainbow trout and European sea bass(7,71).

PPAR are a family of nuclear receptors that have three isoforms in mammals known as PPARα, PPARβ and PPARγ that play key roles in regulation of lipid metabolism(72). It is believed that PPAR control metabolic pathways that support FA β-oxidation (especially PPARα), tissue lipid deposition and lipogenesis (especially PPARγ), and the overall lipid homoeostasis (especially PPARβ), mainly by means of the action of its ligands (such as FA and their derivatives, and also glucose)(72,73). In this study, despite the down-regulation of *PPARα,β* gene expression in the liver and of *PPARβ* in the intestine (only in VO diets) by dietary carbohydrates, gene expression of *CPT1*, a marker of mitochondrial FA β-oxidation, was unaffected both in the liver and intestine of fish fed CH+ diets. In previous studies, a synchronised regulation between piscine *PPARα,β* gene expression and the activity or expression of other enzymes involved in FA β-oxidation, such as acyl-CoA oxidase and L-3-hydroxyacyl-CoA dehydrogenase, was described(74,75). However, present results are not completely unexpected, as in a number of studies the expression of the *CPT1* gene was not nutritionally regulated or directly linked to the FA β-oxidation capacity(24,37,76). Furthermore, FA β-oxidation and lipogenesis are two pathways usually regulated in opposite directions(77,78). Therefore, as lipogenic potential increased in liver of fish fed CH+ diets, it is possible that FA β-oxidation might have been repressed. Additional data on the activity or expression of the other FA β-oxidation related enzymes would be necessary to confirm this hypothesis.

In the above discussion, differences in metabolic responses were assumed to be related to differences in dietary carbohydrate (0 *v.* 17–18 %). However, a potential effect of dietary protein cannot be discarded, particularly in relation to regulation of gluconeogenesis and lipogenesis, as there is increasing evidence that protein and amino acids also modulate these pathways(79–82).

Effects of dietary lipid source

In the present study, gilthead sea bream performed as well as in previous studies with gilthead sea bream juveniles in which

