

## Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles

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### Abstract

This study aimed to evaluate the effects of dietary lipid source and carbohydrate content on the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. For that purpose, four diets were formulated with fish oil (FO) and vegetable oils (VO) as the lipid source and with 20 or 0% gelatinised starch as the carbohydrate source, in a 2×2 factorial design. Liver and intestine antioxidant enzyme activities (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD)), hepatic and intestinal lipid peroxidation (LPO), as well as hepatic oxidative stress index (OSI), were measured in fish fed the experimental diets for 73 d (*n* 9 fish/diet). Carbohydrate-rich diets promoted a decrease in hepatic LPO and OSI, whereas the lipid source induced no changes. Inversely, dietary lipid source, but not dietary carbohydrate concentration, affected LPO in the intestine. Lower intestinal LPO was observed in VO groups. Enzymes responsive to dietary treatments were GR, G6PD and CAT in the liver and GR and GPX in the intestine. Dietary carbohydrate induced GR and G6PD activities and depressed CAT activity in the liver. GPX and GR activities were increased in the intestine of fish fed VO diets. Overall, effects of diet composition on oxidative status were tissue-related: the liver and intestine were strongly responsive to dietary carbohydrates and lipid sources, respectively. Furthermore, different metabolic routes were more active to deal with the oxidative stress in the two organs studied.

**Key words:** Antioxidant enzymes: Feedstuffs: Lipid oxidative damage: Carbohydrates: Vegetable oils

Substitution of fishmeal (FM) and fish oil (FO) in aquafeeds by more readily available and sustainable alternative ingredients such as plant feedstuffs (PF) has become a priority issue in aquaculture<sup>(1)</sup>. However, dietary replacement of fishery by-products by PF may have negative health-related effects, especially in carnivorous species. For instance, vegetable oils (VO) were reported to induce alterations of the immune response in different fish species<sup>(2–7)</sup>, change the fatty acid profile of immune cells or modify eicosanoid production<sup>(2,5,6,8)</sup>. Inflammation and pathological morphological modifications in the intestine and steatosis in the liver were also reported in several species fed VO-rich diets<sup>(9–13)</sup>. Inadequate dietary nutrient balance, high fibre or carbohydrate content and the presence of anti-nutritional factors in PF are possible causes for such effects<sup>(14,15)</sup>.

Oxidative stress occurs when balance between the generation of reactive oxygen species (ROS) exceeds that of ROS removal, thus inducing oxidative damage. ROS are produced at

a controlled rate during normal cell metabolism<sup>(16)</sup>. However, under stressful conditions such as nutritional alterations, ROS may dramatically increase and induce oxidative stress. Accordingly, variations in diet macronutrient sources<sup>(7,17–25)</sup>, dietary protein:non-protein ratios<sup>(26–29)</sup> or non-protein energy sources<sup>(30)</sup> have been linked to modifications of fish susceptibility to lipid peroxidation (LPO).

The knowledge about the effects of macronutrients on oxidative stress is still limited and the available data are contradictory. Regarding the effect of dietary lipid source, VO were reported to either decrease<sup>(7,18,20,23,24)</sup>, increase<sup>(22)</sup> or promote no effects<sup>(31)</sup> in fish hepatic and/or muscular LPO susceptibility. High dietary protein and/or lipid concentrations were observed to increase muscular LPO susceptibility<sup>(26,28)</sup>, whereas dietary carbohydrates decreased LPO in muscles and the liver<sup>(27,28,30)</sup>.

To prevent oxidative damage, organisms have evolved effective antioxidant systems, consisting of both non-enzymatic (GSH, vitamins and carotenoids, among others) and enzymatic

**Abbreviations:** CAT, catalase; FO, fish oil; G6PD, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; LPO, lipid peroxidation; OSI, oxidative stress index; SOD, superoxide dismutase; VO, vegetable oil.

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mechanisms. Enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR) play key roles in the antioxidant defence mechanism<sup>(32,33)</sup>. Available literature suggests that dietary lipid source has no effect on the liver and muscle activities of SOD, CAT and/or GPX<sup>(22,31,34)</sup> or decreased SOD activity in VO-rich diets<sup>(35)</sup> in a number of fish species.

Concerning macronutrient modifications, it was reported that in common dentex (*Dentex dentex*) high dietary lipid concentrations induced an increase in hepatic SOD activity at high dietary protein concentrations, and of GPX, independent of dietary protein concentrations<sup>(28)</sup>. The authors also observed an interaction between dietary protein and carbohydrate or lipid concentrations in muscular GR and hepatic SOD and CAT activities. In yellow catfish (*Pelteobagrus fulvidraco*), a decrease in CAT and GPX activities as dietary carbohydrate:lipid ratio increased was also observed<sup>(30)</sup>. In contrast, partial substitution of dietary proteins with carbohydrates induced no major alterations in antioxidant enzyme activities in Senegalese sole (*Solea senegalensis*) liver<sup>(29)</sup>.

