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Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles

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Abstract

The effects of dietary non-protein energy levels on growth, oxidative status and condition were studied in juveniles of Senegalese sole (*Solea senegalensis*). Four isonitrogenous diets with four energy levels were used: A—low (11% lipid, raw carbohydrate); B—intermediate (11% lipid, digestible carbohydrate); C—intermediate (21% lipid, raw carbohydrate); and D—high (21% lipid, digestible carbohydrate). Survival, relative growth rate (RGR) and feed conversion rate were not significantly affected by the dietary treatments. The fatty acid composition of the fish muscle varied little among the treatments, but trans 18:2$n-6$, total $n-6$ and EPA/DHA ratio, tended to be higher in fish fed diets with low lipid level. Cellular energy allocation (CEA) results (indicative of metabolic status and net energy budgets) showed significant differences in liver, but not in muscle samples. Livers of fish fed diet C contained the lowest carbohydrate, protein and CEA values, but the highest cellular energy consumption. Fish fed diet A had the highest CEA for growth, followed by fish fed diets B and D and then diet C. The liver and muscle peroxidation and antioxidant activity were measured by thiobarbituric acid reactive substances (TBARS) test and the enzymatic activities levels of catalase (CAT) and superoxide dismutase (SOD). TBARS values were higher for fish fed diets with high lipid content. However, no clear relation was found between HUFA level and TBARS value. The activity levels of the antioxidant enzymes CAT and SOD were higher in livers of fish fed diets with a high lipid level. Furthermore, CAT and SOD activity and TBARS values were influenced by the type of dietary starch in the diet. Higher oxidation rates were observed in fish fed

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diets containing raw carbohydrate. These data suggest that lipid and carbohydrate energy sources affect the oxidative status of Senegalese sole. Diets containing low levels of lipid and digestible starch reduce the susceptibility of the fish to oxidation and may enhance growth rate.

Keywords: Dietary energy; Nutrition; Oxidative status; Reactive oxygen species, ROS; Senegalese sole juveniles

1. Introduction

The Senegalese sole is a candidate species for intensive marine aquaculture in the Mediterranean (Dinis et al., 1999). Several aspect of its culture has been developed and optimised (weaning techniques and larvae culture), concluding in significant advances over the last years (Howell, 1997; Dinis et al., 1999). Nevertheless, information is lacking on the nutritional aspects of post-larvae and juveniles of this species. Most marine species tend to use protein as the preferential energy and anabolic intermediate for growth (Cowey and Walton, 1989). However, it is well known that for most cultivated species (particularly salmonids), a significant protein sparing can be achieved by increasing the dietary digestible energy levels in the diet, through the incorporation of fats and digestible carbohydrates (Cho and Kaushik, 1990; Dias et al., 1998). Carbohydrates are cheaper and more available than proteins making their inclusion in the diet attractive. However, the ability of carnivorous fish to digest and metabolise carbohydrates is poorly developed (Wilson, 1994). Therefore, lipids rather than carbohydrates have been used as the major non-protein energy sources for growth in coldwater and marine fish (Sargent et al., 1999). There is, however, a scarcity of information in the literature dealing with the metabolic pathways of carbohydrates and lipids, and how this affect the growth and fish condition. A thorough knowledge of the fish's metabolic potential relative to its nutritional requirements, physiological condition and fish health is needed as a prerequisite to improving the nutritional profile of the diet.

Under normal physiological conditions animal cells produce reactive oxygen species (ROS), such as O_2^-, H_2O_2 and hydroxyl radicals, as well as ozone. Other molecules normally included as ROS species are derived from the reaction of carbon-centred radicals (alkyl radicals R) with molecular oxygen (alkoxyl radicals RO; peroxyl radicals ROO and organic hydroperoxides ROOH) (Halliwell and Gutteridge, 2000). At the same time, antioxidant defences, such as NADH/NADPH, glutathione (GSH), protein sulphydryl (−SH) groups and free radical scavenging enzymes such as superoxidase dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Winston and Di Giulio, 1991) remove ROS. An imbalance between the generation and removal of ROS can produce oxidative stress (Tocher et al., 2002). Fish tissues, particularly bio-membranes rich in $n-3$ polyunsaturated fatty acids (PUFA) are highly susceptible to oxidation by ROS (Sargent et al., 1999). ROS also oxidizes most cellular constituents such as DNA, proteins and lipids (Janssens et al., 2000) causing damage to, and cross-linking of molecules, resulting in reduced enzymatic activity and affecting cellular integrity (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 2000).

There is little known about how the level and nature of dietary energy affects the oxidative status of fish. Nutrition influences oxidation and antioxidation defense mechanisms, and the

composition of the diet should be evaluated in terms of its effect on the balance between ROS generation and breakdown. The source of dietary energy (protein, lipid or digestible carbohydrate) has been shown to modulate lipid oxidation in muscle homogenates of rainbow trout and European sea bass (Alvarez et al., 1998, 1999; Lopez-Bote et al., 2001). Furthermore, lipid and polyunsaturated fatty acids are implicated in peroxidation, while exogenous dietary micronutrients such vitamin E act as antioxidant protection (Tocher et al., 2002).