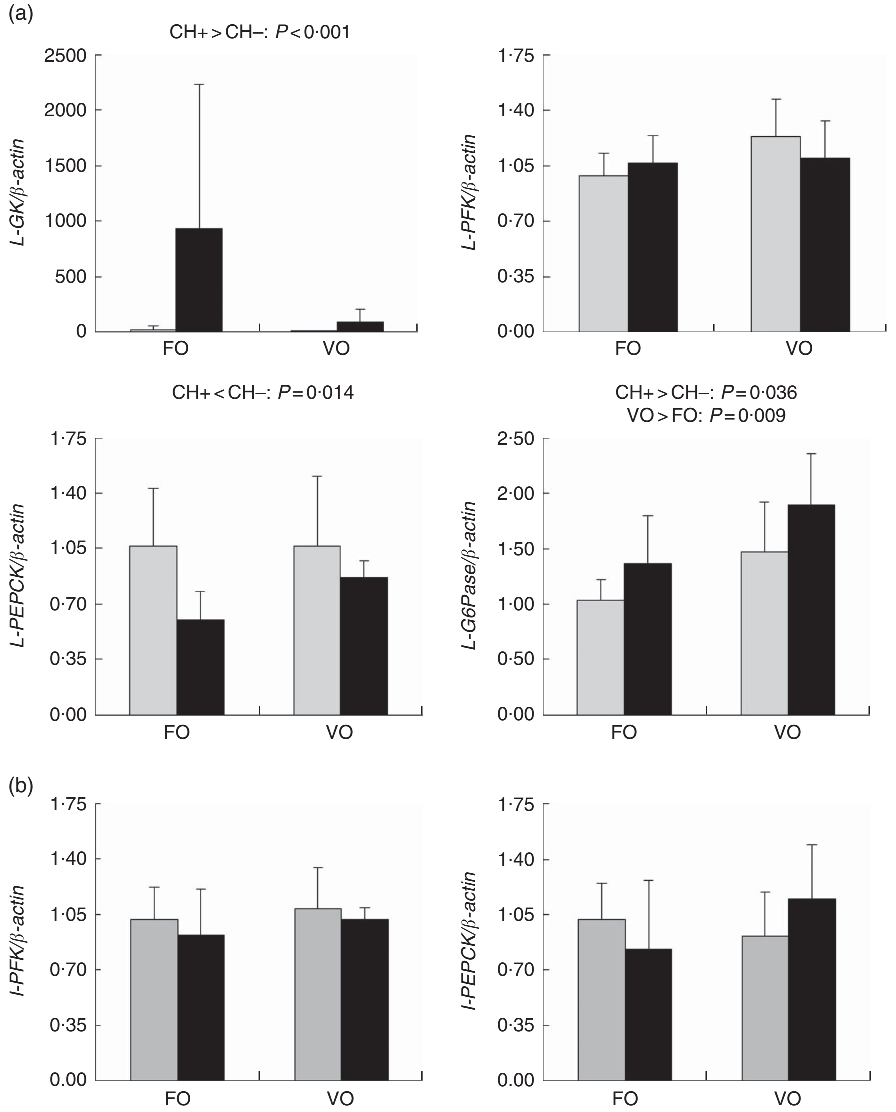

Fig. 1. mRNA levels of genes involved in glycolysis (*glucokinase (GK)*, *6-phosphofructokinase (PFK)*) and gluconeogenesis (*phosphoenolpyruvate carboxykinase (PEPCK)*, *glucose 6-phosphatase (G6Pase)*) in the (a) liver and (b) intestine of gilthead sea bream fed the experimental diets. Expression values are normalised by *β-actin*-expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0 % (▨, CH–) or 20 % (■, CH+) gelatinised maize starch. Relative fold difference between treatments are presented as means (*n* 6), with their standard deviations. Significant differences at $P < 0.05$ (two-way ANOVA).

dietary FO was partially (70 %) replaced by VO in FM[83,84] or plant protein[85,86]-based diets. However, plasma cholesterol and PL concentrations were lower in fish fed the VO diets. Replacing FO with VO usually increases dietary phytosterol and reduces cholesterol content. Such dietary modifications have been reported to induce a decrease in plasma cholesterol and LDL-cholesterol both in humans[87,88] and in fish[35–37,89].

Phytosterols are structurally similar to cholesterol and may induce a relative cholesterol deficiency by mechanisms that interfere with cholesterol absorption such as competition for space in mixed micelles or competition with cholesterol transporters (such as ABC transporters)[87,90]. For instance, in Atlantic salmon, dietary inclusion of soyabean meal and soya saponins decreased the expression of *ABCG5* in the distal intestine[91] and in the liver[92]. In the present study, however, no repression of *ABCG5* expression related to diet composition was observed at hepatic or intestinal levels. Furthermore, no induction in the expression of the selected genes (*CYP51A1* and *DHCR7*) involved in the cholesterol biosynthesis was recorded in the intestine and liver of fish fed the VO diets. This differs from other studies in which hepatic or intestinal gene expression of key enzymes involved in cholesterol biosynthesis pathways (such as 3-hydroxy-3-methylglutaryl-CoA reductase, isopentenyl-diphosphate Δisomerase, *CYP51A1* or *DHCR7*) increased in response to the decreased plasma cholesterol concentration in fish fed plant-based[91–93] or VO[7,10,39] diets. Whether the lack of activation of cholesterol biosynthesis at the molecular level as a response to the reduced plasma cholesterol concentration that was observed in the present study is due to variations in the dietary cholesterol supply or due to species specific sensitivities to alternative ingredients, such as VO, remains to be clarified.

