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Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets

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Abstract

Seven isoproteic and isolipidic semi-purified diets were formulated to assess specific nutrient deficiencies in sulphur amino acids (SAA), *n*-3 long-chain PUFA (*n*-3 LC-PUFA), phospholipids (PL), P, minerals (Min) and vitamins (Vit). The control diet (CTRL) contained these essential nutrients in adequate amounts. Each diet was allocated to triplicate groups of juvenile gilthead sea bream fed to satiety over an 11-week feeding trial period. Weight gain of *n*-3 LC-PUFA, P–Vit and PL–Min–SAA groups was 50, 60–75 and 80–85 % of the CTRL group, respectively. Fat retention was decreased by all nutrient deficiencies except by the Min diet. Strong effects on N retention were found in *n*-3 LC-PUFA and P fish. Combined anaemia and increased blood respiratory burst were observed in *n*-3 LC-PUFA fish. Hypoproteinaemia was found in SAA, *n*-3 LC-PUFA, PL and Vit fish. Derangements of lipid metabolism were also a common disorder, but the lipodystrophic phenotype of P fish was different from that of other groups. Changes in plasma levels of electrolytes (Ca, phosphate), metabolites (creatinine, choline) and enzyme activities (alkaline phosphatase) were related to specific nutrient deficiencies in PL, P, Min or Vit fish, whereas changes in circulating levels of growth hormone and insulin-like growth factor I primarily reflected the intensity of the nutritional stressor. Histopathological scoring of the liver and intestine segments showed specific nutrient-mediated changes in lipid cell vacuolisation, inflammation of intestinal submucosa, as well as the distribution and number of intestinal goblet and rodlet cells. These results contribute to define the normal range of variation for selected biometric, biochemical, haematological and histochemical markers.

Key words: **Haematology: Methionine: Essential fatty acids: Phospholipids: Phosphorus: Minerals: Vitamins**

Clinical haematology and basic blood biochemistry are common diagnostic tools to assess health and welfare in humans and most livestock production systems[1,2]. In fish, although there is experimental evidence that circulating electrolytes, metabolites and hormones highly reflect impaired growth performance, stress condition and disease outcome, the use of such analyses as diagnostic tools is poorly established in practice. This is due to the paucity of reliable information on reference values of haematology and blood biochemistry parameters in healthy and well-nourished fish. Some attempts have been made to compile available data in fish blood biochemistry and haematology[3–6]. However, for the majority of well-established farmed finfish, grown under different rearing conditions with diverse physiological status, validated data are lacking. Besides, important gaps on reliable clinical biomarkers are arising with the advent of new fish feeds with a maximised replacement of fishmeal (FM) and fish oil (FO) by alternative feedstuffs of terrestrial or marine origin. Similarly, for the histopathological scoring of relevant target tissues (liver, intestine), there is evidence of clinical signs of liver steatosis, accumulation of intestinal lipid droplets or intestine submucosa inflammation arising from lipid-related metabolic disorders[7,8], but a direct link to a specific nutrient or a group of nutrients is lacking.

Therefore, there is an urgent need for reliable reference values, but also for the definition of blood and histopathological parameters that have specificity, sensitivity and diagnostic value for nutritional deficiencies. Thus, our experimental setup with a feeding trial of semi-purified diets formulated for a given nutritional deficiency in a typically marine fish such as the gilthead sea bream considered the following two major steps: (i) functional validation of a set of clinical data based on body composition, organosomatic indices, and blood haematology

Abbreviations: ALP, alkaline phosphatase; CTRL, control; FA, fatty acids; FM, fishmeal; FO, fish oil; GH, growth hormone; IGF, insulin-like growth factors; Min, minerals; *n*-3 LC PUFA, *n*-3 long-chain PUFA; PAS, periodic acid–Schiff; PL, phospholipids; SAA, sulphur amino acids; Vit, vitamins; VO, vegetable oils.

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and biochemistry for the initial assessment of nutrient deficiencies in methionine (Met), essential fatty acids (EFA) such as the *n*-3 long-chain PUFA (*n*-3 LC PUFA), phospholipids (PL), P and micronutrients (minerals, vitamins); and (ii) histopathological scoring of liver and intestine sections as a complementary diagnostic tool. The studied nutrient deficiencies were chosen because they are constraining factors in practical marine fish feeds with a maximised FM/FO replacement. In parallel, current work is underway for the definition of the normal range of variation of selected biomarkers, integrating the data reported here with our own data in the framework of the ARRAINA (Advanced Research Initiatives for Nutrition and Aquaculture) EU project, where fish were fed through the production cycle with varying inclusion levels of FM and FO (from 40 % in the control (CTRL) diet to 7·5 % in the extreme low FM/FO diet).

Methods

Diets

Seven isonitrogenous (51–52 % of DM) and isolipidic (14·5–15·5 % of DM) diets were formulated. They were produced in a semi-industrial scale (Sparos LDA) (Table 1). All diets contained casein (20 %), casein hydrolysate (5 %), gelatin (5·8 %) and soya protein concentrate (34·5 %) as protein sources, and were supplemented with L-threonine (0·02 %). Taurine (0·3 %), betaine (0·3 %) and glucosamine (0·4 %) were added as attractants, and ethoxyquin (0·1 %) as the antioxidant in all diets. DL-Methionine was supplemented at 0·4 % in all diets, except in the diet designed to be deficient in sulphur amino acids (SAA diet). FO was added at 13·9 % in all diets, except in the fatty acid (FA)-deficient diet (*n*-3 LC-PUFA diet), in which FO was totally replaced by a blend of vegetable oils (VO) in order to reduce the EPA and DHA contents to trace levels (Table 2). Soya lecithin (2 %) was added as the unique source of PL in all diets, except in the PL-deficient diet. Calcium phosphate (2·2 %) was added in all diets, except in the P-deficient diet. Mineral premix based on available data on mineral requirements of fish[9] was included at 2·2 % in all diets, except in the diet designed to be mineral deficient (Min diet). Vitamin premix based on NRC[10] was added in all diets at an incorportaion level of 2 %, except in the diet designed to be vitamin deficient (Vit diet).

Feeding trial and fish sampling

Juvenile sea bream of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimatised to laboratory conditions for one month before the start of a 12-week trial (May–July) in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC). Following the acclimatisation period, fish of 15 g initial mean body weight were randomly distributed into 500 L tanks in triplicates of 35 fish each. Fish were fed to visual satiety one (12 h)/two times (9 h, 14 h)

Table 1. Ingredients and chemical composition of the experimental diets

	Diet						
	CTRL	SAA	*n*-3 LC-PUFA	PL	P	Min	Vit
Ingredient (%)							
Casein	20	20	20	20	20	20	20
Casein hydrolisate	5	5	5	5	5	5	5
Gelatin	5·78	5·78	5·78	5·78	5·78	5·78	5·78
Soya protein concentrate (Soycomil PC)	34·5	34·5	34·5	34·5	34·5	34·5	34·5
DL-Methionine	0·4	0	0·4	0·4	0·4	0·4	0·4
L-Threonine	0·02	0·02	0·02	0·02	0·02	0·02	0·02
Dextrine	11·2	11·2	11·2	11·2	11·2	11·2	11·2
Soya lecithin	2	2	2	0	2	2	2
Fish oil	13·9	13·9	0	13·9	13·9	13·9	13·9
Vegetable oil	0	0	13·9	0	0	0	0
Cellulose	0	0·4	0	0	2·2	1·5	1·5
$CaHPO_4.2H_2O$ (18 % P)	2·2	2·2	2·2	2·2	0	2·2	2·2
Mineral premix-INRA*	2	2	2	2	2	0·5	2
Vitamin premix-INRA†	2	2	2	2	2	2	0·5
Taurine	0·3	0·3	0·3	0·3	0·3	0·3	0·3
Betaine	0·3	0·3	0·3	0·3	0·3	0·3	0·3
Glucosamine	0·4	0·4	0·4	0·4	0·4	0·4	0·4
Ethoxyquin (75 %)	0·1	0·1	0·1	0·1	0·1	0·1	0·1
Proximate composition							
DM (%)	92·4	92·5	95·4	93·3	95·5	92·2	92·8
Crude protein (% DM)	51·8	51·5	50·8	51·1	50·8	52·0	52·5
Crude fat (% DM)	15·0	15·1	15·5	15·6	14·4	14·7	14·7
EPA + DHA (% DM)	2·68	2·68	0·03	2·83	2·60	2·64	2·37

CTRL, control; SAA, sulphur amino acids; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; PL, phospholipids; Min, minerals; Vit, vitamins.