All the organisms require energy for growth, reproduction and for maintaining their basal metabolism. Additional energy is required for dealing with stress and if this is not provided via the diet, growth and reproduction can be compromised (DeCoen et al., 1995; De Coen and Janssen, 2003). The cellular energy allocation test (CEA) has been used in toxicology studies to measures the energy budget which can be used to assess the overall condition of an organism and/or organ reflecting the metabolic status of an animal (Calow and Sibly, 1990). Previous studies with daphnids, molluscs, mysids and fish have confirm that the CEA test can be considered as an indicator of the animal's overall condition or of the metabolic status of a particular organ (DeCoen et al., 1995; Nguyen, 1997; Verslycke and Janssen, 2002; De Coen and Janssen, 2003).

The objective of this work was to determine the effect of dietary non-protein energy sources and respective levels on growth performance, fish condition, CEA and oxidative status in juveniles of the Senegalese sole.

2. Materials and methods

2.1. Experimental diets

Four isonitrogenous diets (crude protein: 52% DM) were formulated containing two different lipid levels (11% and 21% DM). Within each dietary lipid level, the degree of digestibility of the carbohydrate was varied by incorporating extruded or crude pea meal (Table 1). Diets were tested in triplicate. All ingredients were finely ground, mixed and dry pelleted using a steamless pelleting mill (model 3000, CPM, San Francisco, USA), fitted with a die of 2.5-mm diameter. Part of the pellets were crumbled and sieved to obtain particles with graded diameters (1.5–2.0 and 2.0–2.5 mm), which were used according to fish size.

2.2. Fish and experimental conditions

Larvae of Senegalese sole (*Solea senegalensis*) were cultured until the juvenile stage (190 days) on a diet of rotifers, *Artemia* nauplii and inert diets (SSF Norwegian Herring and Oil, Ind. Res. Industry, Norway). For the trial, fish (initial mean body weight: 23.6 ± 1.2 g) were randomly allotted to 12 white rectangular PVC tanks (50 × 50 × 90 cm) with a volume of 35 l, at 54 fish per tank. The tanks were supplied with well-aerated, recirculated seawater (salinity 30 ± 1 ‰, 20 ± 1 °C), passing through a mechanical filter, protein skimmer, biological filter, activated carbon, sand filter and UV unit. The fish were exposed to a 12-h light/12-h dark photoperiod. The parameters NH_4^+, NO_2^- and NO_3^- were measured weekly. Fish were fed the experimental diets by automated belt feeders (18 h day^{-1}) over 67 days.

Table 1
Formulation and proximate composition of the experimental diets

Experimental diets	A	B	C	D
Ingredients (%)				
Fish meal LT	47.6	47.6	52.2	52.2
Fish protein concentrate[a]	6.9	6.9	6.8	6.8
Fish oil	2.1	2.1	11.8	11.8
Raw Aquatex[b]	41.4		27.2	
Cooked Aquatex[c]		41.4		27.2
Vitamins mix[d]	1.0	1.0	1.0	1.0
Mineral mix[e]	1.0	1.0	1.0	1.0
Proximate composition				
Dry matter (DM, %)	92.0	91.5	91.4	92.1
Crude protein (%DM)	51.7	51.3	51.5	51.9
Crude fat (%DM)	11.0	11.2	21.5	21.3
Starch (%DM)	19.0	18.6	11.1	10.5
Ash (%DM)	10.3	10.0	10.9	10.3
Gross energy (kJ/g DM)	21.3	21.4	23.3	23.4

[a] CPSP 90 from Sopropêche, France: 85% CP, 10% lipid.

[b] Peas meal, Aquatex 8074 from Sotexpro, France: 22.5% CP, 1% lipid, 49% raw starch.

[c] Peas meal, Aquatex 8071 from Sotexpro, France: 23.7% CP, 1% lipid, 50% gelatinised starch.

[d] Vitamin mixture (IU or mg/kg diet): DL-alpha tocopheryl acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 5 mg; ascorbic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg.

[e] Mineral mixture (g or mg/kg diet): calcium carbonate (40% Ca), 2.15 g; magnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25% Cu), 0.3 g; manganese sulphate (33% Min), 0.3 g; dibasic calcium phosphate (20% Ca, 18% P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g.

Every 2 weeks, samples of 10 fish per tank were weighed and biomass estimations were used to calculate the daily feed ration. However, when excess of uneaten food remained, adjustments were made to the rations. At the beginning and end of the experiment, the total biomass per tank was weighed and 15 fish per treatment were used for cellular energy allocation (CEA) homogenates. At the end of the trial, samples of muscle were taken from 18 fish per treatment, frozen in liquid nitrogen and stored at − 80 °C prior to analysis of total lipid content and fatty acid composition. Liver and muscle samples were also collected from 12 other fish per treatment and stored at − 80 °C for the measurement of thiobarbituric acid reactive substances (TBARS), catalase (CAT) activity and superoxide dismutase (SOD) activity. Livers of six other fish per tank were also removed for histological examination.

