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ORIGINAL ARTICLE

Modulation of blackspot seabream (*Pagellus bogaraveo*) intermediary metabolic pathways by dispensable amino acids

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Abstract The objective of the present work is to investigate the main metabolic pathways by which dispensable amino acids (DAA) are diverted towards lipid formation in blackspot seabream. For that purpose, a control diet was formulated to contain 45% of crude protein (7.2 g N/100 g dry matter) mainly supplied by fish meal (45P). In two other diets, 22.2% of the dietary nitrogen (1.6 g N/100 g dry matter) was replaced by an equivalent amount of nitrogen provided by two different mixtures of DAA: alanine and serine (diet AS) or aspartic and glutamic acid (diet AG). A fourth diet (diet 35P) only containing 35% of crude protein (5.6 g N/100 g dry matter) was included in order to analyze the possible additive effects of DAA. Compared to fish fed diet 35P, blackspot seabream appear to make a more efficient use of the nitrogen provided by alanine and serine than that provided by aspartic and glutamic acids in terms of growth. Contrary to fish fed AG, fish fed AS attained similar specific FAS activities as 45P fed fish, suggesting a further role of alanine and serine on this lipogenic pathway. Dietary nitrogen reduction (45P vs. 35P) or its replacement by a mixture of aspartic and glutamic acids (diet AG) were shown to up-regulate phosphoenolpyruvate carboxykinase (PEPCK) but without, however, any effect on plasma glucose levels. Dietary nitrogen level and nature seems to exert a complex regulation on energetic pathways through the gluconeogenesis/tricarboxylic acids cycle interaction.

Keywords Blackspot seabream · Dietary nitrogen replacement · DAA · Intermediary metabolism

Abbreviations

AA	Amino acids
CHOL	Cholesterol
DAA	Dispensable amino acids
IAA	Indispensable amino acids
FAA	Free amino acids
FA	Fatty acid
FAs	Fatty acids
HSI	Hepatosomatic index
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
TAG	Triacylglycerol
VSI	Viscerosomatic index

Introduction

The dietary protein requirements for maximum growth of fish are higher than those of other terrestrial vertebrates (NRC 1993; Wilson 2002), with a major part of this nitrogen being used for energy supply (Cho and Kaushik 1985). Given the current constrains on fish meal

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availability and cost, much effort is devoted to the evaluation of alternative protein sources in aquaculture feeds (Gatlin et al. 2007; Kaushik and Hemre 2008; Tacon and Metian 2008). Although the importance of indispensable amino acids (IAA) profiles (Wilson 2002; Kaushik and Seiliez 2010) and the IAA/dispensable amino acids (DAA) ratio (Akiyama et al. 1997; Green et al. 2002; Gómez-Requeni et al. 2003; Rollin et al. 2003; Peres and Oliva-Teles 2006; Silva et al. 2009) have been well studied, DAA have received much less attention in fish nutrition. The specific effects of DAA to overall protein nutrition have been emphasized since long in mammals (Harper 1974; Jackson 1983; Laidlaw and Kopple 1987) and also in fish (Hughes 1985; Fauconneau 1988; Mambrini and Kaushik 1994; Schuhmacher et al. 1995). Diets completely lacking DAA leads to reduced growth rate in rats, chicks and fish (Frost and Sandy 1951; Stucki and Harper 1961, 1962; Allen and Baker 1974; Schuhmacher et al. 1995). DAA, such as alanine (Ala), serine (Ser), aspartic (Asp) or glutamic (Glu) acids, seem to have a sparing effect on the use of IAA (Cowey and Sargent 1979; Ronnestad et al. 2001; Abboudi et al. 2009). Hence, emerging developments in amino acid (AA) nutrition suggests that the traditional indispensable or dispensable AA classification should be revaluated (Reeds 2000).