Overall, current knowledge about the effects of alternative feed ingredients, which may be rich in carbohydrates or VO, on the oxidative status of animals is still very scarce. Thus, in this study, we evaluated the effect of the dietary lipid source and carbohydrate manipulation on oxidative defence mechanisms in the liver and intestine of European sea bass (*Dicentrarchus labrax*) juveniles.

## Methods

### Experimental diets

Four diets differing in carbohydrate content (0 and 20% gelatinised starch, CH– and CH+, respectively) and lipid source (fish oil (FO) or a VO blend, FO and VO, respectively) were formulated (Table 1). The dietary carbohydrate content was increased at the expense of dietary protein. The VO blend was composed of 20% rapeseed, 50% linseed and 30% palm oils and replaced approximately 70% of dietary lipids, which were provided by cod liver oil and FM. All the ingredients were finely ground, well-mixed and dry-extruded in a laboratory pellet mill (California Pellet Mill), through a 3-mm die. The pellets were air-dried for 24 h and stored in a refrigerator (4°C) until used.

### Animals, experimental conditions and sampling

This experiment was directed by accredited 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 thermo-regulated recirculation water system, equipped with twelve fibre-glass cylindrical tanks of 300 litre water capacity, and supplied with continuous flow of filtered seawater.

After 2 weeks of adaptation to the experimental conditions, twelve groups of twenty European sea bass juveniles (initial body weight 74.0 (SD 1.5) g) were established and randomly

**Table 1.** Ingredients and chemical composition of the experimental diets

Lipid source...	Experimental diets			
	FO		VO	
Carbohydrates...	CH–	CH+	CH–	CH+
Ingredients (% dry weight)				
Fishmeal*	86.5	64.5	86.5	64.5
Gelatinised maize starch†	0	20	0	20
Cod liver oil‡	10	12	0	0
Vegetable oil blend§	0	0	10	12
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	89.5	90.4	91.3	91.0
Crude protein (CP)	62.4	46.6	62.4	47.1
Crude lipid (CL)	18.4	18.4	18.2	18.3
Gross energy (kJ/g)	26.2	25.8	26.7	25.6
Starch	0.8	18.7	0.8	17.5
Ash	17.6	14.0	17.6	13.8
Cholesterol	0.5	0.4	0.4	0.3

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

\* Steam-dried low-temperature fishmeal; Pesquera Diamante (CP: 71.1% DM; CL: 8.8% DM).

† C-Gel Instant-12018; Cerestar.

‡ Labchem, Laborspirit Lda.

§ 30% Palm oil (Colmi), 50% linseed oil (Sociedade Portuguesa de Drogas) and 20% rapeseed oil (Huilerie Emile Noël SAS).

|| Vitamins (mg/kg diet): retinyl acetate, 18 000 IU (6.19 mg)/kg diet; cholecalciferol, 2000 IU (0.04 mg)/kg diet; α-tocopherol 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).

distributed into the tanks. The experimental diets were randomly assigned to triplicate groups of fish (*n* 3 tanks/diet). During the trial, salinity averaged 35 (SD 1.0) g/l, dissolved oxygen was kept near saturation and water temperature was regulated to 25.4 (SD 0.5)°C. The photoperiod was the natural one for June–August. The trial lasted 73 d, and during that period fish were hand-fed twice a day, 6 d/week, to apparent visual satiety. Food intake and mortality were recorded daily, and fish in each tank were bulk-weighted at the end of the trial, after 1 d of feed deprivation to empty gut contents. To eliminate handling stress, the fish were continued to be fed for one more week, and subsequently 18 h after the last meal (previous day afternoon meal) three fish per tank (*n* 9 fish/diet) were killed with a sharp blow to the head; the liver and intestines were removed, immediately frozen in liquid N<sub>2</sub> and stored at –80°C until enzymatic analyses.

### Diet analysis

Chemical analyses of the experimental diets were carried out according to Association of Official Analytical Chemists<sup>(36)</sup> as follows: in brief, DM after drying at 105°C until constant weight;

ash by incineration in a muffle furnace at 450°C for 16 h; protein content ( $N \times 6.25$ ) by the Kjeldahl method after acid digestion using a Kjeltex digestion and distillation units (models 1015 and 1026, respectively; Tecator Systems); starch content according to Beutler<sup>(37)</sup>; gross energy by direct combustion in an adiabatic bomb calorimeter (Parr model 6200; Parr Instruments); and lipid content by petroleum diethyl ether extraction (Soxtec HT System; Höganäs). Fatty acid methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride in methanol (14%) according to Santha & Ackman<sup>(38)</sup> and were analysed by GC (Varian 3900; Varian) as described by Castro *et al.*<sup>(39)</sup>.