* Supplied the following (g/kg mix): calcium hydrogen phosphate 500, calcium carbonate (40 % Ca) 215, sodium chloride 40, ferrous sulphate (21 % Fe) 20, manganese sulphate 3, zinc sulphate 4, copper sulphate 3, cobalt (II) chloride (25 % Co) 0·02, potassium iodine 0·04, sodium selenite 0·03, sodium fluoride 1, magnesium hydroxide (60 % Mg) 124 and potassium chloride 90.

† Supplied the following (g/kg mix, except as noted): retinyl acetate 1, DL-cholecalciferol 2·5, DL-*α* tocopheryl acetate 5, menadione sodium bisulphite 1, ascorbic acid 20, thiamin 0·1, riboflavin 0·4, pyridoxine 0·3, vitamin B_{12} 10 mg, nicotinic acid 1, pantothenic acid 2, folic acid 0·1, biotin 10 mg, choline chloride 200, inositol 30.

Table 2. Fatty acid composition of the experimental diets (% total fatty acid methyl esters)
(Mean values of two determinations)

	Diet						
Fatty acid	CTRL	SAA	*n*-3 LC-PUFA	PL	P	Min	Vit
14 : 0	6·81	7·06	0·51	7·38	7·03	7·06	7·50
15 : 0	0·61	0·74	0·24	0·79	0·76	0·68	1·01
16 : 0	17·56	18·11	15·48	18·53	18·58	18·47	19·13
16 : 1*n*-7	7·36	7·44	0·24	7·60	7·29	7·31	7·46
16 : 2	0·91	0·92	0·12	1·06	1·02	1·23	1·13
16 : 3	1·25	1·25	0·06	1·29	1·20	1·26	1·21
16 : 4	1·37	1·37	0·08	1·41	1·31	1·36	1·30
17 : 0	0·48	0·49	Tr	0·52	0·51	0·51	0·53
18 : 0	3·36	3·47	4·68	3·48	3·62	3·56	3·69
18 : 1*n*-9	10·87	10·79	30·57	10·68	10·84	10·76	10·95
18 : 1*n*-7	2·49	2·52	0·98	2·57	2·52	2·52	2·57
18 : 2*n*-6	3·98	3·87	16·46	2·09	3·81	3·81	3·66
18 : 3*n*-3	1·10	1·07	27·44	1·10	1·13	1·09	1·01
18 : 3*n*-6	0·30	0·30	0·09	0·31	0·29	0·30	0·29
18 : 4*n*-3	2·21	2·22	Tr	2·26	2·14	2·23	2·07
20 : 0	0·29	0·28	0·32	0·29	0·30	0·29	0·33
20 : 1*n*-9	2·32	0·81	0·38	2·51	2·46	2·43	2·48
20 : 1*n*-7	0·21	0·21	Tr	0·22	0·21	0·21	0·22
20 : 2*n*-6	0·15	0·15	0·07	0·15	0·15	0·15	0·15
20 : 3*n*-3	0·07	0·07	Tr	0·07	0·07	0·07	0·06
20 : 3*n*-6	0·07	0·05	Tr	0·05	0·05	0·05	0·06
20 : 4*n*-6	0·90	0·91	Tr	0·92	0·88	0·91	0·85
20 : 4*n*-3	0·61	0·61	Tr	0·62	0·60	0·61	0·58
20 : 5*n*-3 (EPA)	12·98	13·01	0·18	13·17	12·56	13·06	11·88
21 : 0	0·08	0·10	Tr	0·08	0·09	0·09	0·08
21 : 5*n*-3	0·48	0·49	Tr	0·49	0·47	0·49	0·44
22 : 0	0·12	0·13	0·19	0·11	0·13	0·13	0·14
22 : 1*n*-11	3·34	3·58	Tr	3·66	3·69	3·61	3·61
22 : 1*n*-9	0·20	0·11	0·05	0·11	0·08	0·11	0·10
22 : 4*n*-6	0·08	0·08	Tr	0·08	0·08	0·09	0·08
22 : 5*n*-3	1·39	1·40	0·10	1·41	1·36	1·40	1·27
22 : 6*n*-3 (DHA)	9·94	9·95	0·07	10·05	9·60	9·98	8·96
Total	94·14	93·81	98·37	95·28	95·10	95·00	96·08
Saturates	29·31	30·38	21·44	31·19	31·03	30·79	32·40
Monoenes	26·79	25·48	32·26	27·34	27·09	26·96	27·39
n-6 LC-PUFA*	0·98	0·99	Tr	1·00	0·95	0·99	0·93
n-3 LC-PUFA*	25·41	25·45	0·38	25·73	24·59	25·55	23·13

CTRL, control; SAA, sulphur amino acids; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; PL, phospholipids; Min, minerals; Vit, vitamins; Tr, trace value < 0·05.
* Fatty acids with at least twenty carbon atoms and more than three double bonds.

per day (6 d/week). The trial was conducted under natural photoperiod and temperature conditions at IATS latitude (40°5 N; 0°10E), and the water temperature was increased from 19°C in May to 24°C at the end of July. Water flow rate was 20 L/min, oxygen content of water effluents was always higher than 85 % saturation and unionised ammonia remained below toxic levels (<0·02 mg/l).

At the end of the trial and following overnight fasting (10–12 h in the morning), 18 fish per dietary treatment (six per tank) were randomly selected and decapitated under anaesthesia with 3-aminobenzoic acid ethyl ester (MS-222, 100 µg/ml). Blood was taken from the caudal vessels with heparinised syringes (less than 5 min for all the fish sampled from each tank). One aliquot was used for measurements of haematological parameters and respiratory burst (RB) activity of blood leucocytes. The remaining blood was centrifuged at 3000 ***g*** for 20 min at 4°C, and the plasma obtained was stored in separate aliquots at –20°C until further assays were performed. Viscera, liver and mesenteric fat were weighed and representative portions of the liver and intestine segments (anterior and posterior) were taken for histological processing. When blood and tissue collection was completed, additional fish (four fish per tank) were taken for whole body composition analyses.

The experimental protocol was approved by the Agencia Estatal Consejo Superior de Investigaciones Científicas, IATS Review Board, and all procedures were in accordance with the national and current EU legislations on the handling of experimental animals.

Chemical composition

Diets and fish for body composition analyses (a pooled sample of ten fish at the beginning and four fish per tank at the end of trial) were ground, and small aliquots were dried to determine moisture content. The remaining samples were freeze-dried and chemical analyses were carried out: DM by drying at 105°C for 24 h, protein (N × 6·25) by the Kjeldahl method and fat after dichloromethane extraction by the Soxhlet method.

