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Protein utilisation and intermediary metabolism of Senegalese sole (Solea senegalensis) as a function of protein:lipid ratio

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Abstract

Previous experiments with Senegalese sole (Solea senegalensis) have demonstrated that dietary lipid levels above 8% impaired growth and did not promote protein retention. We hypothesised that this low ability to use high-lipid diets may depend on the dietary protein level. In the present study, a 2 × 2 factorial design was applied where two dietary lipid (4–17% DM) and two dietary protein (below and above the requirement levels, 48 and 54% DM) levels were tested in juveniles for 114 d. Growth performance was not improved by the increase in dietary fat, irrespectively of the dietary protein levels. Protein retention was similar among the diets, although fish fed the diets with high lipid content resulted in significantly lower protein gain. Among the enzymes involved in amino acid catabolism, only aspartate aminotransferase activity in the liver was affected by the dietary lipid levels, being stimulated in fish fed high-lipid diets. Moreover, phosphofructokinase 1 activity was significantly elevated in the muscle of Senegalese sole fed 4% lipid diets, suggesting enhanced glycolysis in the muscle when the dietary lipid supply was limited and dietary starch increased. The results confirmed that high-lipid diets do not enhance growth, and data from the selected enzymes support the assumption that lipids are not efficiently used for energy production and protein sparing, even when dietary protein is below the protein requirement of the species. Furthermore, data suggest a significant role of glucose as the energy source in Senegalese sole.

Key words: Lipid levels: Protein levels: Fatty acid oxidation: Protein catabolism

The balance between dietary protein and energy has a major impact on protein utilisation[1,2], affecting the ratio between protein oxidation (energy) and synthesis (growth). It also determines body reserve storage that varies depending on the respective proportions of macronutrients supplied by the diet. Dietary lipids appear to be a more efficient non-protein energy source for fish than carbohydrates, especially in salmonids and other carnivorous species. Besides their higher energy value and their high digestibility, dietary lipids are generally preferentially oxidised compared with carbohydrate sources[3,4]. Consequently, over the past decades, the use of high-lipid diets in fish aquaculture has become a common practice due to the role of dietary lipids as a non-protein energy source, enhancing growth, protein retention and reducing organic matter and N losses[2,5]. However, previous studies have indicated that the lipid metabolism of Senegalese sole (*Solea senegalensis*) differed from the general trend.

Senegalese sole is a marine fish with high protein requirements, a feature shared with most of the marine fish species, especially flatfish[6–11]. According to Rema *et al.*[9], at a fixed dietary lipid level of 12%, Senegalese sole diets should include a high crude protein level (53% DM) to maintain good overall growth performance. In flatfish species, a protein-sparing effect of dietary lipids has been observed in turbot (*Scophthalmus maximus*)[12] and Atlantic halibut (*Hippoglossus hippoglossus*)[13]; however, in Senegalese sole, increasing the lipid levels from 11 to 21%[14] did not improve growth. Borges *et al.*[15] has recently demonstrated a low lipid tolerance of Senegalese sole, and recommended a dietary lipid inclusion up to 8% for optimal growth and feed utilisation

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; FAS, fatty acid synthase; GDH, glutamate dehydrogenase; HAD, 3-hydroxyacyl-CoA dehydrogenase; PFK, phosphofructokinase.

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efficiency at a protein level of 57 % (DM basis). We hypothesised that the low ability to use high-lipid diets may depend on the dietary protein level, with dietary protein at high levels (above 50 % DM basis) preferentially oxidised over non-protein energy sources. In many teleost species, an improvement in protein retention can be achieved when low-protein diets are used as long as the dietary non-protein energy fraction increases[5,16,17].

Measurements of enzyme activity associated with growth parameters are generally useful tools to study fish metabolic adaptation to dietary supply[18]. The activity of key hepatic enzymes involved in protein catabolism has been pointed out as a relevant indicator of the metabolic utilisation of dietary protein by fish[19,20]. Moreover, non-protein energy and dietary protein levels can generate different responses in hepatic lipogenic enzymes[17,21]. With the increase in non-protein energy supplied either as lipids or carbohydrates, changes in β-oxidation and glycolytic pathways are expected. Mitochondrial β-oxidation is the major energy-producing pathway from fatty acid oxidation, providing an important source of energy for tissues, especially the heart and the skeletal muscle[22]. Assessment of the major sites of lipid catabolism and synthesis may provide further insight into the apparent weak utilisation of dietary lipids in Senegalese sole.