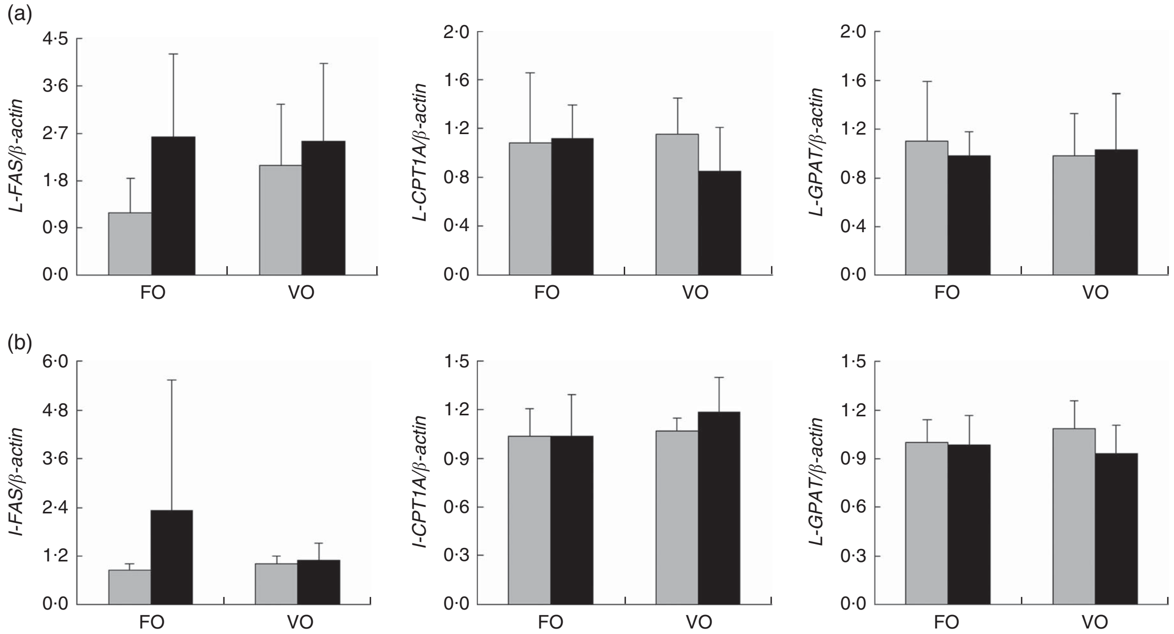

Fig. 2. mRNA levels of genes involved in lipogenesis (*fatty acid synthase (FAS)*), β-oxidation (*carnitine palmitoyltransferase 1A (CPT1A)*) and phospholipid synthesis (*glycerol-3-phosphate acyltransferase (GPAT)*) in the (a) liver and (b) intestine of gilthead sea bream fed the experimental diets. Expression values are normalised by *β-actin*-expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0 % (□, CH–) or 20 % (■, CH+) gelatinised maize starch. Relative fold difference between treatments are presented as means (*n* 6), with their standard deviations. Significant differences at $P < 0.05$ (two-way ANOVA).