Total lipids for analyses of dietary FA acid composition were extracted by the method described by Folch *et al.*[11], with

chloroform–methanol (2:1, v/v) containing 0·01 % butylated hydroxytoluene (BHT) as antioxidant. After the addition of nonadecanoic FA (19:0) as internal standard, total lipids were subjected to acid-catalysed transmethylation for 16 h at 50°C using 1 ml toluene and 2 ml of 1 % (v/v) sulphuric acid in methanol[12]. Fatty acid methyl esters (FAME) were extracted with hexane: diethyl ether (1:1) and purified by TLC (Silica gel G 60, 20 × 20 cm glass plates, Merck), using hexane:diethyl ether:acetic acid (85:15:1·5) as the solvent system. FAMEs were then analysed with Fisons Instruments GC 8000 Series (Fisons) Gas Chromatograph as described elsewhere[13]. Individual FAMEs were identified by comparison with well-characterised sardine oil (Marinol; Fishing Industry Research Institute) and the FAME 37 mix from Supelco. BHT and internal standards (19:0) were obtained from Sigma-Aldrich. All solvents used for lipid extraction and FA analyses were HPLC grade and were obtained from Merck.

Haematology

Hb concentration was determined using a HemoCue B-Haemoglobin Analyser® (AB Leo Diagnostic), which uses a modified azide methaemoglobin reaction for Hb quantification; haematocrit (Hct) was measured after centrifugation of the blood in heparinised capillary tubes at 13 000 ***g*** for 10 min. RBC counts were made in a Neubauer chamber using an isotonic solution (1 % NaCl). Erythrocyte osmotic fragility test was carried out in hypotonic NaCl solutions with haemolysis read at 540 nm. Median corpuscular fragility (MCF) was defined as the concentration of NaCl (g/l) causing 50 % lysis.

Blood biochemistry

Plasma glucose was measured by the glucose oxidase method (Thermo Fisher Scientific). Plasma TAG were determined using lipase/glycerol kinase/glycerol-3-phosphate oxidase reagent. Total plasma cholesterol was determined using cholesterol esterase/cholesterol dehydrogenase reagent (Thermo Fisher Scientific). HDL and LDL/VLDL-cholesterol were determined using a kit (EHDL-100) from BioAssay Systems, based on an improved polyethylene glycol precipitation method in which HDL and LDL/VLDL are separated and cholesterol concentrations are determined using cholesterol esterase/cholesterol dehydrogenase reagent. Total plasma proteins were measured with the Bio-Rad protein reagent (Bio-Rad) with bovine serum albumin as the standard.

Changes in plasma enzyme activities of alanine aminotransferase (ALAT, *EC* 2.6.1.2) (EALT-100), aspartate aminotransferase (ASAT, *EC* 2.6.1.1) (EASTR-100) and glutamate dehydrogenase (GLDH, *EC* 1.4.1.2) (DGLDH-100) were measured using colorimetric assay kits (BioAssay Systems). Plasma alkaline phosphatase (ALP, *EC* 3.1.3.1) activity was determined using a fluorimetric assay kit (QFAP-100, BioAssay Systems).

Plasma levels of creatinine (DICT-500), choline (ECHO-100), calcium (DICA-500), chloride (DICL-250), magnesium (DMG-250) and phosphate (DIPI-500) were measured using colorimetric assay kits (BioAssay Systems). Total antioxidant capacity was measured as Trolox activity using a microplate assay kit (709001) (Cayman Chemical). Plasma lysozyme activity was measured by a turbidimetric assay[14] adapted to microplates, as previously described[15]. Induction of RB activity in blood leucocytes was measured directly from heparinised blood, following the method described by Nikoskelainen *et al.*[16] with some modifications[17].

Plasma growth hormone (GH) was determined by a homologous gilthead sea bream RIA as reported elsewhere[18]. The sensitivity and midrange (ED50) of the assay were 0·15 and 1·8 ng/ml, respectively. Plasma insulin-like growth factors (IGF) were extracted by acid–ethanol cryoprecipitation[19], and the concentration of IGF-I was measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish[20]. The sensitivity and midrange of the assay were 0·05 and 0·7–0·8 ng/ml, respectively.

Histology

For histological examination, pieces of the liver, anterior (a piece immediately after the piloric caeca) and posterior (a piece immediately before the rectum) intestinal segments were fixed in 10 % buffered formalin, embedded in paraffin, 4-µm sectioned and stained with Giemsa and periodic acid–Schiff (PAS). Histochemical reactivity in tissues was evaluated by grading staining using the following scale: negative (–), slight (+), moderate (++) and marked (+++).

Statistical analysis

Data on growth performance and blood haematology and biochemistry were analysed by one-way ANOVA followed by the Student Newman–Keuls test ($P<0·05$). All the analyses were performed with SPSS 17.0 program (SPSS Inc.).

Results

Growth performance

Data on growth, somatic indices and body composition are shown in Table 3. As a general rule, nutrient-deficient diets reduced significantly feed intake, growth rates and feed efficiency in fish fed P and *n*-3 LC-PUFA diets. This resulted in a weight gain of 50 % (*n*-3 LC-PUFA), 60–75 % (P, Vit) and 80–85 % (PL, Min, SAA) of CTRL fish.

Mesenteric fat index (MSI) was markedly reduced in Vit fish. The same was observed in PL and *n*-3 LC-PUFA groups, although there were no statistically significant differences. Conversely, MSI was significantly increased in P fish. Hepatosomatic index (HSI) was also altered by dietary treatments, and it was largely increased in *n*-3 LC-PUFA fish. The opposite was found in Min fish and in a lower extent in Met and Vit fish.

Dietary treatment also altered whole body composition with low protein and lipid content in fish fed P and Vit diets, respectively. This feature was related to a strong decrease in N retention in P and *n*-3 LC-PUFA fish. Lipid retention was significantly reduced by nutrient deficiencies in all experimental groups with the exception of Min fish.

Blood analyses

Data on blood analysis are shown in Table 4. Hb concentration, Hct and RBC counts were significantly lower in *n*-3 LC-PUFA

Table 3. Effect of nutrient deficiencies on growth performance of gilthead sea bream fed to visual satiety from May to July (13 weeks) (Mean values with their standard errors)

	Diet														
	CTRL		SAA		*n*-3 LC-PUFA		PL		P		Min		Vit		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P**
Initial body weight (g)	15·1	0·06	15·1	0·21	15·2	0·14	15·0	0·25	15·1	0·11	15·0	0·04	15·2	0·08	0·977
Final body weight (g)	85·5^{a}	1·58	72·5^{b}	1·41	51·2^{d}	0·17	71·7^{b}	1·80	60·2^{c}	0·66	71·5^{b}	2·12	66·1^{b,c}	0·65	<0·001
Feed intake (g DM/fish)	65·9^{a}	1·46	54·7^{b}	1·36	37·9^{d}	0·68	49·6^{b,c}	2·16	51·4^{b,c}	0·91	51·6^{b,c}	1·73	46·9^{c}	0·59	<0·001
Viscera (g)	6·39^{a}	0·27	5·34^{b}	0·23	4·40^{d}	0·21	5·80^{a,b}	0·19	5·79^{b}	0·18	5·54^{a,b,c}	0·20	4·90^{c,d}	0·16	<0·001
Mesenteric fat (g)	1·14^{a}	0·13	0·92^{a,b,c}	0·12	0·58^{c}	0·09	0·79^{b,c}	0·08	1·27^{a}	0·11	1·04^{a,b}	0·11	0·49^{c}	0·06	<0·001
Liver (g)	1·09^{a}	0·06	0·80^{c}	0·03	0·98^{a,b}	0·04	1·01^{a,b}	0·05	0·87^{b,c}	0·02	0·77^{c}	0·02	0·79^{c}	0·03	<0·001
VSI (%)†	7·12^{b,c}	0·20	7·03^{b,c}	0·22	7·81^{b}	0·20	7·43^{b,c}	0·16	8·95^{a}	0·23	7·10^{b,c}	0·19	6·92^{c}	0·16	<0·001
MSI (%)‡	1·27^{b}	0·13	1·19^{b,c}	0·14	0·98^{b,c}	0·12	0·99^{b,c}	0·10	1·97^{a}	0·16	1·32^{b}	0·13	0·70^{c}	0·08	<0·001
HSI (%)§	1·23^{b,c}	0·04	1·05^{c,d}	0·02	1·76^{a}	0·08	1·29^{b}	0·04	1·36^{b}	0·04	0·99^{d}	0·02	1·10^{c,d}	0·04	<0·001
SGR (%)‖	1·97^{a}	0·02	1·82^{b}	0·04	1·38^{d}	0·01	1·77^{b}	0·05	1·57^{c}	0·01	1·77^{b}	0·03	1·67^{b}	0·01	<0·001
FE (%)¶	1·07^{a}	0·01	1·08^{a}	0·01	0·95^{b}	0·02	1·10^{a}	0·01	0·88^{c}	0·01	1·09^{a}	0·01	1·08^{a}	0·01	<0·001
Whole body composition (% wet weight)															
Moisture	67·6^{b}	0·21	68·3^{b}	0·07	69·9^{a}	0·31	69·70^{a}	0·07	66·3^{c}	0·08	68·2^{b}	0·27	70·3^{a}	0·34	<0·001
Crude protein	17·5^{a}	0·16	16·4^{a,b}	0·29	16·5^{a,b}	0·3	16·5^{a,b}	0·25	15·8^{b}	0·08	17·5^{a,b}	0·17	16·9^{a,b}	0·38	0·017
Crude lipid	9·28^{a}	0·32	8·71^{a,b}	0·10	8·03^{a,b}	0·13	8·25^{a,b}	0·51	8·41^{a,b}	0·52	9·03^{b}	0·24	6·95^{b}	0·08	0·005
Retention (% intake)															
N	37·0^{a}	0·63	33·4^{a,b}	0·49	31·7^{b,c}	0·46	34·5^{a,b}	0·31	27·7^{c}	0·45	36·0^{a}	0·45	36·1^{a}	1·33	<0·001
Lipid	64·3^{a}	1·19	58·8^{b}	0·92	46·9^{c}	0·73	57·4^{b}	0·58	50·1^{c}	0·84	65·6^{a}	1·29	46·6^{c}	1·98	<0·001