2.3. Fatty acid composition

The total lipid content and polyunsaturated fatty acid profile of muscle tissue were determined using standard analytical methods. Lipids were extracted according to Folch et al. (1957), following the modification of Ways and Hanahan (1964). FAME were prepared for gas chromatography according to Coutteau and Sorgeloos (1995) and identified by a

Chrompack CP9001 gaschromatograph equipped with temperature programmable on-column injector (TPOCI). Ten percent of an internal standard 22:$n-6$ was added prior to the reaction. The injections were performed on a polar 50-m capillary column BPX70 (SGE Australia) with a diameter of 0.32 mm and layer thickness of 0.25 μm, connected to a 2.5-m methyl deactivated pre-column.

2.4. Homogenated preparation

Homogenated samples were used for CEA as well as for the different enzymatic tests. Whole body fish were sampled at the beginning of the experiment and liver and muscle at the end. Samples were measured weighted, cut into a small pieces and then homogenized. A preparation of the homogenates was made using an ice Bain Marie and four up-and-down strokes in a Polytron PTA 10S homogeniser. A known volume of buffer (0.1 M Tris–HCl pH 8.5, 15% (w/v) Poly Vinyl Pyrrolidone, 153 μM $MgSO_4$ and 0.2% (w/v) Triton X-100) was added to blend the sample. Homogenated samples were stored at −80 °C.

2.5. Cellular energy allocation

The cellular energy allocation (CEA) test is based on a biochemical assessment of the metabolic balance of the test organism (DeCoen et al., 1995). The energy consumption (Ea) and the energy reserves available for metabolism (Ea) were quantified biochemically and integrated as an indicator of the metabolic condition. The energy consumption was estimated using the electron transport activity (at the mitochondrial level) in the homogenates, while reserve energy for metabolism was assessed by measuring total lipids, protein and sugar contents. The difference between Ea and Ec represented the energy available for growth (DeCoen et al., 1995; De Coen and Janssen, 2003).

Total carbohydrate and protein contents were determined from 200 μl of homogenate. Proteins were precipitated by adding 15% of trichloroacetic acid (TCA) and incubated at −20 °C for 10 min. The samples were centrifuged (3000 × g, 10 min, 4 °C) and the pellets were washed with 5% TCA. The remaining pellets were resuspended in NaOH, incubated at 60 °C for 30 min and neutralized with HCl. The total protein content was then measured using Bradford's (1976) reagent. The absorbance was measured at 590 nm using bovine serum albumin as standard.

Carbohydrates were analysed using both supernatant fractions after centrifuging and washing the samples. For microplating method, the extract of samples, phenol 5% and concentrated H_2SO_4 were added a proportion 1:2:4 to the microplate. After 30-min incubation at room temperature, the absorbance was measured at 492 nm using glucose as standard.

Lipids were extracted according to the method used by Bligh and Dyer (1959). The extract was obtained from 500 μl of homogenated sample, which was previously added to 500 μl of chloroform and 500 μl methanol spectrophometric grade (both). After centrifugation (10,000 × g, 5 min 4 °C), the top phase was separated and 500 μl of H_2SO_4 was added to 100 μl of lipid extract, and then charred for 15 min at 200 °C. The burned residue was then diluted in 1 ml of distilled water and total lipid content was determined by measuring the absorbance at 370 nm using Tripalmitin as standard.

The energy consumed at mitochondrial level was measured as electron transport activity (ETS) based on the spectrophotometric determination of INT (p-Iodonitro Tetrazolium Violet; Sigma, USA) reduction according to DeCoen et al. (1995) and De Coen and Janssen (2003). The homogenates of the samples were centrifuged at 4 °C, 3000 × *g* for 10 min. After centrifugation, 50 μl of extract was added into the microplate well together with 150 μl of buffered substrate solution (BSS; 0.13 M Tris–HCl and 0.3% (w/v) Triton X-100, pH 8.5) and 50 μl NADPH solution (1.7 mM NADH and 250 μl NADPH). The reaction was induced by adding 100 μl INT (p-IodoNitro Tetrazolium 8 mM). Absorbencies were measured kinetically using a spectrophotometer at 20 °C for 10 min. The amount of formazan formed was calculated using $\varepsilon = 15{,}900/M/cm$.

2.6. The CEA calculations

The different energy available fractions (Ea) carbohydrate, protein and lipid of a tissue sample were calculated as the sum of the energy contents. These values were obtained by multiplying the nutrient contents by the respective energetic equivalents using enthalpy combustion (17.5 kJ/g carbohydrates, 24 kJ/g protein and 39.5 kJ/g lipids) (DeCoen et al., 1995). The energy consumed (Ec) was determined using cellular respiration rate (ETS) data, based on the stoichiometrical relationship that for each 2 μmol of formazan, 1 μmol of O_2 was consumed. The estimated oxygen consumption was transformed into energetic equivalents assuming an average lipid, protein and carbohydrate mixture leading to 484 kJ/mol O_2 (DeCoen et al., 1995; De Coen and Janssen, 2003). The total CEA (energy for growth, in this study) is equivalent to the differences between Ea and Ec ($CEA = Ea - Ec$). In this study, the CEA methodology was used to assess energy metabolism of fish after 67 days of feeding the experimental diets. The difference between Ea and Ec is an estimation of the energy budget available for growth during a given time period.