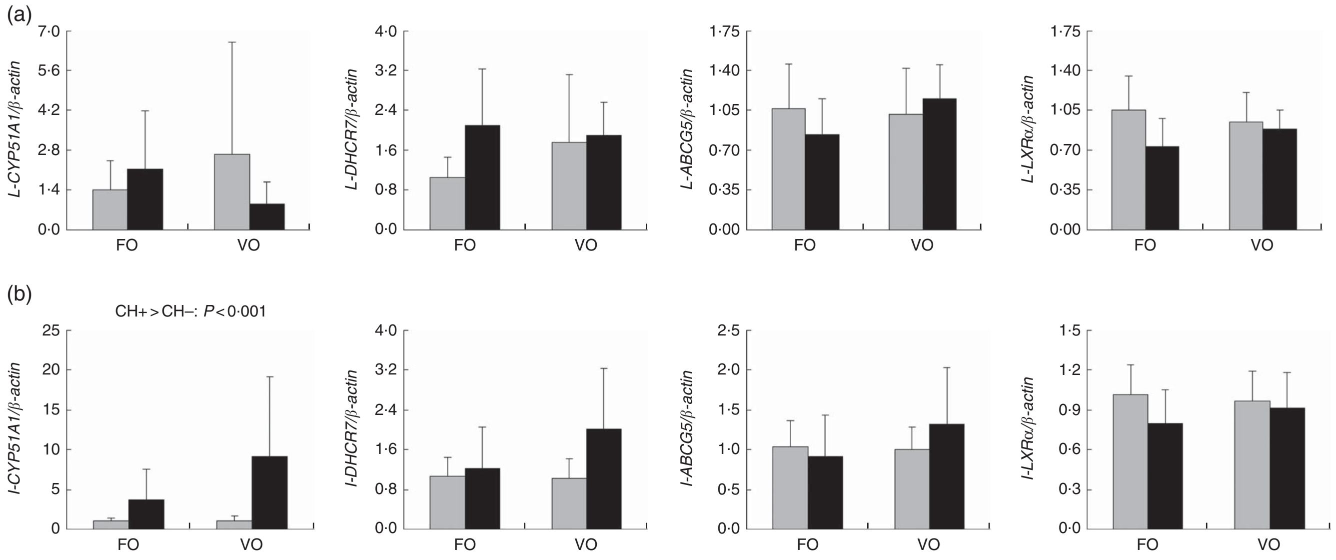

Fig. 3. mRNA levels of genes involved in cholesterol metabolism (lanosterol 14-*α* demethylase, cytochrome P450 51 (*CYP51A1*); 7-dehydrocholesterol reductase (*DHCR7*); liver X receptor *α* (LXR*α*)) and transport (ATP binding cassette G5 (*ABCG5*)) in the (a) liver and (b) intestine of gilthead sea bream fed the experimental diets. Expression values are normalised by *β*-actin-expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0 % (□, CH–) or 20 % (■, CH+) gelatinised maize starch. Relative fold difference between treatments are presented as means (*n* 6), with their standard deviations. Significant differences at $P < 0.05$ (two-way ANOVA).

An intestinal and hepatic induction of expression or increased activity of enzymes involved in PL biosynthesis has been reported in carnivorous fish fed VO[38] or plant feedstuff-based diets[93]. However, the biosynthesis rate seems to be insufficient to avoid an accumulation of lipid droplets in the enterocytes[94,95]. This condition is usually associated with a PL deficit that promotes an impairment in lipoprotein assembly and export from enterocytes and reduces PL concentration in plasma[7,35,36].

In the present study, despite the lower plasma PL concentration in VO groups, a nutritional regulation of transcript levels of *GPAT* was not observed in the intestine or the in liver of gilthead sea bream fed the experimental diets. *GPAT* is involved in the first steps of glycerol-3-phosphate