### Enzymatic activity assays

Liver and whole intestine samples were 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 the 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 the aliquots were stored at -80°C until analysis.

All enzyme assays were carried out at 37°C. Changes in absorbance were monitored using a microplate reader (ELx808; BioTek Instruments) to determine enzyme activities. The optimal substrate and protein concentrations for maximal activity measurement of each enzyme were established by preliminary assays. The molar extinction coefficients used for H<sub>2</sub>O<sub>2</sub> and NADPH were 0.039 and 6.22 mm<sup>-1</sup> × cm<sup>-1</sup>, respectively.

The specific assay conditions for each enzyme were as follows.

SOD (*EC* 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals<sup>(40)</sup>. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.8), 0.1 mM-EDTA, 0.1 mM-xanthine, 0.012 mM-cytochrome C and 0.025 IU/ml xanthine oxidase. Activity was reported as units per mg of protein. One unit of activity was defined as the amount of enzyme necessary to produce 50% inhibition of ferricytochrome C reduction rate.

CAT (*EC* 1.11.1.6) activity was determined according to Aebi<sup>(41)</sup> by measuring the decrease in H<sub>2</sub>O<sub>2</sub> concentration at 240 nm. The reaction mixture contained 50 mM-potassium phosphate buffer (pH 7) and 10 mM-H<sub>2</sub>O<sub>2</sub> freshly added.

GPX (*EC* 1.11.1.9) activity was assayed as described by Flohé & Günzler<sup>(42)</sup>. The GSSG generated by GPX was reduced by GR, and NADPH consumption rate was monitored at 340 nm. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.1), 1 mM-EDTA, 3.9 mM-GSH, 3.9 mM-sodium azide, 1 IU/ml GR, 0.2 mM-NADPH and 0.05 mM-H<sub>2</sub>O<sub>2</sub>.

GR (*EC* 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales *et al.*<sup>(43)</sup>. The reaction mixture consisted of 0.1 M-sodium phosphate buffer (pH 7.5), 1 mM-EDTA, 0.63 mM-NADPH and 0.16 mM-GSSG.

Glucose-6-phosphate dehydrogenase (G6PD; *EC* 1.1.1.49) activity was assayed by measuring the reduction of NADP<sup>+</sup> at 340 nm as previously described by Morales *et al.*<sup>(44)</sup>, using a reaction mixture containing 50 mM-imidazole-HCl buffer (pH 7.4), 5 mM-MgCl<sub>2</sub>, 2 mM-NADP and 1 mM-glucose-6-phosphate.

Except for SOD, whose units of expression were described above, all other enzyme activities were expressed as units (CAT) or milliunits (G6PD, GPX and GR) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate/min under the above assay conditions. Protein concentration was determined according to Bradford<sup>(45)</sup> using a protein assay kit (Sigma) and bovine serum albumin as the standard.

### Total glutathione, GSH and oxidative stress index

A portion of liver was homogenised in nine volumes of ice-cold solution containing 1.3% 5-sulphosalicylic acid (w/v) and 10 mM-HCl. The procedure was carefully performed always on ice in order to avoid the GSH. Homogenates were centrifuged at 14 000 *g* for 10 min at 4°C, and the resulting supernatants were immediately analysed.

Total glutathione (tGSH) and GSSG were measured following the method described by Griffith<sup>(46)</sup> and Vandeputte *et al.*<sup>(47)</sup> with modifications. Both tGSH and GSSG analyses were carried out at 37°C, and the changes in absorbance as a consequence of the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were monitored at 405 nm using a microplate reader (ELx808; BioTek Instruments). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The molar extinction coefficient used for DTNB was 13.6 mm<sup>-1</sup> × cm<sup>-1</sup>.

tGSH was determined using a reaction mixture containing 133 mM-phosphate buffer with 5.8 mM-EDTA at pH 7.4, 0.71 mM-DTNB, 0.24 mM-NADPH and 1.2 IU/ml GR.

GSSG was measured using an aliquot from the solution obtained after 60 min of incubation of 100 μl of the sample with 2 μl vinylpyridine and 6 μl 1.5 M-triethanolamine. The reaction mixture contained 122 mM-phosphate buffer with 5.4 mM-EDTA at pH 7.4, 0.71 mM-DTNB, 0.24 mM-NADPH and 1.2 IU/ml GR.