The objective of the present study was to test the hypothesis that the limited capacity of Senegalese sole to utilise dietary lipids is related to dietary protein levels. To this end, a 2 × 2 factorial design was applied where two dietary lipid (4–17 % DM) and two dietary protein (just below and above the requirement levels, 48 and 54 % DM) levels were tested in Senegalese sole juveniles. The dietary lipid levels were chosen based on previous experiments[15] where sole fed with diets containing 4 % lipid displayed significantly higher growth rates than those fed higher lipid levels (12, 16 and 20 % lipid, DM basis). At the end of the trial, growth performance, body composition and feed utilisation were analysed. In addition, the activity of selected hepatic enzymes was determined as an indicator of intermediary metabolism. Therefore, the activities of selected enzymes involved in amino acid catabolism (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and glutamate dehydrogenase (GDH)), fatty acid oxidation (3-hydroxyacyl-CoA dehydrogenase (HAD)), fatty acid synthesis (fatty acid synthase (FAS)) and glucose catabolism (phosphofructokinase 1 (PFK-1)) were assessed.

Materials and methods

Experimental diets

In the present study, four experimental diets were formulated to contain two protein levels, below and above the requirement levels (48 and 54 % DM), and two lipid levels, previously shown to result in significantly different growth rates (4 and 17 % DM) (Table 1). The increase in the lipid levels was achieved by adding fish oil and lowering the amount of wheat meal, whereas the two protein levels were obtained by mixing different quantities of wheat meal and wheat gluten. As a consequence, all diets had varying carbohydrate levels that resulted in different starch contents. Low-fat/low-protein diets had higher starch contents (26–20 *v.* 17–14 % DM). The ingredients and proximate composition are presented in Table 1. The ingredients were finely ground, mixed and pelleted dry without steaming using a laboratory pelleting machine (C-300 model; California Pellet Mill) with a 2·0 mm die.

Growth trial

The present study was conducted under the supervision of an accredited expert in laboratory animal science (following Federation of Laboratory Animal Science Associations (FELASA) category C recommendations) and according to the European Economic Community animal experimentation guidelines directive of 24 November 1986 (86/609/EEC).

The study was performed at the experimental facilities of Centro de Investigação Marinha e Ambiental (CIIMAR), Porto, Portugal, with Senegalese sole (*S. senegalensis*) juveniles supplied by a commercial fish farm (Coelho & Castro). After arrival at the experimental unit, fish were acclimatised to the new facilities for 2 weeks. For each treatment, three homogeneous groups of twenty fish (average initial body weight 17 (SD 1·8) g) were grown in white fibre glass tanks

Table 1. Ingredients and proximate composition of the experimental diets with the different protein and lipid levels

	Dietary treatments			
Protein levels …	48 %		54 %	
Lipid levels …	4 %	17 %	4 %	17 %
Ingredients (g/kg)				
Fishmeal LT	300	300	300	300
CPSP G	80	85	85	85
Soyabean meal 48	120	119	125	106
Maize gluten	90	90	90	90
Wheat meal	360·9	217·9	271·9	140·9
Wheat gluten	39	60	118	148
Fish oil	0	118	0	120
Choline chloride	1	1	1	1
Lutavit C35	0·3	0·3	0·3	0·3
Lutavit E50	0·5	0·5	0·5	0·5
Vitamin* and mineral mix†	2·5	2·5	2·5	2·5
Betaine	0·7	0·7	0·7	0·7
DCP	5	5	5	5
Proximate composition (% DM)				
DM	92·1	93·3	93·1	96·0
Ash	7·9	7·5	7·8	7·4
Crude protein	48·4	48·6	55·5	54·1
Crude fat	4·4	17·6	4·7	17·3
Starch	26·0	16·5	20·1	13·5
Gross energy (kJ)	20·1	22·8	20·4	23·0

LT, low temperature; CPSP G, fish soluble protein concentrate (hydrolysed fish-meal); Lutavit C35, vitamin C; Lutavit E50, vitamin E; DCP, dibasic calcium phosphate.

* Vitamins (per kg diet): vitamin A, 10 000 IU (retinol, 3000 mg); vitamin D_3, 2125 IU (cholecalciferol, 53 mg); vitamin K_3, 12·5 mg; vitamin B_{12}, 0·025 mg; vitamin B_1, 10 mg; vitamin B_2, 25 mg; vitamin B_6, 12·5 mg; folic acid, 12·5 mg; biotin, 0·86 mg; inositol, 300 mg; nicotinic acid, 85 mg; pantothenic acid, 37·50 mg.

† Minerals (per kg diet): Mn (manganese oxide), 25 mg; I (potassium iodide), 1·88 mg; Cu (copper sulphate), 6·25 mg; Co (cobalt sulphate), 0·13 mg; Zn (zinc oxide), 37·5 mg; Se (sodium selenite), 0·31 mg; Fe (iron sulphate), 75 mg.