2.7. TBARS test

The susceptibility of the muscle and liver homogenates to induced lipid oxidation was assayed by the TBARS test. Five hundred microliters of homogenate of the sample was mixed with 400 μl of thichloroacetic acid 15% (TCA) and 800 μl of thiobarbituric acid (TBA 0.67% diluted in NaOH solution 0.3 N). After heating at 95 °C for 20 min and then cooling, the protein precipitate was removed by centrifugation (2000 × *g*, at 4 °C, for 5 min). The supernatant was recovered and measured in a fluorescence spectrophotometer (Em 555 and Ex 515) Spectramax 190, Molecular Devices, Sunnyvale, CA 94089. The sample concentrations were calculated from a standard curve established with TBA–malondialdehyde (MDA). Results are expressed as nmol of MDA/mg protein. Protein measurements were done according to Lowry et al. (1951).

2.8. Catalase (EC 1.11.1.6)

The method of Maral et al. (1977) for CAT assay was adapted for 96-well microplates. Homogenate samples were centrifuged (4000 × *g*, at 4 °C, for 15 min). The microplate

wells were filled with 200 μl of supernatants previously diluted 50 ×. The measurements were carried out on a PC-controlled microplate luminometer (Microlumat EG&G Berthold, Germany) at 25 °C, equipped with two 50-μl injectors. A first injection of 50 μl H_2O_2 solution induced a reduction of the CAT contained in the sample. After 30 min, 50 μl of a solution containing luminol (20 mmol l^{-1}) and horseradish peroxidase (HRP) (11.6 units ml^{-1}) was injected. The luminol injection produces an emission of light whose intensity was proportional to the amount of H_2O_2 remaining in the sample. CAT activity in the samples was estimated with a standard curve, prepared with purified bovine liver CAT. Results are expressed as international units per mg protein. Protein measurements were done according to Lowry et al. (1951).

2.9. Superoxide dismutase (EC 1.15.1.1)

The microplate test for SOD activity was developed according to Nakano (1990). A chemiluminescent reaction occurs when the imidazolopyrazinone molecule (Methyl-coelenterazine mCLZ) reacts with superoxide (O_2^-) generated by xanthine oxidon–hypoxanthine system. Total SOD activity can be estimated by the degree to which light production is limited, in conditions where the enzymatic activity generated by the O_2^- is the rate-limiting factor of the luminescence. In these circumstances, SOD competes with mCLZ for O_2^- and the luminescence becomes weaker as the activity of SOD in the sample increases (Janssens et al., 2000).

Homogenate samples were centrifuged (4000 × *g*, at 4 °C, for 15 min). The microplate wells were filled with 50 μl of supernatants previously diluted (5 ×) with phosphate buffer saline (PBS containing 1% triton X-100) and with 155 μl PBS containing a mixture of 0.6 mmol l^{-1} EDTA and 0.01% bovine serum albumin (BSA), 5 μl 0.8 μmol mCLZ and 5 μl of xanthine oxidase (27.56 munits ml^{-I}). The measurements were carried out on a PC-controlled microplate luminometer (Berthold Microlumat EG&G) at 25 °C. The reaction was initiated by injecting 50 μl of hypoxanthine solution (34 mg ml^{-1}). The records were taken every 20 s. The SOD concentrations were calculated using a standard curve established with SOD purified from bovine erythrocytes in PBS-Triton buffer. The SOD activities are expressed in international units per mg protein. Protein measurements were performed according to Lowry et al. (1951).

2.10. Histological analysis

In order to study the hepatocytes structure and glycogen contents, the liver samples were fixed in Bouin fixative, dehydrate in graded ethanol series and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) and diastase-PAS, according to Pearse (1985).

2.11. Statistical analysis

Inter-treatment differences for total biomass, individual weight, liver weight, total fatty acids and FAME fraction, TBARS, CAT and SOD were analysed by one-way analysis of

Table 2
Growth performance, feed utilization, hepatosomatic index and TBARS contents in juvenile Senegalese sole fed the experimental diets over 10 weeks (IBW = 23.6 ± 1.2 g)

	Dietary treatment			
	A	B	C	D
FBW (g)	43.79 ± 2.1	39.76 ± 2.67	37.72 ± 1.35	40.57 ± 2.59
Weight gain (%IBW/day)	1.38 ± 0.17	1.39 ± 0.4	1.1 ± 0.21	1.2 ± 0.25
RGR (%BW/d)	0.88	0.84	0.69	0.77
FCR	2.46 ± 0.27	2.56 ± 0.58	3.12 ± 0.44	2.87 ± 0.62
HSI	0.97 ± 0.04^{b}	1.26 ± 0.09^{a}	1.19 ± 0.05^{a}	1.21 ± 0.02^{a}
TBARS (nmol/mg protein)				
Liver	3.40e − 10 ± 5.52e − 11^{b}	7.81e − 10 ± 4.39e − 10^{b}	4.47e − 09 ± 3.90e − 09^{b}	1.69e − 08 ± 1.09e − 09^{a}
Muscle	5.11e − 10 ± 1.07e − 10^{b}	6.36e − 10 ± 1.87e − 10^{b}	1.44e − 09 ± 6.23e − 10^{a}	5.38e − 09 ± 6.97e − 10^{a}