The results were calculated using the standard curves of GSH and GSSG for tGSH and GSSG measurements, respectively. GSH level was calculated by subtracting GSSG from tGSH values. The results are expressed as nmol per g of tissue.

Oxidative stress index (OSI) was calculated as follows:

$$\text{OSI (\%)} = 100 \times (2 \times \text{GSSG}/\text{tGSH}).$$

### Lipid peroxidation

Concentration of malondialdehyde (MDA) was determined as a marker for LPO following the methodology described by Buege & Aust<sup>(48)</sup>. An aliquot of supernatant from the homogenate (100 μl) was mixed with 500 μl of a previously prepared solution containing 15% (w/v) TCA, 0.375% (w/v) thiobarbituric acid (TBA), 80% (v/v) HCl 0.25 N and 0.01% (w/v) butylated hydroxytoluene. The mixture was heated to 100°C for 15 min. After being cooled to room temperature and centrifuged at 1500 *g* for 10 min, the absorbance was measured at 535 nm in the supernatant. MDA concentration was expressed as nmol MDA per g of tissue, calculated from a calibration curve.

### Statistical analysis

Data are presented as mean values and standard deviations. Data were checked for normality and homogeneity of variances

and when appropriate were normalised (log, inverse and  $\arcsin(\sqrt{(x/100)})$  transformations). Statistical evaluation of the data was carried out using a 2x2 factorial arrangement of treatments in a completely randomised experimental design (two-way ANOVA), with carbohydrate concentration and lipid source as fixed factors. The significance level of 0.05 was used for rejection of the null hypothesis. All the statistical analyses were carried out using SPSS 21.0 software package (IBM Corp.) for Windows.

**Results**

The diets presented small differences in the proportions of total SFA, which were slightly higher in the VO diets, and of MUFA, which were higher in the FO diets (Table 2). Within MUFA, higher concentrations of oleic acid (18 : 1n-9) were found in the VO diets, whereas the opposite occurred for palmitoleic acid (16 : 1n-7), eicosenoic acid (20 : 1n-9) and erucic acid (22 : 1n-9). Linoleic acid (LA, 18 : 2n-6), n-6 PUFA, was considerably higher in the VO diets. Regarding n-3 PUFA, VO diets were particularly rich in linolenic acid (LNA, 18 : 3n-3) and poor in EPA(20 : 5n-3) and DHA) (22 : 6n-3).

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

Lipid source...	Experimental diets			
	FO		VO	
Carbohydrates...	CH-	CH+	CH-	CH+
<b>SFA</b>				
14 : 0	5.2	5.0	2.7	2.0
15 : 0	0.4	0.4	0.2	0.2
16 : 0	14.5	13.6	17.8	17.2
17 : 0	0.3	0.3	0.3	0.2
18 : 0	2.9	2.7	3.7	3.5
20 : 0	0.2	0.1	0.3	0.3
∑SFA	23.7	22.2	25.3	23.8
<b>MUFA</b>				
16 : 1n-7	7.5	7.5	3.3	2.4
18 : 1n-9	16.7	17.7	24.6	27.1
20 : 1n-9	5.9	7.0	0.5	0.5
22 : 1n-9	4.6	5.5	0.1	0.1
∑MUFA	34.9	37.8	28.5	30.0
<b>n-6 PUFA</b>				
18 : 2n-6	1.5	1.7	9.2	10.8
18 : 3n-6	0.2	0.2	0.1	0.1
20 : 2n-6	0.3	0.3	0.1	0.1
20 : 4n-6	0.8	0.7	0.5	0.4
∑n-6 PUFA	5.9	5.7	11.7	12.5
<b>n-3 LC-PUFA</b>				
18 : 3n-3	0.9	1.0	17.4	21.3
18 : 4n-3	2.3	2.4	0.7	0.5
20 : 4n-3	0.7	0.7	0.3	0.2
20 : 5n-3	10.9	10.5	5.6	4.2
21 : 5n-3	0.5	0.5	0.2	0.2
22 : 5n-3	1.6	1.5	0.8	0.6
22 : 6n-3	13.9	13.1	7.3	5.4
∑n-3 LC-PUFA	30.8	29.7	32.4	32.4
<b>SFA:PUFA</b>	0.6	0.6	0.6	0.5
<b>n3:n6</b>	10.5	10.1	3.2	2.9

FO, fish oil, VO, blend of vegetable oils; carbohydrate content, 0% (CH-) or 20% (CH+) gelatinised maize starch; n-3 LC-PUFA, n-3 long-chain PUFA.

Results of the feeding trial were not the aim of this study and are presented elsewhere<sup>(49)</sup>.