CTRL, control; SAA, sulphur amino acids; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; PL, phospholipids; Min, minerals; Vit, vitamins.
Data on body weight, feed intake, growth indices and body composition are the mean values with their standard errors of the mean of triplicate tanks. Data on viscera, mesenteric fat and liver weight are the mean values with their standard errors of the mean of twenty fish.
^{a,b,c,d} Mean values with unlike superscript letters in each row indicate significant differences among dietary treatments (Student Newman–Keuls test, $P<0·05$)
* Result values from one-way ANOVA.
† Viscerosomatic index (VSI) = (100 × viscera weight)/fish weight.
‡ Mesenteric index (MSI) = (100 × mesenteric fat weight)/fish weight.
§ Hepatosomatic index (HSI) = (100 × liver weight)/fish weight.
‖ Specific growth rate (SGR) = 100 × (ln final body weight–ln initial body weight)/d.
¶ Feed efficiency (FE) = wet weight gain/dry feed intake.

fish than in CTRL fish. This feature was related to a greater osmotic fragility, evidenced by the significant increase of MCF values from 6·6 g/l in CTRL fish to 7·4 g/l in *n*-3 LC-PUFA fish. Other experimental groups did not show any statistically significant alterations in haematological parameters.

Blood biochemistry was altered in a nutrient-specific manner, and strong hypotriglyceridaemia, hypocholesterolaemia and hypoproteinaemia with decreased plasma levels of creatinine were found in *n*-3 LC-PUFA fish, but also in Vit fish. Hypoproteinaemia was a sign of SAA and PL deficiency, whereas hypertriglyceridaemia and hypercholesterolaemia were characteristic features of P fish. Low plasma choline levels were found in SAA, Min and Vit fish.

Plasma electrolytes were highly refractory to dietary treatment in our experimental conditions, with the exception of calcium and phosphate in fish fed diets not supplemented with the vitamin premix and inorganic P, respectively. Likewise, enzyme activities of ALAT, ASAT and GLDH were not modified by dietary treatment, whereas ALP activity was significantly decreased in PL fish but increased in P and Min fish groups. Lysozyme activity was not altered by any dietary treatment. In contrast, RB was triggered in a consistent manner in *n*-3 LC-PUFA fish. Plasma antioxidant capacity was also increased by nutrient deficiencies, although this feature was especially evident in fish fed P, Min and Vit diets.

Regarding growth factors, circulating levels of GH highly reflected the impairment of growth performance and the highest plasma concentration was observed in *n*-3 LC-PUFA fish, followed by fish fed P and Vit diets. The opposite was found for circulating levels of IGF-I, and the lowest IGF-I concentration was found in *n*-3 LC-PUFA and P fish groups.

Histopathological traits

The histological examination of the liver and intestine showed different features, which are summarised in Table 5. Representative differential microphotographs are also provided in Figs. 1–3. The highest level of fat accumulation either in the liver or in the anterior intestine was observed in fish fed *n*-3 LC-PUFA, but without reaching steatosis. Accumulation of glycogen in the liver (revealed by PAS staining) was observed in fish fed SAA, P and Vit diets, but it was not extreme. No fat accumulation was observed in the posterior intestine in any of the groups. Goblet cell content and number varied with the diet, and a clear decrease in the number of neutral mucins (stained with PAS) was observed in the anterior intestine of fish fed *n*-3 LC-PUFA, PL, P and Min diets. In all fish groups, the number of PAS+ goblet cells was lower in the posterior intestine compared with the anterior intestine, and only PL and Vit fish had slight staining. The number of Giemsa-stained goblet

Table 4. Effect of nutrient deficiencies on basic plasma biochemistry of sea bream fed to visual satiety from May to July (13 weeks) (Mean values with their standard errors of ten fish)