It was recently shown that lipid retention was high in blackspot seabream (*Pagellus bogaraveo*) at low dietary lipid levels (10%), suggesting the conversion of nutrients other than lipid (protein and/or carbohydrates) into body fat (Figueiredo-Silva et al. 2010). Lipogenic and glycolytic regulation appears to be more related to dietary protein level than to dietary starch type in this species (Figueiredo-Silva et al. 2009). In addition, the protein source strongly affected lipogenesis in this species, with excessive plant protein leading to up-regulation of FA synthesis de novo and to high lipid retention (Figueiredo-Silva et al. 2010). Thus, the dietary protein level and source seems to play a major role on lipid metabolism in this species. When excess protein/AA is supplied in the diet, only part of it will be used to make new proteins and the remainder is deaminated and converted into energy compounds such as fat or glucose (Wilson 2002). The modification of body lipid content as well as lipogenesis by protein source has been demonstrated in other teleosts (Gómez-Requeni et al. 2003; Kaushik et al. 2004; Dias et al. 2005) as in higher vertebrates (Iritani et al. 1986, 1996; Kayashita et al. 1996; Padmakumarannair et al. 1998). The mechanisms underlying the modulation of FA synthesis de novo by protein/AA source are ill-defined, but dietary AA composition has been cited as one of the majors factors (Herzberg 1991). In fish, the incorporation of ^{14}C-alanine or ^{14}C-glutamate into triacylglycerols was shown to be much higher than that coming from ^{14}C-glucose (Nagai and Ikeda 1972; Henderson and Sargent 1981; Shikata and Shimeno 1997). However, the knowledge on the dietary DAA involvement on intermediary metabolic pathways in fish is scarce and mainly devoted to glucose metabolic pathways. Since some DAA constitute important substrates for endogenous glucose synthesis (Moon and Foster 1995), a high level of dietary DAA was hypothesized as a possible explanation for the prolonged postprandial hyperglycaemia observed in rainbow trout. However, Kirchner et al. (2003a) failed to show any effect of a dietary DAA surplus on hepatic glucose metabolism in rainbow trout.

Recognizing the propensity of blackspot seabream to turn protein into lipid deposits, we hypothesized that dietary DAA content will up-regulate the lipogenic potential in this species. Our objective was also to investigate the main metabolic pathways by which DAA are diverted into lipid formation. For that purpose, dietary nitrogen was partially replaced (22%) by two different mixtures of DAA. The two different mixtures of DAA were chosen for their preferential links with distinct metabolic pathways: alanine and serine (pyruvate precursors) or aspartic and glutamic acids (intermediates of the tricarboxylic acids cycle, TCA).

Materials and methods

Diets

The basal diet was formulated to contain 45% of protein (7.2 g N/100 g dry matter) supplied by fish meal (48% feed basis), CPSP G (5% feed basis) and wheat gluten (2.5% feed basis) (diet 45P). In two other diets, 22.2% of dietary nitrogen (1.6 g N/100 g dry matter) was replaced by an equivalent amount of nitrogen provided by two different mixtures of DAA: alanine and serine (diet AS) or aspartic and glutamic acid (diet AG). DAA nitrogen was added to the diets at the expense of fish meal nitrogen and the proportions between the two DAA of each mixture kept similar to that found in fish meal. A fourth diet (diet 35P) containing only 35% of protein (5.6 g N/100 g dry matter) was also included to determine if any growth/physiologic effect detected was due to nitrogen replacement and not due to shortage of dietary fish meal nitrogen supply (e.g., IAA). The four diets were formulated to contain similar energy content (20 kJ g^{-1} DM). All ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and were finely ground, mixed and dry pelleted through a 2.4 mm die at 50°C (California Pellet Mill, C-300 model). Ingredient and proximate composition of the diets are presented in Table 1, dietary fatty acid (FA) profiles in Table 2, and the dietary AA profiles in Table 3.

Table 1 Ingredient and proximate composition of the four diets (% as feed) fed to blackspot seabream juveniles

	Diets			
	45P	AS	AG	35P
Ingredients (%)				
Fish meal[a]	48.00	31.50	31.50	31.50
CPSP G[b]	5.00	5.00	5.00	5.00
Wheat gluten	2.50	4.25	5.00	2.50
Fish oil	4.00	5.00	5.00	5.00
Gelatinized corn starch[c]	25.00	23.25	22.50	25.00
Wheat bran	14.50	19.10	13.84	30.00
Vitamins and mineral mix[d]	0.25	0.25	0.25	0.25
Choline chloride[e]	0.10	0.10	0.10	0.10
Lutavit E50[f]	0.05	0.05	0.05	0.05
Lutavit C35[g]	0.05	0.05	0.05	0.05
Betafin S1[h]	0.05	0.05	0.05	0.05
Europellin[i]	0.50	0.50	0.50	0.50
L-Alanine[j]		6.24		
L-Serine[j]		4.66		
L-Aspartic acid[j]			6.19	
L-Glutamic acid[j]			9.97	
Proximate composition				
Dry matter, DM (%)	90.4	91.8	91.9	89.6
Crude protein (N x 6.25, % DM)	45.2	45.8	45.3	34.8
Crude fat (% DM)	10.4	10.2	9.7	10.8
Ash (% DM)	9.6	6.7	6.6	7.9
Gross energy (kJ/g DM)	20.4	20.3	20.0	20.7