In brief, growth performance and feed efficiency were not affected by dietary treatments. Final body mass averaged 221.7 (SD 5.0)g, feed efficiency (wet weight gain(g)/dry feed intake (g)) averaged 0.8 (SD 0.03) and feed intake (g/kg average body mass per d) averaged 16.6 (SD 0.7). Liver fatty acid composition is summarised in Table 3. Accordingly, liver samples of fish fed VO-based diets were characterised by higher concentrations of LA (18 : 2n-6), γ-LNA (GLA; 18 : 3n-6) and LNA (18 : 3n-3) and by lower concentration of arachidonic acid (ARA; 20 : 4n-6), EPA (20 : 5n-3) and DHA (22 : 6n-3) as well as n-3 PUFA:n-6 PUFA ratio. Except for ARA concentrations, lower in groups fed the CH+ diets, no other differences were found for hepatic n-6 PUFA concentrations. Regarding n-3 PUFA, groups fed the CH+ diets presented similar LNA concentrations but lower EPA and DHA concentrations compared with those fed CH- diets. Unsaturation index was lower in fish fed the CH+ diets. On the other hand, differences between lipid sources on unsaturation index were only evident in the CH- group. During the trial, no mortality occurred.

Overall, both in the liver and in the intestines, there were no interactions between dietary carbohydrate and lipid source in the activities of oxidative stress enzymes or in LPO values. In the liver, dietary lipid source did not affect the activities of oxidative stress enzymes or LPO values, whereas dietary carbohydrates promoted a decrease in LPO values and in CAT activity and an increase in GR and G6PD activities (Table 4). Moreover, in the liver, SOD and GPX activities were not affected by dietary carbohydrates. In the intestine, the dietary carbohydrate level did not affect the activities of oxidative stress enzymes or LPO values, whereas dietary VO promoted a decrease in LPO and an increase in GPX and GR activities (Table 5). No effects on activities of CAT, SOD or G6PD in relation to diet composition were observed in the intestine.

In the liver, higher GSH content and lower GSSG levels and OSI were observed in the carbohydrate groups (Fig. 1), but no effects of dietary lipid source on glutathione (total, reduced and oxidised) or OSI were observed.

**Discussion**

In this study, low susceptibility to LPO was observed in the liver of fish fed carbohydrate-rich diets, which is in agreement with previous observations on muscles of this and other fish species<sup>(27,28)</sup>. The beneficial effect of dietary carbohydrates on lipid oxidative damage may be related to the nature of the glucose molecule, which can scavenge ROS by itself<sup>(50)</sup>, or through increased activity of the pentose phosphate pathway<sup>(27,28,50,51)</sup>. The pentose phosphate pathway, via G6PD, generates reducing power in the form of NADPH, which is required for the activity of two H<sub>2</sub>O<sub>2</sub>-scavenging pathways involving CAT and GPX<sup>(43,52)</sup>. In fact, along with lower hepatic LPO levels, higher G6PD activity was observed in European sea bass fed carbohydrate-rich diets. Furthermore, dietary carbohydrates also promoted an increase in hepatic GR activity, an enzyme that also requires NADPH to regenerate reduced glutathione

**Table 3.** Liver fatty acid (FA) profile (% of total FA) of European sea bass fed the experimental diets (Mean values and standard deviations; *n* 6 fish)

Lipid source (LS)...	Experimental diets										<i>P</i> *		
	FO				VO								
	CH–		CH+		CH–		CH+		CH	LS	CH × LS		
Carbohydrates (CH)...	Mean	SD	Mean	SD	Mean	SD	Mean	SD					
FA													
<i>n</i> -6 PUFA													
18 : 2 <i>n</i> -6	1.3	0.5	0.9	0.3	3.5	0.9	3.1	0.8	0.153	<0.001	0.922		
18 : 3 <i>n</i> -6	0.1	0.1	0.1	0.1	0.2	0.03	0.2	0.1	0.700	0.001	0.376		
20 : 4 <i>n</i> -6	0.7	0.1	0.3	0.1	0.5	0.1	0.2	0.02	<0.001	<0.001	0.336		
<i>n</i> -3 PUFA													
18 : 3 <i>n</i> -3	0.4	0.1	0.3	0.1	4.6	1.2	4.5	1.3	0.093	<0.001	0.131		
20 : 5 <i>n</i> -3	5.4	0.9	2.9	0.6	2.6	0.6	1.4	0.2	<0.001	<0.001	0.054		
22 : 6 <i>n</i> -3	9.1	2.6	3.6	0.9	3.8	1.3	1.8	0.4	<0.001	<0.001	0.775		
Ratios													
<i>n</i> -3: <i>n</i> -6	7.5	1.2	5.4	0.6	2.7	0.2	2.3	0.1	<0.001	<0.001	0.482		
Unsaturation index	146	20	92	11	109	15	91	9	<0.001	0.004	0.008		

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

\* Significant differences at *P* < 0.05 (two-way ANOVA).