	CTRL		SAA		*n*-3 LC-PUFA		PL		P		Min		Vit		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P**
Hb (g/dl)†	7·16^{a,b}	0·47	6·62^{a,b}	0·30	5·90^{b}	0·36	7·09^{a,b}	0·42	7·79^{a}	0·47	7·19^{a,b}	0·34	8·13^{a}	0·47	0·008
Haematocrit (%)	32·8^{a}	1·68	34·1^{a}	1·33	27·4^{b}	0·88	30·5^{a,b}	0·97	34·9^{a}	1·69	36·2^{a}	1·63	32·8^{a}	1·48	0·001
Erythrocytes × 10^{-6}/ml	2·66^{a}	0·07	2·53^{a}	0·09	2·07^{b}	0·05	2·31^{b}	0·13	2·53^{a,b}	0·11	2·80^{a}	0·08	2·79^{a}	0·09	<0·001
MCF (g/l)	6·62^{a}	0·21	ND		7·37^{b}	0·24	6·90^{a,b}	0·14	ND		ND		ND		0·048
Glucose (mg/dl)†	56·1	2·86	58·3	2·86	56·0	2·13	52·3	1·40	57·5	3·10	57·3	1·83	50·6	1·96	0·099
TAG (mM)	0·87^{a}	0·09	0·94^{a}	0·10	0·53^{b}	0·05	0·86^{a}	0·09	2·22^{c}	0·26	0·86^{a}	0·06	0·62^{a,b}	0·08	<0·017
Total cholesterol (mg/dl)†	152·2^{a}	10·7	130·5^{a,b}	3·91	65·62^{c}	7·36	113·6^{b}	5·52	192·5^{d}	9·67	139·9^{a}	9·66	90·7^{e}	5·23	<0·001
HDL-cholesterol (mg/dl)†	119·0^{a}	9·38	123·6^{a}	5·68	64·2^{b}	3·76	110·7^{a,d}	6·39	159·7^{c}	8·44	120·1^{a}	11·9	89·9^{d}	6·96	<0·001
VLDL/LDL-cholesterol (mg/dl)†	34·1^{a,b}	9·31	18·9^{a,b}	1·58	16·5^{a}	1·39	22·8^{a,b}	3·33	52·2^{b}	6·38	23·2^{a,b}	2·16	18·4^{a}	2·62	0·012
Total proteins (g/l)	33·8^{a}	1·81	28·4^{b}	0·66	24·1^{b}	2·05	26·3^{b}	1·37	38·1^{a}	1·85	35·3^{a}	1·86	26·1^{b}	2·23	<0·001
ALAT (U/l)	3·21	0·46	3·15	0·24	2·29	0·29	2·83	0·35	2·78	0·37	2·56	0·27	2·36	0·41	0·362
ASAT (U/l)	19·5	1·31	19·9	1·86	15·1	4·03	24·1	7·62	16·5	2·43	25·6	5·96	17·1	1·86	0·507
GLDH (U/l)	2·25	0·17	1·95	0·13	2·02	0·25	1·63	0·29	1·43	0·30	1·59	0·24	1·18	0·32	0·078
ALP (U/l)	78·8^{a}	6·34	80·6^{a}	4·12	79·2^{a}	5·41	60·8^{b}	2·41	101·1^{c}	8·52	92·6^{c}	6·33	76·2^{a,c}	4·65	0·002
Creatinine (mg/dl)†	0·12^{a}	0·01	0·10^{a}	0·01	0·06^{b}	0·01	0·10^{a}	0·01	0·12^{a}	0·01	0·10^{a}	0·01	0·05^{b}	0·01	<0·001
Choline (μM)	15·1^{a}	1·30	10·6^{b,c}	0·6	15·5^{a}	1·61	13·2^{a,b}	1·11	13·8^{a,b}	0·97	10·4^{b,c}	0·77	9·00^{c}	0·57	<0·001
Ca (mg/dl)†	11·2^{a}	0·50	11·2^{a}	0·26	9·78^{a}	0·52	10·4^{a}	0·28	11·8^{a}	0·76	10·9^{a}	0·32	6·64^{b}	1·64	<0·001
Chloride (mg/dl)†	448·6	6·60	445·0	7·75	472·6	5·82	464·9	10·9	457·4	12·5	451·2	5·22	451·2	12·9	0·370
Mg (mg/ml)	1·82	0·17	1·81	0·05	1·67	0·04	1·71	0·09	1·75	0·21	1·89	0·10	1·78	0·14	0·923
Phosphate (mg/dl)†	14·7^{a}	0·40	13·1^{a}	0·45	14·01^{a}	0·52	13·7^{a}	0·0·45	7·94^{b}	0·25	12·9^{a}	0·32	14·8^{b}	0·55	<0·001
Antioxidant capacity (Trolox mM)	0·25^{a}	0·03	0·37^{b}	0·03	0·41^{b}	0·03	0·43^{b}	0·03	0·61^{c}	0·03	0·65^{c}	0·06	0·68^{c}	0·03	<0·001
Lysozyme (U/l)	97·7	25·98	14·4	5·01	70·7	35·90	98·7	16·56	51·5	18·30	35·5	14·37	68·0	18·40	0·122
Respiratory burst (iRLU)	337·0^{a}	54·6	336·5^{a}	37·7	1933·5^{b}	254·9	479·0^{a}	104·1	332·50^{a}	39·2	326·0^{a}	32·3	365·0^{a}	37·7	<0·001
GH (ng/ml)	5·13^{a}	48	7·35^{a,b}	0·94	14·0^{c}	0·23	10·0^{b}	2·04	13·3^{c}	2·16	10·1^{b}	1·31	12·3^{c}	0·97	<0·001
IGF-I (ng/ml)	96·7^{a}	0·85	78·7^{b}	5·15	45·1^{c}	4·34	69·1^{b}	4·68	53·1^{c}	3·88	66·6^{b}	2·61	64·6^{b}	4·61	<0·001

CTRL, control; SAA, sulphur amino acids; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; PL, phospholipids; Min, minerals; Vit, vitamins; MCF, median corpuscular fragility; ND, not determined; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GLDH, glutamate dehydrogenase; ALP, alkaline phosphatase; GH, growth hormone; IGF, insulin-like growth factors.

^{a,b,c} Unlike superscript letters in each row indicate significant differences among dietary treatments (Student Newman–Keuls test, $P<0·05$)

* Result values from one-way ANOVA.

† To convert Hb from g/dl to g/l, multiply by 10. To convert glucose from mg/dl to mmol/l, multiply by 0.0555. To convert cholesterol, HDL-cholesterol and VLDL/LDL-cholesterol from mg/dl to mmol/l, multiply by 0.0259. To convert creatinine from mg/dl to μmol/l, multiply by 88.4. To convert Ca, chloride and phosphate from mg/dl to g/l, multiply by 10.

Table 5. Summary of the histological features observed in the liver and anterior (AI) and posterior (PI) intestine of fish fed control (CTRL) and nutrient-deficient diets*

Tissue	Trait	CTRL	SAA	*n*-3 LC-PUFA	PL	P	Min	Vit
Liver	Lipid droplets	–	+	++	+/–	+	+	–
	Glycogen PAS+	–	++	+	–	++	+	++
AI	Goblet cells PAS+	++	++	–	–	–	–	+
	Lipid droplets	–	–	+++	++	–	–	++
	Submucosal granulocytes	–	–	–	+	–	+	++
	Lymphocytes	–	–	–	–	–	–	+
PI	Goblet cells PAS+	+	–	–	+	–	–	+
	Goblet cells Giemsa+	++	++	+	++	–	–	–
	Submucosal granulocytes	–	–	–	–	–	+	+
	Epithelial lymphocytes	–	–	–	+	–	–	+
	Rodlet cells	–	–	–	+	+	++	+

SAA, sulphur amino acids; *n*-3 LC-PUFA, *n*-3 long-chain PUFA; PL, phospholipids; Min, minerals; Vit, vitamins; PAS, periodic acid–Schiff.
* The intensity of the features was graded from absence (–) to the highest observed level (+++).