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(50 cm × 35 cm). Each tank was supplied with filtered and heated (20 ± 1°C) seawater (30‰), at a flow rate of 1·5 litres/min. The most important water parameters (temperature, dissolved O_2, salinity, pH and nitrogenous compounds) were monitored during the entire trial and maintained at levels within the limits recommended for marine species. Fish were exposed to an artificial photoperiod of 12 h light. At the beginning and end of the experiment, individual weights and the length of fish were recorded. Fish were fed *ad libitum* by automatic feeders six to eight meals per d (24 h) over a period of 114 d. All tanks were daily monitored and feed distribution adjusted according to feed losses in each tank[15].

At the beginning of the feeding trial, ten fish from the initial stock were sampled and stored at −20°C until whole-body analysis. At the end of the trial, three fish per tank were sampled 24 h after the last meal for the same purpose. All fish sampled were euthanised by a sharp blow on the head. Liver and muscle were removed from twelve fish per tank 6 h postprandial and immediately frozen in liquid N_2, and thereafter stored at −80°C. From those, two liver samples were used for total lipid analysis and the remaining ten liver samples for determination of enzyme activity.

Analytical methods

Frozen samples from each tank were cut without thawing into pieces avoiding drip losses, minced altogether using a meat mincer and pooled homogeneously. After homogenisation, a part of each sample was used to determine the moisture content (105°C for 24 h to constant weight). The rest of the samples were subsequently freeze-dried before further analyses.

Feed and freeze-dried whole-body samples were analysed for DM (105°C for 24 h), ash (550°C for 6 h, Nabertherm L9/11/B170; Nabertherm GmbH), crude protein (N × 6·25, Nitrogen Analyser, Leco FP-528; Leco), crude lipid (petroleum diethyl ether extraction, Soxtherm Multistat/SX PC; Gerhardt) and gross energy (adiabatic bomb calorimeter, C2000; IKA). Starch was measured following the amyloglucosidase–amylase method[23].

Liver samples were analysed individually for total lipid levels following Folch *et al.*[24]. HAD (*EC* 1.1.1.35) activity was measured in the liver and muscle according to Kobayashi *et al.*[25]. ALAT (*EC* 2.6.1.2), ASAT (*EC* 2.6.1.1) and GDH (*EC* 1.4.1.2) activities were determined as follows: a frozen sample of either the liver or muscle was homogenised, diluted at 1:10 in ice-cold buffer (30 mM-HEPES, 0·25 mM-saccharose, 0·5 mM-EDTA, 5 mM-K_2HPO_4 and 1 mM-dithiothreitol, pH 7·4). The homogenate was then centrifuged at 900 ***g*** for 10 min, and the supernatant used for ALAT, ASAT and GDH determinations. For GDH, the supernatant was sonicated for 1 min (pulse 1 s, amplitude 50) and centrifuged again at 15 000 ***g*** for 20 min. ALAT and ASAT were assayed using kits from Enzyline (ALAT/GPT, reference no. 63 313; ASAT/GOT, reference no. 63 213) at 37°C and followed by at 340 nm. GDH activity was measured using 10 mM-L-glutamic acid at 37°C, as described previously by Bergmeyer[26].

FAS activity was measured in the liver by an isotopic method using [^{14}C]acetyl-CoA according to Hsu & Butterworth[27]. Liver was homogenised in three volumes of ice-cold buffer (0·02 M-Tris–HCl, 0·25 M-sucrose, 2 mM-EDTA, 0·1 M-NaF, 0·5 mM-phenylmethylsulphonyl fluoride and 0·01 M-b-mercaptoethanol, pH 7·4). The homogenates were centrifuged at 15 000 ***g*** for 20 min at 4°C and the activity of FAS was measured on the supernatant.

PFK-1 activity was assayed in the muscle in a final volume of 0·2 ml containing 100 mM-Tris–HCl (pH 8·25), 5 mM-$MgCl_2$, 50 mM-KCl, 0·15 mM-NADH, 4 mM-ammonium sulphate, 12 mM-2-mercaptoethanol, 10 mM-fructose-6-phosphate, fructose bisphosphate aldolase (0·675 U/ml, *EC* 4.1.2.13), triose-phosphate isomerase (5 U/ml, *EC* 5.3.1.1), glycerol-3-phosphate dehydrogenase (2 U/ml, *EC* 1.1.99.5) and 5 µl crude extract. The PFK-1 reaction was measured after the addition of 1 mM-fructose-6-phosphate at 30°C, followed by a decrease in absorbance at 340 nm.

One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 µmol of substrate per min at the assay temperature. Enzyme activities were expressed per mg soluble protein. Protein concentration was measured according to the Bradford method[28], using a protein assay kit (Bio Rad) with bovine serum albumin as a standard.

Statistical analysis

Statistical analyses followed methods outlined by Zar[29]. All data were tested for homogeneity of variances by Bartlett's tests, and then subjected to a two-way ANOVA with dietary protein and lipid levels as main effects using STATISTICA 9.0 (Statsoft). When protein × lipid interactions were significant ($P \leq 0{\cdot}05$), individual means were compared using Tukey's test. Significant differences were considered when $P \leq 0{\cdot}05$.