**Table 4.** Specific activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein) and lipid peroxidation (LPO) (nmol malondialdehyde per g tissue) in the liver of European sea bass fed the experimental diets (Mean values and standard deviations; *n* 9 fish)

Lipid source (LS)...	Experimental diets										<i>P</i> *		
	FO				VO								
	CH–		CH		CH–		CH+		CH	LS	CH × LS		
Carbohydrates (CH)...	Mean	SD	Mean	SD	Mean	SD	Mean	SD					
G6PD	269	44	539	154	295	34	572	56	<0.001	0.089	0.724		
GPX	57	15	52	15	56	21	49	20	0.342	0.703	0.894		
GR	7.6	2.2	9.5	2.9	7.6	3.0	9.1	1.4	0.044	0.832	0.778		
CAT	618	90	518	68	620	78	552	56	0.002	0.474	0.520		
SOD	232	29	220	58	220	40	178	45	0.071	0.080	0.325		
LPO	11	1.0	9.0	1.6	12	2	8.7	0.7	<0.001	0.591	0.232		

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

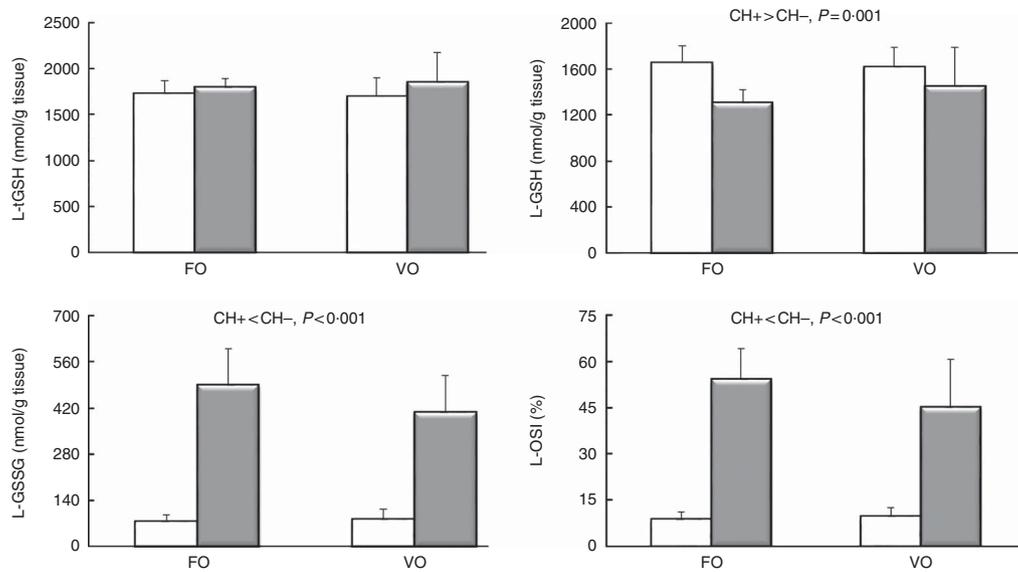
\* Significant differences at *P* < 0.05 (two-way ANOVA).

**Table 5.** Specific activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), superoxide dismutase (SOD), catalase (CAT) (U/mg protein) and lipid peroxidation (LPO) (nmol malondialdehyde per g tissue) in the intestine of European sea bass fed the experimental diets (Mean values and standard deviations; *n* 9 fish)

Lipid source (LS)...	Experimental diets										<i>P</i> *		
	FO				VO								
	CH–		CH+		CH–		CH+		CH	LS	CH × LS		
Carbohydrates (CH)...	Mean	SD	Mean	SD	Mean	SD	Mean	SD					
G6PD	16	13	27	13	19	20	22	18	0.258	0.857	0.477		
GPX	122	75	99	40	185	87	190	66	0.748	0.009	0.613		
GR	18	6	15	3	22	6	20	6	0.250	0.029	0.782		
CAT	89	26	84	20	94	22	88	15	0.475	0.570	0.932		
SOD	196	43	194	30	201	50	145	15	0.052	0.087	0.051		
LPO	75	24	51	17	47	15	46	13	0.076	0.025	0.107		

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

\* Significant differences at *P* < 0.05 (two-way ANOVA).