Values are means ± S.D.
Within a row, means without a common superscript letter differ significantly ($P < 0.05$).
IBW, Initial body weight.
FBW, Final body weight.
RGR, Relative growth rate: $(e^{g} - 1) \times 100$, with g = (Ln FBW − Ln IBW)/67 days.
FCR, Feed conversion ratio: (dry feed intake/wet weight gain).
HSI, Hepatosomatic index: (liver weight/body weight) × 100.
TBARS, Thiobarbituric acid reactive substances values (mol/mg protein).

variance (ANOVA) and, when pertinent, by Tukey's honest significant difference (HSD). The homogeneity of the variances of means was checked with the univariate tests of Cochran (Sokal and Rohlf, 1995).

3. Results

3.1. Growth performance, feed utilization and hepatosomatic index

Data on growth performance, feed utilization and hepatosomatic index (HSI) are presented in Table 2. At the end of the experimental 67-day period, a two-fold increase in mean fish weight was recorded for all treatments. Relative growth rate (RGR) values ranged from 0.69% to 0.88% day^{-1}, with highest values being recorded for fish fed diets containing low lipid levels (diets A and B). Feed conversion rate varied between 2.46 and 3.12. However, neither RGR nor FCR was significantly affected by the dietary treatments ($P>0.05$). The HSI varied from 0.97% to 1.26%. The HSI value observed in fish fed diet A was significantly lower than that found in fish fed any other diet ($P<0.05$).

3.2. Fatty acid composition

The FAME composition analyses for the muscle tissue showed few significant differences between the dietary treatments. The polyunsaturated fatty acid 18:2$n-6-c$ fraction and total $n-6$, tended to be higher in diets A and B (low lipid level diets).

Table 3
Identified fatty acids composition (%) on muscle of juvenile Senegalese sole as affected by the different experimental diets; $n=18$ per treatment

	Dietary treatments			
	A	B	C	D
Sum saturated	26.10 ± 0.46	26.50 ± 0.66	25.80 ± 0.92	23.40 ± 3.95
Sum monounsaturated	22.90 ± 0.37	24.80 ± 2.47	23.00 ± 0.38	21.50 ± 3.75
Polyunsaturated				
18:2$n-6-t$	0.15 ± 0.0	0.20 ± 0.03	0.18 ± 0.02	0.20 ± 0.04
18:2$n-6-c$	3.80 ± 0.08^{ab}	3.98 ± 0.47^{a}	2.80 ± 0.03^{bc}	2.70 ± 0.61^{c}
18:3$n-6$	0.10 ± 0.0	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.03
18:3$n-3$	1.10 ± 0.20	1.10 ± 0.21	0.92 ± 0.05	0.90 ± 0.27
18:4$n-3$	1.30 ± 0.08	1.50 ± 0.28	1.40 ± 0.09	1.50 ± 0.37
20:4$n-6$ (ARA)	1.30 ± 0.06	1.10 ± 0.30	1.50 ± 0.09	1.21 ± 0.03
20:5$n-3$ (EPA)	4.80 ± 0.24	5.00 ± 0.32	5.50 ± 0.35	5.10 ± 0.66
22:5$n-3$	4.70 ± 0.07	4.90 ± 0.11	5.20 ± 0.33	5.10 ± 0.83
22:6$n-3$ (DHA)	26.10 ± 0.55	22.60 ± 3.48	25.01 ± 0.31	21.20 ± 1.71
Total $n-3$	36.50 ± 0.80	32.00 ± 6.70	36.80 ± 0.81	32.60 ± 3.50
Total $n-6$	6.10 ± 0.07^{a}	5.60 ± 0.48^{ab}	5.50 ± 0.16^{a}	4.70 ± 0.49^{b}
ARA/EPA ratio	0.27	0.22	0.27	0.24
EPA/DHA ratio	5.43^{a}	4.47^{b}	4.53^{b}	4.13^{b}

Values are means ± S.D.
Within a row, means without a common superscript letter differ significantly ($P<0.05$).

The total amount of $n-6$ polyunsaturated fatty acid was significantly lower in the muscle of fish fed diet D when compared to the other diets ($P<0.05$). The EPA/DHA ratio for diet A was significantly higher than diets B, C and D (Table 3).

3.3. Cellular energy allocation

Table 4 summarizes the average energetic content for each component. Significant differences were detected between all the macromolecules (carbohydrates, protein and lipid) in the liver samples. Fish fed on diet A had the highest macromolecules (carbohydrates, protein and lipid) and as consequence, the highest overall energy available content, followed by diets D, B and C in the liver samples. Fish fed diet C generally showed the lowest values for all the macromolecules except for lipids and overall energy available content The energy consumed by respiration was highest for diet A (Table 4). CEA results in liver samples showed significant differences, with the higher value in fish fed diet A, while diets B and D grouping together and with the lower value in diet C ($P<0.05$). Higher values of carbohydrate, protein and energy consumption were measured for liver than for muscle samples (Table 4).