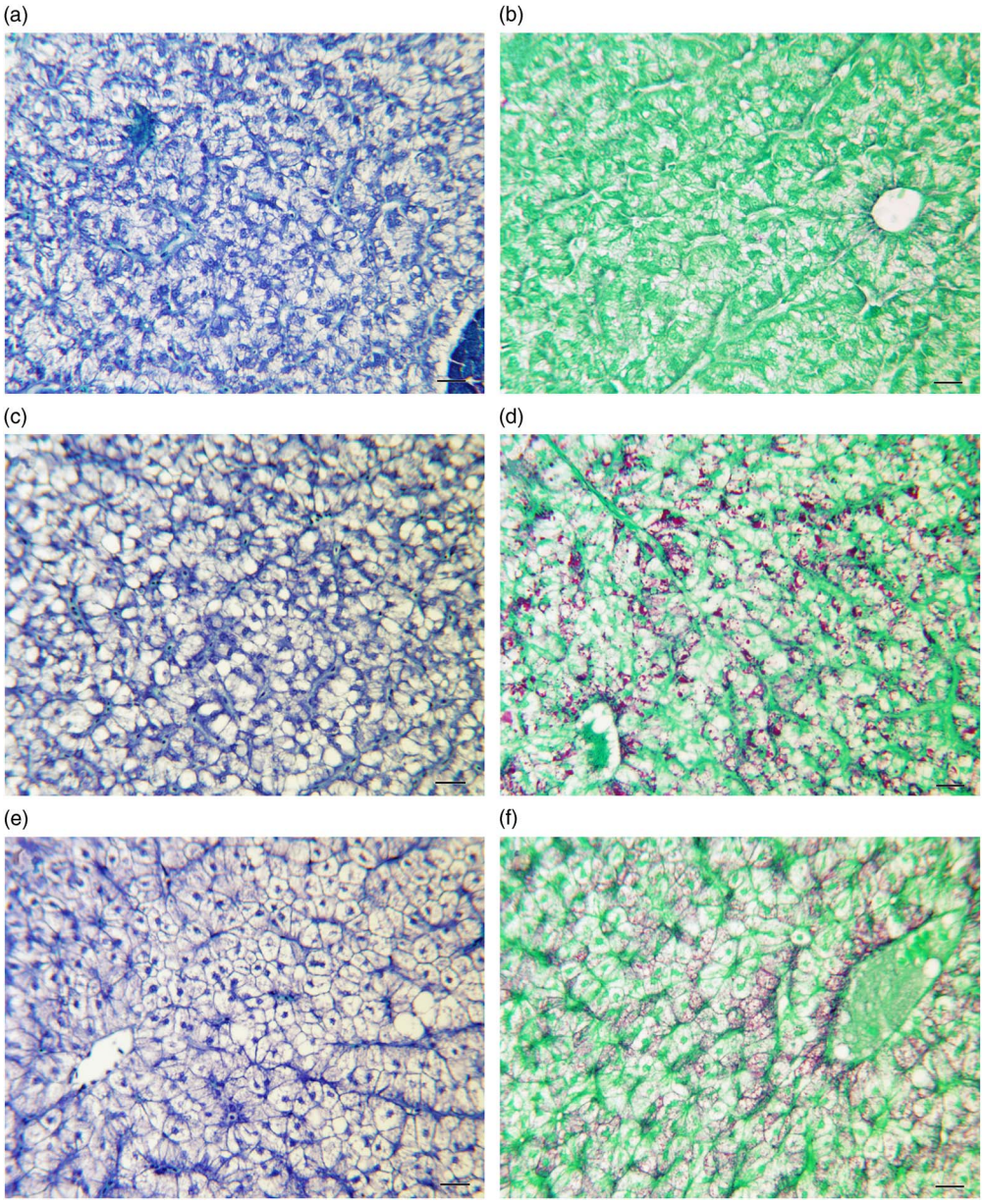

Fig. 1. Histochemical staining of liver sections from gilthead sea bream fed the control diet (a, b), the *n*-3 long-chain PUFA diet (c, d) or the sulphur amino acids diet (e, f). Stainings: Giemsa (a, c, e), periodic acid–Schiff (b, d, f). Scale bars = 20 μm.

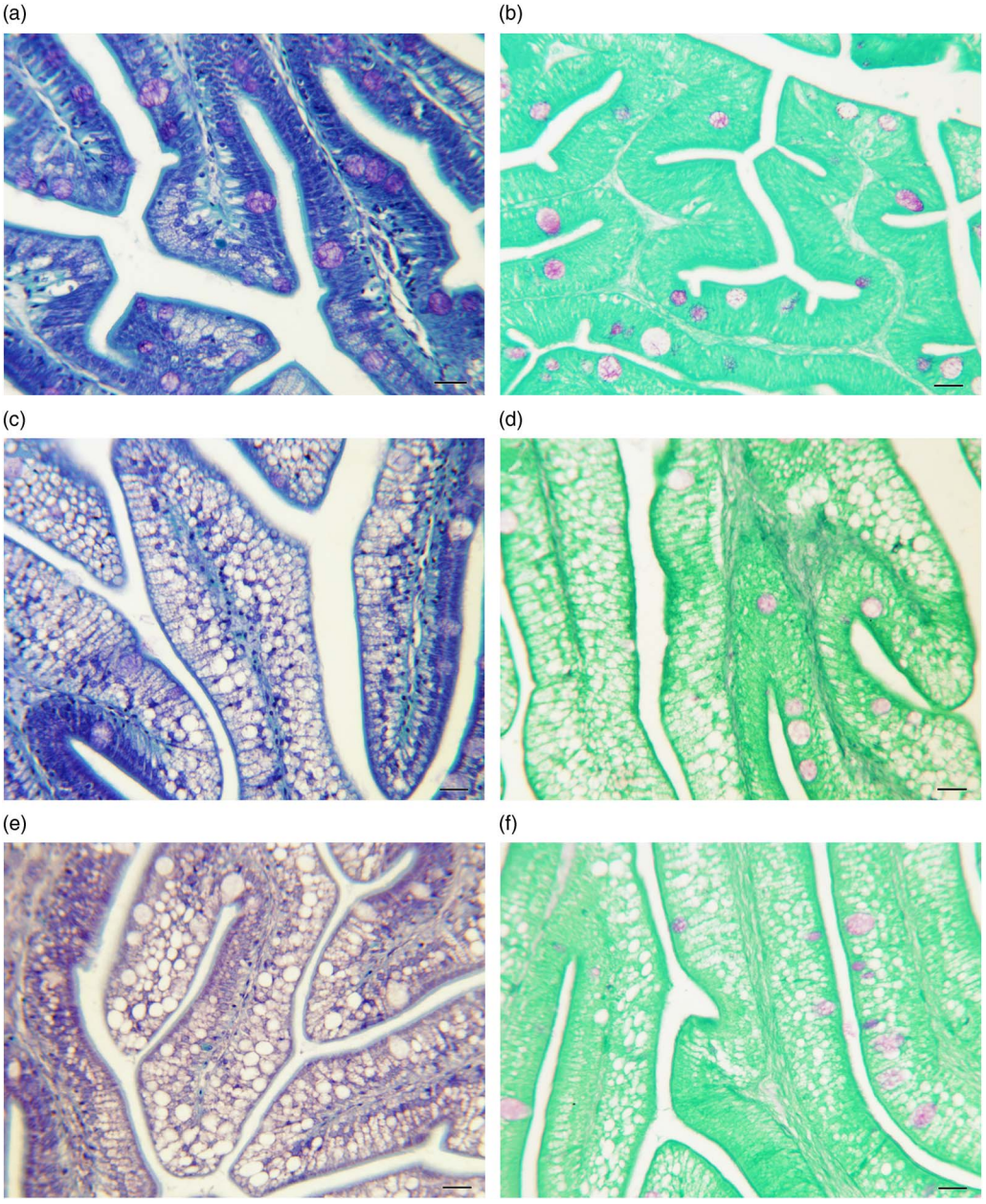

Fig. 2. Histochemical staining of anterior intestine sections from gilthead sea bream fed the control diet (a, b), the *n*-3 long-chain PUFA diet (c, d) or the vitamin diet (e, f). Stainings: Giemsa (a, c, e), periodic acid–Schiff (b, d, f). Scale bars = 20 μm.

cells was also decreased in P, Min and Vit fish. The staining of the epithelial layer of the posterior intestine was biphasic in the samples from all the experimental diets, except SAA and PL, in which it was homogeneous as in CTRL fish. The number of granulocytes in the submucosae in the anterior intestine and posterior intestine was not outstanding, except in Vit fish in the anterior intestine. Vit was the only diet in which intra-epithelial lymphocytes were in higher number than in the CTRL diets in both intestinal segments. Another remarkable feature was the high number of rodlet cells in the posterior intestine epithelium of PL, P, Min and Vit fish, with the highest level in Min fish.