**Fig. 1.** Total glutathione (tGSH), GSH, GSSG and oxidative stress index (OSI) in the liver (L) of European sea bass fed experimental diets. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0% (■, CH-) or 20% (□, CH+) gelatinised maize starch. Values are means (*n* 9 fish), with standard deviations represented by vertical bars. Significant differences at *P* < 0.05 (two-way ANOVA).

(GSH) to its oxidised form (GSSG). Consistent with this observation, higher concentration of GSH and lower concentration of GSSG were observed in the liver of fish fed carbohydrate-rich diets. GSH is an essential substrate for GPX, but as it is also an effective ROS scavenger it plays an important role by preventing some of the deleterious effects of oxidative stress<sup>(53)</sup>. Accordingly, liver OSI, a biomarker of the cellular redox state, was also lower in groups fed carbohydrate-rich diets. Studies using OSI to assess the effects of oxidative stress in fish are scarce<sup>(54–58)</sup> and mainly report a huge variation in OSI values (from 0.6 to 30%) at the hepatic level<sup>(55–58)</sup>. As several variables (such as fish species, abiotic factors, fish size, time elapsed from the last meal or nutritional history) may regulate OSI values, comparisons among studies or establishment of reliable reference values for this parameter are difficult. Furthermore, a number of parameters or techniques have traditionally been used as measurements of oxidative stress. Among the most studied are antioxidant defence enzymes, TBA reactive substances (TBARS), concentrations of tGSH and GSSG and resulting OSI. In this study, the response of most parameters analysed (including OSI) was well correlated. However, other studies<sup>(55,58)</sup> have demonstrated that OSI was not a suitable biomarker of oxidative stress under stressful conditions. As stated by Peres *et al.*<sup>(59)</sup>, reliable parameters are those that respond to stressful conditions but have low variation under normal conditions. This highlights the importance of taking into consideration the response of more than one parameter involved in the oxidative status<sup>(55,58)</sup>.

On the other hand, the carbohydrate-rich diets promoted a lower hepatic CAT activity. Previously, Lygren & Hemre<sup>(50)</sup> also reported that hepatic CAT and SOD activities decreased in Atlantic salmon (*Salmo salar*) fed high-carbohydrate diets. These observations suggest that the lower LPO observed in fish fed the carbohydrate diets was mainly related with the higher concentrations of reducing equivalents such as NADPH and

GSH and that activation of primary antioxidant enzymes, which are more energy-demanding, was not required.

Fatty acid composition of reserve lipids in tissues (liver, muscle, intestine, etc.) reflects dietary fatty acid profile<sup>(60)</sup>. In the present study, the reduced *n-3:n-6* ratios (2.3 to 2.7 in VO groups *v.* 7.5 to 5.4 in FO groups) in the hepatic fatty acid profile of European sea bass fed VO-blend diets are consistent with previously reported values (1.9 to 2.5 in VO groups *v.* 4.3 in FO group) for this species also fed similar VO-blend based diets<sup>(5)</sup>. FO is rich in long-chain PUFA (LC-PUFA), whereas VO is rich in PUFA, and it is well established that due to their high degree of unsaturation LC-PUFA are more susceptible to peroxidation than PUFA or MUFA, thus promoting the formation of more free radicals<sup>(16)</sup>. Accordingly, in this study, higher hepatic LC-PUFA content was observed in European sea bass fed the FO-based diets than the VO-rich diets<sup>(49)</sup>, although liver oxidative status was not affected by dietary lipid source. In fact, although unsaturation index was lower in fish fed the carbohydrate-rich diets, differences related to lipid sources were only evident in the CH- group (lower in VO diet). Similarly, partial replacement of FO by VO did not induce changes in hepatic lipid oxidative damages and antioxidant enzyme activities of Atlantic cod (*Gadus morhua*) or rockfish (*Sebastes schlegelii*)<sup>(31,34)</sup> or in LPO concentration in Japanese sea bass (*Lateolabrax japonicus*)<sup>(23)</sup>.

In contrast to what was observed in the liver, in the present study, the dietary lipid source affected the intestinal oxidative status in European sea bass juveniles. Overall, oxidative lipid stress measured as LPO concentration was higher in fish fed the FO-based diets than the VO-rich diets. Studies addressing the effect of dietary lipid source on fish oxidative status were mainly focused on liver and muscle tissues<sup>(7,18,20,22–24,31,35)</sup>. To the best of the authors' knowledge, the impact of dietary lipid source on intestinal oxidative status of fish was only evaluated in Japanese sea bass, and it was limited to the assessment of LPO

concentration<sup>(23)</sup>. In that study, dietary inclusion of palm oil led to decreased intestinal LPO concentration. Available studies dealing with the modulator role of nutrition on intestinal antioxidant enzymatic mechanisms were mainly dedicated to the role of amino acids<sup>(61,62)</sup>, anti-nutritional factors<sup>(63)</sup> and NSP<sup>(64)</sup>.