Results

Feed intake varied significantly with the dietary treatments and was lower with the high-lipid diets so that gross energy intake was similar in the four groups. Given the large differences in dietary lipid levels (17 *v.* 4% of DM), lipid intake was significantly higher in fish fed the 17% lipid diets despite the reduction in feed intake. As expected, there was an interaction between the lipid and protein levels for starch intake. The diets with a low lipid level (higher dietary starch content) clearly presented higher starch intake compared with the high-fat diets. Protein intake was affected either by the lipid or protein level and was significantly higher ($P < 0{\cdot}05$) in the diets containing 54% crude protein with the highest values recorded for fish fed the diet 54%/4%.

At the end of the trial, all treatments resulted in a threefold increase in body weight (Table 2) that ranged from 52 g (54%/17%) to 60 g (54%/4%). The daily growth index and the final body weight were significantly affected by the dietary lipid levels, with the highest values recorded for the diets with the low lipid/high starch level ($P \leq 0{\cdot}05$). The feed conversion ratio was affected by both the protein ($P < 0{\cdot}05$) and lipid

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Table 2. Effect of the different dietary protein/lipid levels on Senegalese sole intake and growth after 114 d (Mean values and standard deviations, *n* 3)

	Dietary treatments										
Protein levels …	48 %				54 %						
Lipid levels …	4 %		17 %		4 %		17 %		Two-way ANOVA		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Lipid	Protein	Lipid × protein
Intake (g or kJ/kg per d)*											
DM	12·32	0·22	11·14	0·55	12·00	0·48	10·17	0·64	<0·05	0·054	0·28
Protein	5·96	0·10	5·41	0·27	6·66	0·26	5·50	0·35	<0·05	<0·05	0·07
Lipids	0·54[b]	0·01	1·96[a]	0·10	0·57[b]	0·02	1·76[a]	0·11	<0·05	0·09	<0·05
Starch	3·20[a]	0·01	1·83[c]	0·01	2·41[b]	0·01	1·47[d]	0·01	<0·05	<0·05	<0·05
Energy (kJ)	247·26	4·35	253·48	12·48	245·14	9·74	233·75	14·70	0·70	0·12	0·20
Growth											
Final body weight	54·59	1·57	53·15	3·90	60·05	3·40	52·32	3·62	<0·05	0·25	0·13
DGI†	1·07	0·03	1·04	0·08	1·17	0·07	1·02	0·07	<0·05	0·30	0·17
FCR‡	1·34	0·03	1·24	0·02	1·23	0·07	1·14	0·03	<0·05	<0·05	0·87
PER§	1·54	0·04	1·66	0·02	1·47	0·08	1·62	0·04	<0·05	0·08	0·52

DGI, daily growth index; FCR, feed conversion ratio; PER, protein efficiency ratio.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* Nutrient intake = nutrient intake/average body weight ((initial body weight + final body weight)/2)/d.
† DGI = $100 \times ((\text{final body weight})^{1/3} - (\text{initial body weight})^{1/3})$/d.
‡ FCR = dry feed intake/weight gain.
§ PER = weight gain/crude protein intake.

levels ($P<0·05$) of the diets with values ranging from 1·14 (54 %/17 %) to 1·34 (48 %/4 %). The protein efficiency ratio was affected by the lipid levels and was higher in fish fed the high-lipid diets (1·66 and 1·62 for 54 %/17 % and 48 %/17 %, respectively).

No differences were found in the hepatosomatic index and liver lipid content between the treatments, with the values ranging from 0·99 to 1·01 and 8·54 to 11·10, respectively. The viscerosomatic index was significantly affected by the lipid levels ($P<0·05$), with the high-lipid diets leading to higher values. Whole-body protein and moisture were significantly affected by the lipid levels, and were higher in fish fed the low-lipid diets compared with fish fed the high-lipid diets (Table 3). The opposite trend was observed for both whole-body energy and lipid content. The dietary lipid levels had a stronger effect on whole-body lipid and energy content than the dietary protein levels.

DM gain was similar among the treatments and varied from 2·24 (48 %/4 %) to 2·51 (48 %/17 %). Protein gain was affected by the lipid levels and was higher in fish fed the low-lipid diets. An interaction between the protein and lipid levels was observed for lipids and energy gain; fish fed the high lipid levels deposited more lipids and, consequently, more energy. DM was affected by either the dietary lipid or protein levels, with the higher values registered for the 54 %/17 % (23·78 %) and the lowest for the 48 %/4 % (18·18 %) diets. Lipids and energy retention (Table 3) were significantly affected by the lipid levels ($P<0·05$), whereas protein retention was neither affected by the lipid levels nor by the protein content. Fish fed the low-lipid diets displayed higher lipid retention than those fed the high-lipid diets. The inverse was observed for energy retention since fish fed the high-lipid diets ingested higher amounts of lipids.