Discussion

Comprehensive approaches have been used to address the total or partial FM/FO replacement in a wide range of finfish including rainbow trout[21] and typically marine fish such as European sea bass[22] and gilthead sea bream. In particular, for gilthead sea bream, the long-term consequences of feeding low FM/FO feeds on growth performance and endocrine status[7,23,24], health and welfare[17,25–30], fish quality[13,31,32] and food safety[33,34] have been considered in a highly integrated manner. However, knowledge on the specific effects and consequences of a given nutrient or a group of nutrients is

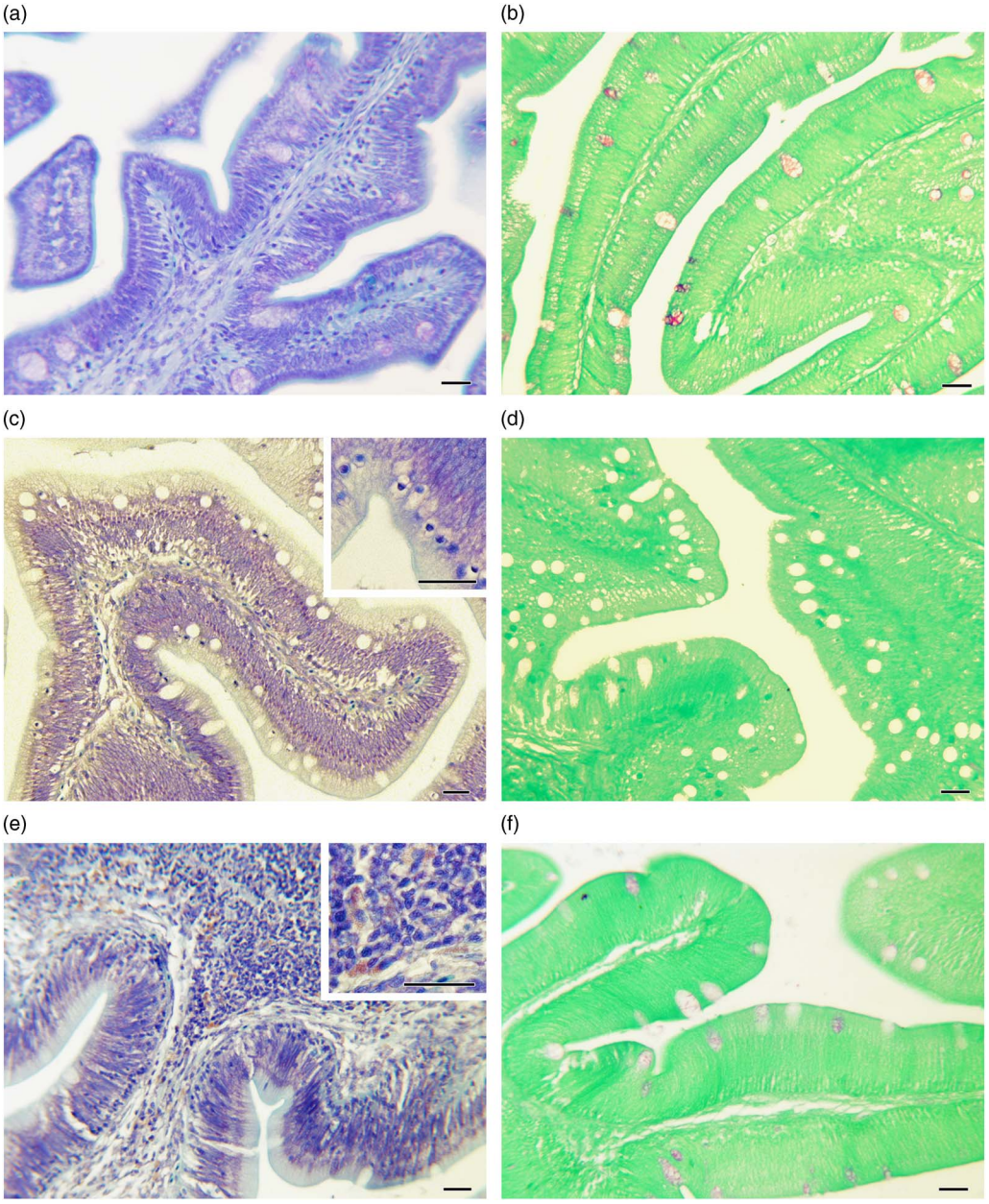

Fig. 3. Histochemical staining of posterior intestine sections from gilthead sea bream fed the control diet (a, b), the *n*-3 long-chain PUFA diet (c, d) or the mineral diet (e, f). Stainings: Giemsa (a, c, e), periodic acid–Schiff (b, d, f). Scale bars = 20 μm. Inset in (c) shows detail of rodlet cells in the epithelium; inset in (e) shows the abundant granulocytes in the submucosa.

mostly lacking. Thus, this is one of the first studies analysing at the same time the effects of dietary deletion or reduction of six different nutrients recognised as essential to fish[10], which contributes to fill the gaps in the diagnosis of the nutritional fish condition under standardised rearing conditions. Of note, we did not have the same degree of deficiency for the six different nutrients tested. Indeed, EPA and DHA contents were reduced to trace levels in the *n*-3 LC-PUFA diet with the total replacement of FO by VO. Likewise, the main source of P in the CTRL diet was the added calcium phosphate, but it was more difficult to induce a severe Met deficiency while preserving the supply of other essential amino acids.

Adipose tissue (AT) is now recognised as an important target tissue for the diagnosis and treatment of most lipid metabolic disorders arising from an excessive lipid influx[35]. Clinically, lipotoxicity not only appears with fattening, but also with hypoxia, blockage of glucocorticoid-sensitive pathways and the acquisition and maintenance of inflammatory phenotypes[36–38]. In humans as well as in other animal models, FO and *n*-3 LC-PUFA of FO are able to reverse these clinical symptoms, decreasing lipolysis and alleviating the inflammatory condition of AT, which in turn reduces the production of lipolytic cytokines, the release of free FA and thereby the risk of hepatic steatosis[39]. This liver syndrome is the result of a

massive synthesis and/or deposition of TG in the form of lipid vacuoles, and it is commonly observed in many fish species challenged with xenobiotics and unbalanced diets[40]. This metabolic derangement is often accompanied by the displacement of the nucleus of hepatocytes and even pyknosis. Relatively little is known about the ultimate mechanism, although the dietary protein source and protein/energy ratio have a major effect on the regulation of lipid metabolism in European sea bass[41,42]. The replacement of FM and FO with plant protein and oil sources also has a number of effects on the regulation of intermediary metabolism in trout[43] and Atlantic salmon[44]. In gilthead sea bream, tissue FA uptake[45,46] and mitochondrial respiration uncoupling[47] are highly affected by FM and FO replacement with plant ingredients. In addition, increases in cell size and lipolytic rates are characteristic features of isolated adipocytes from fish fed either plant proteins or VO[48,49], and lipoid liver degeneration is frequently observed as a metabolic disturbance in gilthead sea bream fed high plant ingredient-based feeds[7,23,50]. Accordingly, the present study shows that the total replacement of FO by VO in FM-free diets (*n*-3 LC-PUFA diet) caused a slight reduction of MSI related to a lipodystrophic phenotype with clinical signs of hypolipidaemia and hepatomegaly (high HSI). The loss of mesenteric AT mass, together with low plasma levels of choline and calcium, was even higher in the fish fed diets with reduced vitamin supply. However, abnormal liver lipid deposition rates were not found by light microscopy in this group of fish. Therefore, the absence or modification of AT mass cannot be used as the only criterion for the risk assessment of liver steatosis.

The main signs of P deficiency in fish nutritional studies are poor bone mineralisation and bad growth performance[9,51], which were correlated in the present study with low plasma phosphate concentrations and high plasma activities of ALP in the absence of apparent skeletal deformities. ALP activity is a well-known bone turnover biomarker[52], but here we also observed an increased plasma ALP activity with the reduced dietary supply of trace minerals (iron, magnesium, zinc, copper and selenium) in Min fish. However, the increase in MSI in combination with hyperlipidaemia allows differentiating the effects of deficiency in P from those due to deficiencies in trace minerals. In this sense, it must be noted than an important role of P in the regulation of lipid metabolism has also been reported in other fish species[53,54].