No significant differences between carbohydrate and proteins were found in the muscle samples (Table 4). Lipid content in muscle samples was relatively constant between the various dietary treatments. The energy available for growth was higher on fish fed diet B followed by C, D and A but no significant differences were clear. Diet C (prepared with raw starch and a high lipid level) promoted significantly higher energy consumed ($P<0.05$).

Table 4
Energetic content on the main macronutrients and energy consumed in liver and muscle of Senegalese sole juveniles fed the various experimental diets, $n=16$ per treatment

	Dietary treatments			
	A	B	C	D
Liver (kJ/g tissue)				
Carbohydrates	14.6 ± 3.29^{a}	12.2 ± 2.96^{ac}	11.2 ± 3.39^{b}	13.1 ± 3.18^{ac}
Protein	82.5 ± 18.96^{a}	68.8 ± 16.74^{ac}	61.0 ± 18.26^{bc}	73.9 ± 18.01^{a}
Lipids	206.1 ± 57.99^{a}	102.7 ± 42.07^{b}	150.4 ± 48.55^{b}	175.0 ± 40.54^{ab}
Energy available	292.2 ± 74.9^{a}	234.5 ± 59.34^{bc}	214.3 ± 67.13^{bc}	252.1 ± 59.21^{ac}
Energy consumed	0.022 ± 0.005^{a}	0.015 ± 0.004^{b}	0.021 ± 0.006^{a}	0.019 ± 0.005^{a}
Cellular energy allocation	292.2 ± 74.9^{a}	234.5 ± 59.35^{ab}	214.3 ± 67.13^{b}	252.1 ± 59.22^{ab}
Muscle (kJ/g tissue)				
Carbohydrates	5.7 ± 0.9	6.2 ± 0.6	6.1 ± 0.8	5.9 ± 0.8
Protein	35.5 ± 5.8	39.1 ± 3.6	37.7 ± 4.9	37.1 ± 5.3
Lipids	241.9 ± 38.0	266.6 ± 24.5	259.5 ± 35.6	252.5 ± 32.4
Energy available	283.1 ± 44.67^{a}	311.9 ± 28.7^{ab}	303.2 ± 41.25^{b}	295.5 ± 38.4^{ab}
Energy consumed	0.005 ± 0.001^{b}	0.004 ± 0.002^{b}	0.007 ± 0.002^{a}	0.005 ± 0.002^{b}
Cellular energy allocation	283.1 ± 44.7	311.9 ± 28.7	303.2 ± 41.3	295.5 ± 38.4

Values are means ± S.D.
Within a row, means without a common superscript letter differ significantly ($P<0.05$).

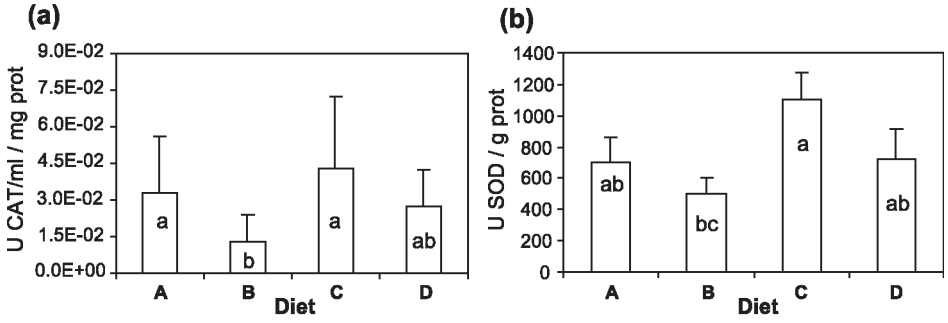

Fig. 1. Activities for catalase (a) and superoxidase dismutase (b) in livers of juvenile Senegalese sole fed the different experimental diets. Different letters inside bars indicate significant differences between dietary treatments ($p < 0.05$), $n = 12$ per treatment.

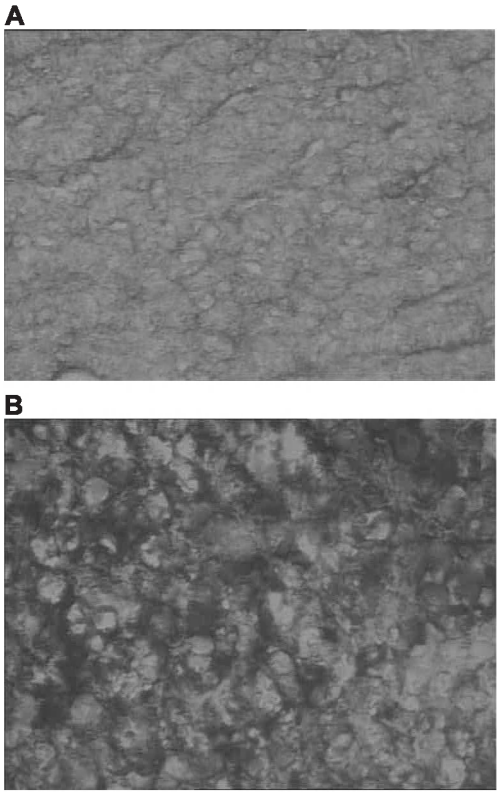

Fig. 2. (A) Hepatocytes of *S. senegalensis* juveniles fed diet A, spherical nucleus centrally located; PAS staining (+). (B) Lipid vacuoles and nuclear migration in the hepatocytes of juveniles fed diet D; PAS staining (+++). (PAS staining, 100 ×).