In the muscle, activity of the enzymes involved in protein catabolism (ALAT, ASAT and GDH) was not significantly affected by the dietary treatments (Table 4). In the liver, ASAT activity was significantly lower in fish fed the low-lipid diets than in those fed the high-lipid diets ($P<0·05$). The activity of HAD in either the liver or muscle was not affected by the protein or lipid levels in the diet. FAS was significantly affected by both the protein and lipid levels. FAS activity was higher in fish fed the low lipid level and the highest dietary protein level. PFK in the muscle was mainly affected by the lipid levels and ranged from 836·9 (48 %/17 %) to 1150·9 mU/mg protein (48 %/4 %) (Table 4).

Discussion

The results of the present study demonstrated that high-fat diets do not promote growth since Senegalese sole fed the high-lipid diets had a lower growth rate, despite the similar energy intake of the four groups. The present study confirms the results from earlier studies showing that increasing dietary lipid levels when dietary protein content is kept constant did not improve growth performance in either juveniles[15] or large-sized sole[30]. Moreover, diets containing lipid levels above 8 % resulted in reduced growth rates in Senegalese sole juveniles[15]. The contrary is observed in salmonids and most marine fish species[5,31–33]. In other flatfish species, the picture is not so clear. In juvenile (140 g)[13] or commercial-sized (1 kg) halibut[34], growth performance was not affected by the inclusion of non-protein energy sources (lipids and carbohydrates), even when dietary protein was below its protein requirement[11]. In juvenile turbot[35,36] and Japanese flounder (*Paralichthys olivaceus*)[37], a decline in growth rate was observed when fish were fed lipid-rich diets with high protein levels, and a possible positive role of

Table 3. Effect of the different dietary protein/lipid levels on Senegalese sole somatic indices (*n* 12) and nutrient utilisation (*n* 3) after 114 d (Mean values and standard deviations)

	Dietary treatments										
Protein levels …	48 %				54 %						
Lipid levels …	4 %		17 %		4 %		17 %		Two-way ANOVA		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Lipid	Protein	Lipid × protein
HSI*	0·99	0·16	1·01	0·19	0·99	0·18	0·99	0·15	0·69	0·72	0·76
Liver total lipids	9·04	1·75	11·1	2·68	8·54	1·47	9·16	2·17	0·14	0·17	0·42
VSI†	2·16	0·33	2·44	0·32	2·23	0·24	2·57	0·36	<0·05	0·34	0·89
Whole body composition‡ (% WW)											
Moisture	76·27	0·73	73·78	0·1	75·95	0·34	74·37	0·53	<0·05	0·64	0·14
Ash	1·82	0·23	1·62	0·09	1·79	0·08	1·70	0·24	0·19	0·82	0·59
Protein	19·17	0·29	18·31	0·29	19·46	0·48	18·41	0·15	<0·05	0·35	0·63
Lipids	3·08^{c}	0·29	6·63^{a}	0·23	3·25^{c}	0·29	5·8^{b}	0·23	<0·05	<0·05	<0·05
Energy (kJ/g)	5·53^{c}	0·01	6·66^{a}	0·04	5·64^{c}	0·06	6·37^{b}	0·12	<0·05	0·07	<0·05
Gain§ (g/kg per d)											
DM	2·24	0·07	2·51	0·11	2·41	0·13	2·42	0·15	0·08	0·62	0·09
Protein	1·88	0·03	1·72	0·12	2·01	0·13	1·72	0·09	<0·05	0·27	0·26
Lipids	0·26^{c}	0·03	0·72^{a}	0·01	0·3^{c}	0·04	0·61^{b}	0·04	<0·05	0·09	<0·05
Energy (kJ)	54·05^{c}	0·86	66·57^{a}	2·69	57·59^{b,c}	2·69	62·56^{a,b}	2·63	<0·05	0·86	<0·05
Retention‖ (% intake)											
DM	18·18	0·44	22·58	0·37	20·1	1·47	23·78	1·16	<0·05	<0·05	0·54
Protein	31·48	0·25	31·77	0·68	30·26	2·54	31·28	1·08	0·45	0·33	0·67
Lipids	48·95	6·67	36·94	1·8	53·25	5·2	34·71	2·63	<0·05	0·70	0·25
Energy (kJ)	21·86	0·43	26·27	0·23	23·51	1·4	26·8	1·41	<0·05	0·10	0·37