Hct and Hb values are general indicators of health, and these haematological parameters change in response to nutrient deficiencies, environmental conditions, growth status and anti-nutritional factors[55,56]. A high incidence of anaemia has been reported in yellowtail and parrot fish fed FM-free diets[57,58]. This has been attributed, at least in part, to taurine deficiency, and thus its supplementation seems to be required when these fish are fed low levels of taurine in FM- or plant protein-based diets[59]. In our study, all the diets were supplemented with taurine (0·3 %), and signs of anaemia, such as low erythrocytes, Hct and Hb values, were found only in fish fed the *n*-3 LC-PUFA diet, which also showed an increased erythrocyte osmotic fragility, as reported in rats with deficiencies in LC-PUFA[60]. Importantly, this clinical sign was not found in the PL fish group, and therefore contributes to better define the sometimes-overlapping signs of nutrient deficiencies in EFA and PL.

Previous studies in gilthead sea bream have already highlighted increased RB of blood leucocytes in fish fed diets with a high replacement of FO by VO[17,25]. This has been confirmed in our study, and importantly RB turned out to be a specific criterion for the diagnosis of *n*-3 LC-PUFA deficiency, as it was notably increased in this group and not with PL or other nutrient-induced deficiencies. Likewise, a close talk between PL and bone metabolism exists, and the decrease in plasma ALP activity is becoming an easy and highly valuable marker of PL deficiencies in gilthead sea bream. Of note, a high PL supply is required during early life stages in fish to improve survival rates and to decrease the incidence of skeletal deformities[61], but PL requirements in juvenile and adult fish are still controversial[62,63].

Plasma transaminases and GLDH are commonly used in clinical chemistry as markers of tissue damage[64], but the current results indicate that they are poorly informative of nutritionally mediated metabolic derangements in gilthead sea bream. In contrast, overall plasma total antioxidant activity was increased in parallel to the reduced growth performance, indicative of a reduced aerobic metabolism and, therefore, of a reduced production of reactive oxygen species. Strong support for this comes from inbreeding selection of rat strains, which demonstrates that most stressful and oxidative risk factors correlate with the low expression of genes required for mitochondrial biogenesis and oxidative phosphorylation[65]. Experimental evidence also indicates that plasma antioxidant capacity is increased by hypoxia exposure, probably due to the concurrent decrease of basal metabolism, mitochondrial respiration uncoupling and oxidative phosphorylation[66]. However, the magnitude and even the direction of the change is poorly predictable when comparisons are made between this and previous feeding trials with practical diets containing FM[17].

Most growth regulatory events in fish are mediated at the hormonal level by the GH/IGF axis, keeping pituitary GH secretion and hepatic/extra-hepatic IGF production under control[67–69]. Therefore, circulating GH and IGF-I are one of the most important endocrine determinants of growth in a vast array of stress and nutritional disorders arising from crowding and handling stress[70], changes in ration size[68,71], dietary protein/energy ratio[72,73] and dietary protein and lipid sources[23,24,74]. This notion was also found here, and importantly a close positive correlation between growth rates and circulating levels of IGF-I was evidenced, regardless of the nutrient deficiency. As expected, an opposite trend was found for GH and growth rates, which would reflect a lowered negative feedback inhibition of IGFs upon pituitary GH release as a result of a transcriptional defect in the signal transduction of GH receptors. This metabolic feature leads to liver GH resistance and reduced hepatic IGF production, in spite of increased plasma levels of GH, as it has been stated previously in a wide range of fish species, including gilthead sea bream[71,75–77].

Histological traits also gave interesting information on the possible pathological outcome of nutrient deficiencies. The highest accumulation of hepatic lipids was found in gilthead sea bream fed the *n*-3 LC-PUFA-deficient diet, followed by SAA, P and Min. However, lipid accumulation did not reach the highest score of steatosis observed by us with other dietary interventions[7,15], or by other authors using diets with an

excess of dietary lipids[78], EFA deficiencies[79] and VO[50,80,81]. Glycogen accumulation was also high in the liver of SAA, P and Vit fish, although the observed glycogen deposition did not reach an extreme condition. Massive accumulation of supranuclear lipid droplets in the intestinal epithelial layer is also considered a sign of inadequate/unbalanced diets due to a reduced metabolisation of absorbed lipids, either because they are not needed or because they are absorbed in a higher amount than needed. This accumulation was not observed in the posterior intestine for any diet, but it was a clear feature in the anterior intestine after feeding *n*-3 LC-PUFA-, PL- and Vit-deficient diets. This epithelial accumulation stands as an early marker of deregulated lipid metabolism compared with lipid accumulation in the liver, as it was visible in PL and Vit fed fish, in which lipoid liver degeneration was not found.

Another histological feature with clear differences among fish was the number of goblet cells and their staining characteristics. In this sense, it is noteworthy that the *n*-3 LC-PUFA diet induced a strong reduction in the number of goblet cells, and the remaining goblet cells were not stained either by Giemsa or by PAS, indicating that mucin content was not neutral or acidic. Neutral mucins were also absent in the anterior intestine as a result feeding the different diets, except SAA and Vit, and in the posterior intestine, except PL and Vit. Previous gilthead sea bream studies have indicated that other models of nutritional- and parasite-induced enteritis also invoke modifications in goblet cell type and number[26,82]. In fact, the 66 % replacement of FO by VO in plant protein-based diets produced a significant decrease in GC with neutral and acidic mucins in the anterior intestine and medium intestine as well as in those with carboxylic mucins and sialic acid in the medium intestine, but no significant changes in the posterior intestine. In European sea bass fed mannan oligosaccharides, the number of goblet cells secreting acidic mucins was increased[83]. In yellow perch fed wheat–gluten–protein-based diets, even supplemented with free lysine, the number of goblet cells was also decreased[84]. In contrast, Atlantic salmon[85], Atlantic cod[86] and carp[87] fed high plant protein-based diets presented goblet cells hypertrophy and hyperplasia.

Rodlet cells are exclusive of teleost epithelial layers and represent a cell type whose function has not yet definitively been established, although considered to be closely linked to the immune system and osmoregulation[88]. Many studies consistently report an association between rodlet cells proliferation/hyperplasia and the presence of a variety of parasites, chemicals and environmental stressors[89,90] and they even have been proposed as biomarkers[89]. However, there is no previous report on the relationship with the diet. In the present study, the number of rodlet cells was increased only in the posterior intestine, notably in fish fed Min diet. The increased presence of rodlet cells could, therefore, be interpreted either as a sign of inflammation or as a sign of osmoregulatory imbalance and reinforce the idea that cell osmoregulation is dependent on trace minerals rather than P uptake. The higher presence of rodlet cells was coincident with other inflammatory markers (intra-epithelial lymphocytes and submucosal granulocytes) in fish fed Min, PL and Vit diets either in the anterior intestine or in the posterior intestine. In any case, the observed cellular inflammation was mild and by no means comparable with that caused by other dietary interventions in Atlantic salmon[91], common carp[87] or gilthead sea bream[15].

In summary, clinical blood biochemistry and tissue histopathology have been proved highly informative to assess the nutritional condition of farmed gilthead sea bream. The final diagnosis outcome might require confirmation by more specific assays, but the generated information is by itself useful for the overall assessment of fish performance and metabolic condition when the measured parameters are referred to a CTRL group or historical data for a given fish strain, life stage and/or rearing condition. All this information is summarised in online Supplementary Table S1 as a set of clinical signs for a given nutritional deficiency in gilthead sea bream. To establish the normal range of variation of these parameters as a function of season and growth performance, the data from this study were combined with those derived from other past and on-going feeding trials with practical diets. The rationale for this is to cover a wide range of variation for marine and plant ingredients without apparent detrimental effects on fish performance through the production cycle. The reference values for these studied biomarkers are shown in online Supplementary Table S2, and will be periodically updated on the basis of the data produced within the ARRAINA project.

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G. F. B.-L. and L. B.-P. performed blood analyses; I. E. and A. S.-B. performed and supervised the histopathological analyses and RBC counts; S. K. formulated the experimental diets; J. P.-S. designed and coordinated the work, wrote the manuscript and took primary responsibility for its final content. All the authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest perceived to bias the study.

Supplementary Material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S0007114515002354

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