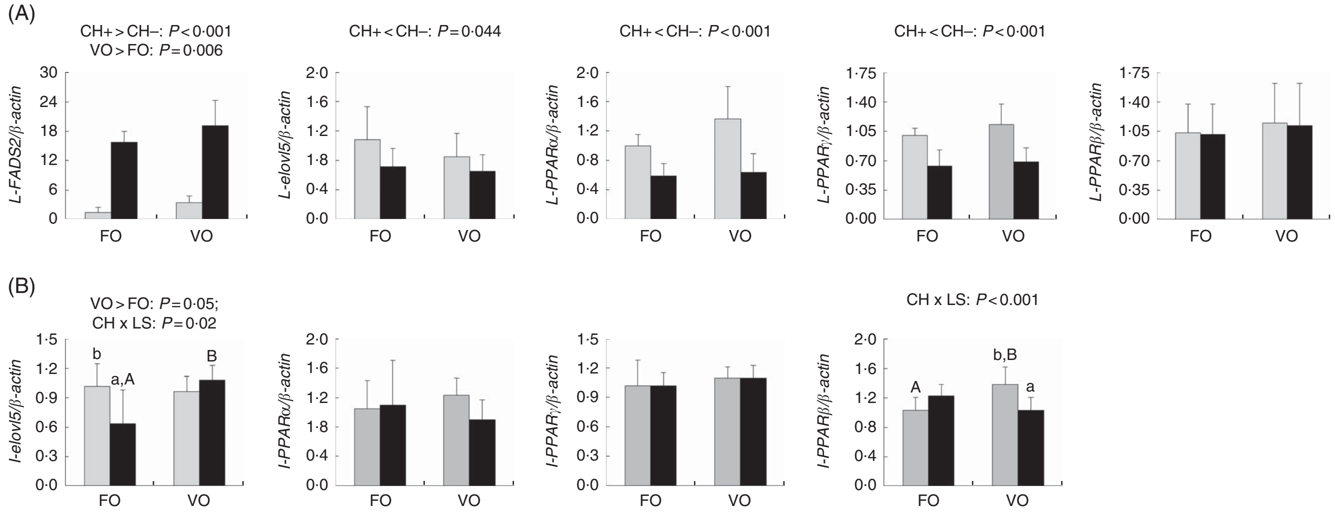

Fig. 4. mRNA levels of genes involved in the long-chain PUFA-biosynthesis pathway (*Δ6 fatty acyl desaturase (FADS2)*, *elongase 5, (elovl5)*) and transcription factors involved in several lipid-related processes (*PPARα,γ,β*) in the (A) liver and (B) intestine of gilthead sea bream fed the experimental diets. Expression values are normalised by *β-actin*-expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0 % (▨, CH–) or 20 % (■, CH+) gelatinised maize starch. Relative fold difference between treatments are presented as means (*n* 6), with their standard deviations. Significant differences at $P < 0.05$ (two-way ANOVA). If interaction was significant, one-way ANOVA was performed for each factor. [a,b,A,B] Means with different capital and small letters indicate significant differences ($P < 0.05$) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different ($P > 0.05$).

pathway, which according to Caballero *et al.*(38) is the main pathway implicated in intestinal PL synthesis in gilthead sea bream. Therefore, further investigation on the nutritional regulation of the enzymes involved in downstream steps of the PL biosynthesis pathways is required to understand PL biosynthesis regulation in gilthead sea bream.

Dietary lipid source is known to regulate lipogenesis and FA bioconversion pathways, which may affect tissue lipid deposition and FA composition. Although in some studies increased hepatic lipogenic enzymes activity and lipid content have been reported in fish fed diets in which dietary FO was replaced with VO(6,19), in other studies such effects were not demonstrated(7,35,36,96). In the present study, dietary lipid source did not affect hepatic and muscular lipid content, and a decrease in whole-body lipid content was reported within CH– groups when FO was substituted by VO. On the other hand, liver and muscle FA composition was strongly influenced by dietary replacement of FO by VO, as previously reported in gilthead sea bream(97) and in other species(7,8,29,98). According to the predicted effects of dietary oils in muscle FA composition of 1-year-old gilthead sea bream with different nutritional backgrounds(97), in the present study liver and muscle lipids increased the content of 18 : 1n-9, 18 : 2n-6 and 18 : 3n-3, whereas total lipid reduced the content of 20 : 5n-3 and 22 : 6n-3 when FO was replaced by VO. Thus, despite the hepatic up-regulation of FADS2 in fish fed the VO-based diets, the low proportion of LC-PUFA or their intermediary products such as 18 : 3n-6 and 18 : 4n-3 in VO groups suggest a limited FADS2 activity. Maximal FADS2 efficiency is thought to be modulated by the levels of substrate and product availability(30). In this sense, in the present study the limited accumulation of FA intermediates could partly have been caused by an inadequate dietary supply of substrate and/or product availability. Accordingly, in a previous study with this species using a diet completely devoid of LC-PUFA and containing olive oil as the sole lipid source, Seiliez *et al.*(47) observed higher transcript levels of FADS2 and increased accumulation of FA intermediates (18 : 2n-9, 20 : 2n-9 and 18 : 3n-6) in fish fed that diet comparatively to fish fed an LC-PUFA-rich diet, which suggested the existence of FADS2 activity.