HSI, hepatosomatic index; VSI, viscerosomatic index; WW, wet weight.
^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
* HSI = 100 × (liver weight/body weight) (%).
† VSI = 100 × (visceral weight/body weight) (%).
‡ Initial body composition was as follows: moisture, 75·65 % WW; ash, 2·49 % WW; protein, 17·22 % WW; lipids, 4·53 % WW; energy, 5·81 kJ/g WW.
§ Nutrient gain = (nutrient in final carcass−nutrient in initial carcass)/average body weight/d.
‖ Retention = 100 × (final body weight × final carcass nutrient content−initial body weight × initial carcass nutrient content)/nutrient intake.

carbohydrates was suggested in Japanese flounder[38]. However, Caceres-Martinez *et al.*[35] found that increasing dietary lipids with a protein level below the protein requirement did not affect turbot growth. The present findings do not support the hypothesis of a protein-sparing effect of dietary lipids in Senegalese sole, regardless of the dietary protein levels. Indeed, the lowest-energy diets (devoid of fish oil addition) were able to promote higher growth rates than the high-lipid diets (high-energy-density diets). Despite the similar energy intake, most of the energy ingested by sole fed the low-lipid diets was delivered by protein and starch, and even so these fish showed the highest growth performance. As mentioned before, low-lipid diets presented higher starch content, which can be the reason for this unexpected higher growth. Incorporation of high lipid levels in the diets for salmonids and most marine fish species is a common practice to improve protein retention[5,17,33,39]. Carbohydrates can also be used to spare protein, although in marine fish, which are mainly carnivorous, the ability to utilise this macronutrient for energy proposes is generally limited when compared with lipids[40,41]. In European seabass and gilthead sea bream, data regarding the efficiency of protein sparing by carbohydrates are contradictory, with studies reporting either a significant protein-sparing effect or no effect at all[42]. The interaction between macronutrients is fairly well documented in fish species. A recent study in rainbow trout has suggested that impaired glucose utilisation in carnivorous species can be dependent on dietary lipid levels[43]. In Senegalese sole, protein retention was not influenced by either the lipid or protein levels in the diet. However, nutrient gain and whole body composition were significantly affected by the composition of the diets. Body protein content and protein gain were lower in fish fed the high-fat/low-starch diets, regardless of the dietary protein levels, providing additional evidence that dietary lipids do not favour protein accretion in Senegalese sole and that carbohydrates probably have a positive effect in nutrient utilisation. Further studies with adequate design are needed to assess the possible link between dietary carbohydrates and lipid intolerance. Overall, the results support the assumption that lipids are not being efficiently used as non-protein energy sources, even when the protein content is below the species requirement. This also could result from impaired lipid absorption that would limit the availability of dietary lipids for metabolic pathways. However, a recent study from Dias *et al.*[44] has suggested a high degree of lipid absorption in the intestine given the fact that energy digestibility of a diet with 55 % protein and 14 % fat was approximately 93 %. Data on the activity of enzymes involved in amino acid catabolism reinforce the hypothesis of a low protein-sparing effect of dietary lipids in Senegalese sole. Dietary amino acids can be used for protein deposition and growth or diverted from this pathway to serve as carbon substrates for gluconeogenesis, lipogenesis, ketogenesis or energy production. When amino acids are not used for tissue protein

Table 4. Effect of the different dietary protein:lipid ratios on Senegalese sole enzyme activity (mU/mg protein) after 114 d (Mean values and standard deviations, *n* 6)

	Dietary treatments										
Protein levels …	48 %				54 %						
Lipid levels …	4 %		17 %		4 %		17 %		Two-way ANOVA		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Lipid	Protein	Lipid × protein
ALAT											
Liver	563·4	212·6	686·4	200·7	576·4	206·7	684·1	191·6	0·20	0·95	0·93
Muscle	19·26	5·15	19·45	5·9	16·08	6·18	16·43	4·6	0·91	0·19	0·97
ASAT											
Liver	1706·4	259·4	2027·8	342·5	1729·1	182·7	2309·4	194·7	<0·05	0·16	0·22
Muscle	131·55	18·05	140·71	51·44	137·73	25·25	124·22	30·68	0·89	0·73	0·46
GDH											
Liver	174·7	39·3	191·3	41·8	207·7	43·3	206·5	35·9	0·66	0·18	0·61
Muscle	1·09	0·13	1·4	0·56	1·12	0·36	1·42	0·54	0·13	0·92	0·96
HAD											
Liver	69·07	14·21	72·52	9·59	61·05	6·86	64·69	11·77	0·44	0·09	0·98
Muscle	6·16	2·96	6·09	1·95	6·84	2·41	6·94	1·66	0·99	0·44	0·93
FAS											
Liver	0·05	0·024	0·01	0·01	0·11	0·06	0·03	0·02	<0·05	<0·05	0·78
PFK											
Muscle	1150·9	156·5	836·8	200·2	1037·2	225·3	928·9	253·3	<0·05	0·91	0·27