3.4. TBARS test

Higher oxidation rates were recorded for fish fed higher lipid levels, which was significant higher for fish treated with diet D ($P < 0.05$) (Table 2). This effect was evident both in liver and muscle samples. However, in liver samples, differences were found when comparing treatments A vs. B and C vs. D separately using one-way ANOVA. Fish fed diets containing digestible starch treatments B and D had lower oxidation levels ($P < 0.05$). Muscle samples evidenced significant lower oxidation in diet B when compared with diet A (one-way ANOVA). Nevertheless, no significant difference was found when comparing oxidative levels in diets C vs. D.

3.5. Catalase and superoxide dismutase

Catalase and superoxide dismutase activities were significantly affected by diet (Fig. 1). Fish fed with higher dietary lipid levels (diets C and D) tended to have a higher CAT and SOD activities ($P < 0.05$). The nature of the starch included in the diet shown, however, to have a significant effect in the fish oxidative status. Fish fed diets prepared with raw starch evidenced a higher oxidation in the liver tissue than those fed digestible starch (Fig. 1). These differences (diet C vs. D) did not however prove statistically significant ($P > 0.05$).

3.6. Histological analysis

Histological examination of the liver sections revealed one or two lipid drops in the hepatocytes. The structure of the liver cells of fish fed diets C and D showed a higher hepatocyte vacuolization than that of fish fed diet A. The increased size of the vacuoles in the hepatocytes produced a migration of the liver cell nucleus. Livers of fish fed diet A showed a lower PAS affinity than those fed diets C and D (Fig. 2).

4. Discussion

4.1. Growth and condition

The different non-protein dietary energy sources and levels used in this study did not have a significant effect on the growth of juvenile Senegalese sole. There was, however, a trend toward faster growth rates and better feed conversion for fish fed with diets containing lower lipid levels.

The HSI is often used as an indicator of condition and nutritional status of fish. Diets C and D showed a significant increment in HSI that could have been influenced by high fat contents in the diet. Gaylord and Gatlin (2000) observed a decrement in HSI when hybrid striped bass (*Morone chrysops* ♀ × *M. saxatilis* ♂) were fed 20% lipids compared with fish fed 5% or 10% lipids. Same authors found liver glycogen inversely related to liver lipid. Lee et al. (2002) recorded significant higher HSI for *Sebastes schlegeli* fed 14% dietary lipid compared to those fed 7% lipid level. These authors suggested that high in lipid might result in impaired liver function and higher HSI. Their results revealed however that

under conditions of low dietary lipid level, the incorporation of digestible starch increased HSI significantly. Rawles and Gatlin (1998) observed that striped seabass produced greater body fat deposition and higher HSI when soluble carbohydrates were added to the diet. Similarly, significant correlations between HSI values and digestible starch intake have been reported in rainbow trout and European seabass juveniles (Kaushik and Oliva-Teles, 1985; Dias et al., 1998). In this study, HSI was associated with high dietary lipid levels. An exception occurs on fish fed diet B, that presented the higher HSI, in spite that diet had low lipid content.

Furthermore, histology revealed that fish fed high-fat diets had more hepatocyte vacuolisation than those fed a diet low in digestible energy. Most of the vacuolisation in Senegalese sole appeared to be due to lipid accumulation. In many cases, the presence of numerous and voluminous lipid droplets in hepatocytes is a physiological response to excess lipid (Mosconic-Bac, 1987; Gallagher, 1996; Caballero et al., 1999; Kestemonst et al., 2001). The displacement of the nucleus to the periphery in livers of fish fed high fat diets may also be a result of the liver adapting to higher levels of lipid in the diet. The higher PAS positive results recorded in juveniles fed diet C suggest that hepatocyte vacuolisation was due to the storage of lipid and glycogen. However, lower PAS positive results for fish fed D imply that vacualisation was mainly due to glycogen storage.

The CEA parameter is used frequently in toxicology studies, and has been shown to be an interesting endpoint providing metabolic insight into the condition of an organism or the effects at the organ level as consequence of the exposition to any stressor (Muyssen et al., 2002; Verslycke and Janssen, 2002; De Coen and Janssen, 2003). CEA values express the net energy budget that is present in an organism or organ. In the present study, the CEA parameter was assessed at the organ level and should therefore be seen as an integrative parameter of the overall energetic status resulting from the various feeding regimes. Fish with a higher CEA are considered to be in better condition, as they have more energy available for growth. Juvenile sole fed diet A, containing low levels of lipid and raw starch had the highest liver CEA value, meaning the highest net energy budget, followed by diets D, B and C, respectively. These CEA results agree with the individual weight, total biomass, and FCR. Therefore, low energy diets and incorporation of digestible carbohydrates in low fat diets had a positive effect on the condition of juvenile sole. In the previous studies, the CEA value has been shown to be a good indicator of sublethal stress and energy budget (DeCoen et al., 1995; Nguyen, 1997; Verslycke and Janssen, 2002).