GPX catalyses the reduction of H<sub>2</sub>O<sub>2</sub> and lipid peroxides (ROOH) into the corresponding stable alcohols (ROH) at the expense of GSH<sup>(32)</sup>. GR catalyses the reduction of GSSG to GSH and its activity is tightly bound to that of GPX, in an attempt to keep the adequate GSSG:GSH ratio necessary for GPX function. In the present study, GPX and GR activities were responsive to dietary treatments and had increased activities in groups fed the VO diets. Although flavonoids and phenolic compounds of VO were not measured in the present study, their potential role in the intestinal GPX and GR activation cannot be discarded. There is evidence that some plant constituents (flavonoids, phenolic compounds) also present in VO have antioxidant functions in mammals<sup>(65–67)</sup>. This antioxidant effect may result from a direct free-radical scavenging action by these compounds or from their role in activation of important antioxidant enzymes<sup>(65–67)</sup>. Accordingly, Nagata *et al.*<sup>(65)</sup> observed *in vitro* that in rat hepatocytes two flavonoids (quercetin and catechin) exert their antioxidant activity against H<sub>2</sub>O<sub>2</sub> through activation of GPX. In human hepatocytes, it was also demonstrated that cocoa flavonoids exert an antioxidant protective effect against tert-butyl hydroperoxide via induction of GPX and GR activities. In fish, studies are not yet available. However, Sitjà-Bobadilla *et al.*<sup>(68)</sup> suggested that replacement of FM by a mixture of plant ingredients in diets for gilthead sea bream (*Sparus aurata*) exerts an antioxidative effect through activation of glutathione metabolism, as plant-containing diets increased muscular GSH and hepatic glutathione redox status, as well as GR activity, in both hepatic and muscular tissues. According to the authors, this increased antioxidant potential related to plant ingredients was probably linked to the presence of phenolic compounds and/or flavonoids. Further, Saera-Vila *et al.*<sup>(69)</sup> reported that FO replacement by VO in plant protein-based diets increased hepatic GSH:GSSG ratio, despite the reduction of absolute glutathione concentrations. According to the authors, this suggests that fish fed these diets may be less susceptible to LPO and oxidative damage. Overall, both the fatty acid profile of VO-based diets and the presence of plants constituents with antioxidant potential may explain the lower intestinal susceptibility to oxidative stress/damages in VO groups.

Transcriptional and proteomic studies have also evidenced that intestinal oxidative status may be affected by changes in dietary lipid source<sup>(70,71)</sup>. Accordingly, Morais *et al.*<sup>(71)</sup> reported that in Atlantic salmon the dietary FO substitution by VO led to an up-regulation of transcripts and proteins involved in detoxification and protection from oxidative stress, CAT and selenoprotein, as well as haemopexin-like protein and peroxiredoxin-1 proteins. On the contrary, a previous study on the same species showed that dietary FO replacement by VO decreased the transcription levels of antioxidant-related genes such as glutathione S-transferase and GR<sup>(70)</sup>.

Similarly to our previous studies on sturgeon (*Acipenser naccarii*) and rainbow trout (*Oncorhynchus mykiss*)<sup>(72)</sup>, in this study, European sea bass intestines also showed LPO damage

values and GPX activity 2- to 5-fold higher compared with the liver. GPX and GR activities were also 2-fold higher in the intestine than in the liver of European sea bass. On the contrary, G6PD and CAT activities were, respectively, more than 10- and 5-fold lower in the intestine than in the liver. These differences between the two organs are a reflex of the distinct antioxidant mechanism strategies that are more relevant in each organ. The intestine being a tissue with high cell turnover is highly susceptible to oxidative status, and seems to require high activity of antioxidant enzymes.

In conclusion, results of the present study indicate that liver and intestine oxidative status balance were regulated through different metabolic pathways, and that dietary macronutrients affected metabolic pathways differently in each tissue. In addition, the observed LPO values suggest that susceptibility to oxidative stress was more important in the intestine than in the liver. Dietary carbohydrates contributed to reduced oxidative stress only in the liver, whereas the intestine was exclusively responsive to the dietary lipid source, with VO having a positive effect on the reduction of oxidative stress. Nowadays a wide range of PF are already used in commercial aquafeeds, including cereals and peas, which are rich sources of starch, and may therefore have a protective effect on liver oxidative stress. Given the importance of the intestine on whole-body homeostasis and health, increased attention should be paid to this tissue in future studies evaluating the impact of dietary alternative ingredients on oxidative stress.

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The authors declare that there are no conflicts of interest.

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