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; HAD, 3-hydroxyacyl-CoA dehydrogenase; FAS, fatty acid synthase; PFK, phosphofructokinase.

synthesis, they are rapidly deaminated through the combined intervention of transaminases such as ALAT and ASAT, and GDH. In seabream (*Sparus aurata*), hepatic ALAT appears to be sensitive to dietary protein levels[20], and in Atlantic salmon (*Salmo salar*) and rainbow trout, high-protein diets promoted the stimulation of liver ALAT[19,45]. In *Mugil capito*, the activity of both ALAT and ASAT increased with the increase in the protein content of the diet[46]. In the present study, the activity of ALAT, ASAT and GDH in the liver of sole was not affected by dietary protein levels. Contrarily to our expectations, the only significant change regarding the activity of the enzymes involved in amino acid catabolism was an increase in ASAT activity in the liver of sole fed the high-lipid diets, at both protein levels tested (48 and 54 % DM), supporting the assumption that high-lipid diets do not promote protein sparing. In seabream, ASAT seems to have a more prominent role in protein mobilisation compared with ALAT, but the activity of both enzymes decreased when fish were fed with high-energy diets, regardless of the protein levels[20]. In the liver of Atlantic salmon, ASAT activity is not dependent on the type of dietary energy sources[19]. With the increase in aspartate transamination in the liver of sole fed the high-lipid diets, higher concentrations of glutamate, the substrate of GDH, were expected and thus an elevated activity of GDH. However, liver GDH activity did not respond to increased ASAT activity.

Muscle tissue represents the largest body compartment and is responsible for most of the energy expenditure in fish. Dietary lipid content did not significantly affect the activity of ALAT, ASAT and GDH in the sole muscle, although there was a significant negative correlation between ALAT activity in the muscle and protein gain ($R -0{\cdot}73$, $P=0{\cdot}01$). Nevertheless, Campos *et al.*[47] observed a decrease in the expression of myogenic regulatory factors and myosins in the muscle of Senegalese sole fed increasing dietary lipid levels, supporting somehow the hypothesis that high lipid levels depress growth by reducing protein accretion.

Whole-body lipids and energy content were significantly affected by both the lipid and the protein levels of the diets. The high-lipid diets led to a significantly higher whole-body lipid and energy content of sole, confirming the effect of elevated lipid intake on whole-body lipid accumulation widely reported in various animals. In sole, lipid gain promoted by the high-lipid diets probably resulted from the storage of dietary fat because the activity of FAS, the enzyme catalysing the end-step of lipid neosynthesis, was depressed in the liver of sole fed the high-lipid diets. The higher body fat content observed in sole fed the high-lipid diets reinforces the assumption that dietary lipids are effectively absorbed once digested. However, lipid retention was significantly lower with the 17 % fat diets than with the 4 % lipid diets regardless of the dietary protein levels, suggesting that part of the dietary fat supply was diverted towards other pathways. Despite the general belief that high-protein diets may promote high lipid deposition in fish[17,45,48], in the present study, lipid gain and whole-body lipid content were the highest in sole fed the high-lipid/low-protein diet, suggesting metabolic disturbances when high fat levels are combined with high protein levels in the diet.

The low activity of FAS suggests a marginal contribution of *de novo* synthesis of lipids for the increment of body lipid content. This could be related to the fact that in sole, as in other flatfish, the capacity to store fat is limited. However, FAS activity was found to be highly sensitive to the composition of nutrient intake. The highest value was found in the liver of sole fed the low-fat/high-protein diet. The inhibition of FAS by increasing levels of dietary lipids is a well-documented phenomenon in several species[17,49,50] including Senegalese

sole[14]. Dias *et al.*[14] showed that a diet with 21 % fat led to a reduced specific activity of FAS compared with a diet with 11 % fat. We should also consider that high-fat diets contained a greater amount of *n*-3 PUFA, which have been proved responsible for decreasing the activity of some lipogenic enzymes, including FAS[51]. In the present study, the higher level of dietary starch in the low-fat diets could have further enhanced FAS activity, as it has been found in rainbow trout[16] and blackspot seabream (*Pagellus bogaraveo*)[48] that digestible carbohydrate favoured lipogenesis. In addition, the higher values of FAS activity in the liver of sole fed the 54 % protein diets suggest that some dietary amino acids could have been used as substrates for *de novo* lipogenesis, but with a marginal consequence on body lipid content.

The preferential sites for lipid deposition are not quite well defined. In flatfish species, more than two-thirds of the lipid content appears to be located in the bone, fins and brain[11,15,52]. Nevertheless, and contrarily to what has been observed in other marine fishes, it seems that liver is not a preferential organ for lipid deposition in Senegalese sole, since the hepatosomatic index and lipid content were not affected by the dietary protein or lipid content. Moreover, the viscerosomatic index was significantly higher in fish fed the high-lipid diets, showing the capacity of the digestive tract to accumulate perivisceral fat. Indeed, a twofold increase in intestinal lipid content was observed previously in Senegalese sole fed diets with 16 and 20 % lipid levels compared with 4 % lipid diets[15].