4.2. Oxidative status

Polyunsaturated fatty acids are essential for growth and development of marine fish, but they also impose a peroxidation burden on the fish. Under normal physiological conditions, there is a continuous production of reactive oxygen species that induce oxidative stress and can result in an inactivation of enzymes and genetic or cellular damage (Mourente et al., 1999). Other factors such as the presence of pro-oxidant or antioxidant compounds may also have an effect (Alvarez et al., 1998). In the present study, despite the different lipid and energy levels of the experimental diets, no significant

differences in fatty acid profile of muscle samples were recorded from fish of each treatment. The exception occurred for linoleic acid and the total $n-6$ fractions as well for EPA/DHA ratio that were significantly higher in those fish fed diets with low lipid level (diet A). These results disagree with those of Alvarez et al. (1998), who reported that muscle samples from trout and sea bass fed with high concentrations of fat, exhibited increased intramuscular concentrations of total and neutral lipids. The low lipid content of Senegalese sole muscle tissue (Dias et al., unpublished) and its highly conservative fatty acid profile suggest that muscle may not be a preferential lipid storage site in this species. Relatively low liver and visceral fat deposition (Dias et al., unpublished) further suggests that other tissues such as fins, head and subdermal adipose tissue, may be important areas of lipid storage for Senegalese sole.

The highest oxidation (TBARS) values were recorded in liver and muscle of fish fed with diets D and C (Table 2). These results were consistent with the high lipid level present in those diets. Gray (1977) found a geometrical relationship between fatty acid susceptibility and rate of oxidation, and with the degree of unsaturation. Stéphan et al. (1995) demonstrated that higher levels fish oil in the diet increase the susceptibility of turbot to fatty acid peroxidation. In addition to oxidation produced by lipid level, the starch type also had an influence. Unfortunately, there are no published works relating the quantity and quality of carbohydrates in the diet to oxidation in the tissues.

As with other animals, fish have antioxidant defence mechanisms, which help to maintain health and prevent oxidation lesions. CAT and SOD are important antioxidant enzymes (Halliwell and Guterridge, 2000; Tocher et al., 2002). The enzymes are commonly used in toxicological tests as stress indicators (Esterbauer and Cheesman, 1991). There was a relationship between CAT and SOD activities as well for TBARS test, and the lipid level and starch type incorporated in the diet. This resulted in higher oxidation rates and augmented enzyme activities. In fish fed raw starch and high lipid levels, SOD and CAT are scavengers of the reactive oxygen species, acting on hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively (Tocher et al., 2002). High rates of oxidation could therefore be expected in diets C and D, which had higher SOD and CAT levels. However, higher ratios of oxidation (TBARS test) and CAT and SOD levels were recorded for diets A and C (made with raw starch) in comparison with fish fed diets B and D. This suggests that lipid and carbohydrate energy sources interact in characterizing oxidative status of the fish, despite the lack of significance in SOD results. It seems that when different sources of energy are supplied in different proportions or with different levels of digestibility, the oxidative status in fish liver and muscle is affected. Similar results have been record in rainbow trout and seabass (Alvarez et al., 1998, 1999). Tocher et al. (2002) compared liver antioxidant enzymes in turbot, halibut and sea bream juveniles. The peroxidation activity and the antioxidant activities levels varied for each species. The addition of oxidized or normal oil as well as the inclusion of different amount vitamin E in the diets also had a significant influence in the oxidative and antioxidative parameters recorded. The same authors observed that effect of the antioxidant enzymes was reduced when vitamin E was added to the diet. The limited information available suggests that lipid peroxidation and antioxidant defences vary between species and with rearing conditions (Mourente et al., 1999). Publications dealing with fish diets with carbohydrates as energy sources in fish, and their effect on oxidative status, are however scarce.

4.3. Non-protein energy in sole diets

Diets with high lipid levels do not seem to be suitable for Senegalese sole. They lead to higher susceptibility to oxidation, poor physiological condition, altered liver histology, and lipid accumulation in liver, viscera and muscle (Dias et al., unpublished). Diets low in lipid levels and containing digestible starch are suitable for culturing *S. senegalensis* juveniles, which the doubling their biomass over a 67-day period. This diet produces fish less susceptible to oxidation, exhibiting good growth rates. However, this diet also seems to depress the activity of antioxidative enzymes. The significance of this in terms of fish physiology and performance needs further investigation. More attention should be paid to the activity of antioxidant enzymes and the oxidative status of the fish. This will provide a better understanding of the effects that nutrition and the environmental conditions have on the fish's physiological condition. Furthermore, studies on the fish carbohydrates metabolism and its interaction with other metabolic pathways are needed, in order to formulate diets of higher quality.

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