In this study, the low efficacy in induction of *FADS2* expression and of nutritional regulation of *elovl5* transcripts by VO observed, along with lack of Δ5 desaturase activity observed *in vitro*(99), may explain the low capacity of conversion of C18 PUFA into LC-PUFA at an appreciable rate in gilthead sea bream.

Dietary carbohydrate content and lipid source interaction

The interactions between dietary carbohydrate content and lipid source that were recorded on the whole-body, liver and plasma lipid content suggest that the overall effect of starch intake on lipid deposition was disturbed by the change in dietary lipid source. Indeed, an increase in lipid retention with dietary starch intake was more evident when fish were fed the VO-based diet. This assumption is supported by the higher, whole-body and liver lipid content in the VOCH+ group than in the VOCH– group and can be related with the coupled increase in the lipogenic potential of dietary carbohydrates (higher liver FAS, ME and G6PD activities) and VO (higher liver G6PD activity). On the other hand, under an FO-based diet regimen, the unaltered lipid retention, liver and whole-body lipid content, but elevated TAG levels following starch intake, suggest a higher lipid mobilisation, transport and/or utilisation in the FOCH+ group than in the FOCH– group. No molecular markers for lipid uptake were assessed in this study in liver and intestine, and the one evaluated here and related to catabolism (*CPT1A*) does not help to understand or clarify this hypothesis because transcriptional regulation of these proteins by nutrients was not recorded.

At the same time, we also noticed that the stimulatory effect of dietary starch on muscle glycogen deposition was attenuated and became non-significant under the VO-based dietary regimen, probably related with the fact that dietary VO *per se*, but not in combination with carbohydrates, had an inductor effect on glycogenesis, as suggested by the higher muscle and liver glycogen levels in the VOCH– group than in the FOCH– group.

Furthermore, it was observed that intake of starch when coupled with dietary VO seemed to enhance the hypocholesterolemic effects of dietary VO as plasma cholesterol concentration decreased with carbohydrate intake only when fish were fed VO-based diet. It is important to note here that, within the molecular actors involved in cholesterol-related processes (*CYP51A1*, *DHCR7*, *ABCG5*, *LXRα*) assessed in liver and intestine in the present study, no molecular difference induced by a nutritional interaction of nutrients that could reflect this phenotype was found. Therefore, the physiological or metabolic mechanisms underlying this finding remain to be demonstrated.

Conclusion

To our knowledge, this is the first study in a marine fish species reporting a transcriptional induction of *FADS2* by dietary carbohydrate, in addition to VO. Although the *n*-3 LC-PUFA biosynthesis pathway was insufficient to compensate for the reduced dietary EPA and DHA in VO-based diets, this study provides new perspectives on the use of nutritional strategies for inducing LC-PUFA biosynthesis in marine fish species.

Furthermore, change in dietary lipid source seemed to modify the overall effect of starch intake on mechanisms involved in cholesterol body pools, lipid and glycogen body allocation. Considering that these metabolites greatly influence the fish quality, the present findings highlight the critical need to assess the potential effects between dietary nutrients on metabolic-related processes involved in tissue metabolites deposition, especially in the context of alternative aquafeeds rich in VO and carbohydrates.

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C. C. carried out the main experimental work and wrote the draft of the manuscript under the direction of the project designer and leaders A. O.-T., G. C. and S. P.; A. D. assisted with the biochemical analyses; and L. L. performed the fatty acid analyses. All authors contributed to and approved the manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S000711451600163X

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