Mitochondrial β-oxidation is the major energy-producing pathway from fatty acids. From the cleavage of fatty acid in acetyl-CoA, with the reduction of NAD^+ to NADH as part of the β-oxidation spiral[22], ATP can be generated to supply energy and cover cellular energy needs. Compared with the liver, white muscle has a lower enzyme specific activity expressed per unit tissue. However, it is worth considering data from white muscle for the assessment of global β-oxidation capacity due to this tissue's large size[53]. The increase in dietary lipids or dietary protein levels did not change the activity of HAD, one of the key enzymes of the β-oxidation pathway, neither in the liver nor in the muscle of sole. Similar results were observed in rainbow trout (*Oncorhynchus mykiss*) from different genetic lines (fat line *v.* lean line) fed high-energy and low-energy diets[3]. In haddock (*Melanogrammus aeglefinus*)[54], there was no significant increase in mitochondrial or peroxisomal β-oxidation activity in various tissues, when the dietary lipid level increased from 12 to 24 %. In demersal fish such as sole and haddock, specific activity from β-oxidation key enzymes is lower compared with salmon or rainbow trout. Sole[14,15] and gadoid[54,55] fishes, in general, have reduced capacity to accumulate lipids. Muscle, which can constitute more than 50 % of the whole body, is a lean tissue with no more than 5 % of fat (wet weight basis). Overall, the low mitochondrial β-oxidation observed in both tissues (liver and muscle) probably reflects the low lipid levels in these tissues. Fish fed the high-fat diets did not enhance fatty acid oxidation despite the higher whole-body lipid levels and fat intake. The regulatory mechanisms involved in the balance between lipid intake, fatty acid oxidation and lipid deposition are intricate with important physiological implications such as obesity[56,57]. When high-lipid diets are chronically ingested, this balance is affected, resulting in lipid accumulation and, consequently, health disorders[58–60]. The regulatory mechanisms of lipid oxidation and deposition are still not fully understood, and lessons can be taken from lean models to reveal these mechanisms. The absence of the response of HAD to the dietary levels of fat could be related to the use of carbohydrate substrates for energy production.

We found that PFK-1 (*EC* 2.7.1.11) activity was significantly enhanced in the muscle of sole fed the low-lipid diets, regardless of the dietary protein levels. As mentioned before, the formulation of the 4 % lipid diets was achieved by increasing the proportions of wheat meal and hence resulted in increased starch levels (Table 1). Although Dias *et al.*[44] found that wheat meal had a lower energy digestibility compared with other ingredients tested, the up-regulation of PFK-1 activity was probably due to higher starch intake in the 4 % lipid diets. PFK-1 catalyses the formation of fructose-1,6-biphosphate from fructose-6-phosphate and MgATP, one of the early rate-limiting steps of glycolysis[61,62]. In omnivorous or herbivorous fish species, metabolic adaptations to high-carbohydrate diets have been described[41]. In the wild, Senegalese sole feeds basically on benthonic invertebrates, such as polychaeta larvae, bivalves, molluscs and small crustaceans[63]. However, despite apparent carnivorous feeding habits, Senegalese sole presents digestive features generally found in species with omnivorous feeding habits[64], i.e. residual stomach acid digestion and proteolysis and a rather long intestine where pH is always above 6. Dias *et al.*[14] reported that hepatic activity of glucokinase and pyruvate kinase, other two key glycolytic enzymes, was not affected by the inclusion of high levels of carbohydrates, irrespective of starch type, when the activity was measured 18–20 h after the meal. Nevertheless, data obtained in the present experiment suggest that glucose is used as a source of energy by the muscle when the lipid supply is limited and the dietary starch content high. In flounder, Lee *et al.*[37,38] also suggested a possible role of carbohydrates in metabolic energy supply when the dietary lipid level is low (7 % lipid in the diets). This requires further verification through experiments focused on the role of carbohydrates as non-protein energy sources in flatfish.

In conclusion, according to the results achieved so far, dietary lipids do not seem to be a good energy source for promoting growth in *S. senegalensis*. There is no evidence of a protein-sparing effect by increasing dietary lipid levels, even when the dietary protein level is lowered from 54 to 48 %. The activity of enzymes involved in key metabolic pathways points towards a lack of metabolic adaptation to high lipid levels, reflecting the high protein requirement of the species. The metabolic fate of dietary lipids remains to be fully understood but could be linked to the competition with carbohydrates for energy supply. Data suggest a possible metabolic adaptation to dietary carbohydrates in Senegalese sole, which contrasts with what is generally observed in Salmonids and most marine species.

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