[a] Fish meal 89.9% DM, protein 74.2% DM, lipids 9.5% DM

[b] CPSP G, fish-soluble protein concentrate, Sopropêche, France

[c] Gelatinized starch, Cerestar, Barcelona, Spain

[d] Vitamins (mg or IU/kg diet): vitamin A 8,000 IU, vitamin D_3 1,700 IU, vitamin K_3 10 mg, vitamin B_{12} 0.02 mg, thiamine 8 mg, riboflavin 20 mg, vitamin B_6 10 mg, folic acid 6 mg, biotin 0.7 mg, inositol 300 mg, nicotinic acid 70 mg, pantothenic acid 30 mg

Minerals (g or mg/kg diet): Mn (manganese oxide) 20 mg, I (potassium iodide) 1.5 mg, Cu (copper sulphate) 5 mg, Co (cobalt sulphate) 0.1 mg, Mg (magnesium sulphate) 500 mg, Zn (zinc oxide) 30 mg, Se (sodium selenite) 0.3 mg, Fe (iron sulphate) 60 mg, Ca (calcium carbonate) 2.15 g, dibasic calcium phosphate 5 g, KCl 1 g, NaCl 0.4 g

[e] Choline chloride, 1,000 mg kg^{-1} diet

[f] Lutavit E50 (BASF): vitamin E 300 mg kg^{-1} diet

[g] Lutavit C35 (BASF): vitamin C 500 mg kg^{-1} diet

[h] Betafin S1 (DANISCO): betain 500 mg/kg diet

[i] Europelin (EUROTEC): Binder

[j] L-Alanine, L-Serine, L-Aspartic acid, L-Glutamic acid provided by Ajinomoto, Paris, France

Table 2 Fatty acid composition of the diets (data expressed as g/100 g total fatty acid)

	Diets			
	45P	AS	AG	35P
14:0	5.2	5.1	5.0	4.8
15:0	0.5	0.5	0.5	0.5
16:0	17.6	17.3	17.2	16.9
17:0	0.4	0.4	0.4	0.4
18:0	3.6	3.4	3.5	3.3
20:0	0.3	0.3	0.3	0.3
Saturates	27.7	27.2	27.0	26.3
16:1	6.3	6.2	6.2	5.9
17:1	0.2	0.2	0.2	0.2
18:1	15.4	16.3	16.3	16.0
20:1	2.7	3.1	3.2	3.0
22:1	2.7	3.2	3.3	3.1
MUFA	27.5	29.1	29.3	28.3
14 PUFA	0.1	0.2	0.2	0.2
16:2 n-4	0.7	0.7	0.6	0.6
16:3 n-4	0.9	0.8	0.7	0.7
16:4 n-1	1.4	1.2	1.2	1.2
18:2 n-6	5.5	7.0	6.4	8.9
18:3 n-6	0.2	0.2	0.2	0.2
20:2 n-6	0.2	0.3	0.3	0.3
20:3 n-6	0.1	0.1	0.1	0.1
20:4 n-6	0.9	0.8	0.8	0.7
n-6	6.9	8.4	7.8	10.2
18:3 n-3	1.1	1.3	1.2	1.5
18:4 n-3	1.6	1.6	1.6	1.6
20:3 n-3	0.1	0.1	0.1	0.1
20:4 n-3	0.7	0.7	0.7	0.7
20:5 n-3	12.7	11.4	11.6	10.9
21:5 n-3	0.5	0.5	0.5	0.5
22:5 n-3	1.9	1.8	1.8	1.7
22:6 n-3	10.6	10.1	10.4	9.8
n-3	29.2	27.5	28.0	26.8
PUFA	39.4	38.7	38.5	39.7
Sat/PUFA	0.7	0.7	0.7	0.7
n-3/n-6	4.2	3.3	3.6	2.6

Growth trial

Experiments were undertaken by trained scientists (following FELASA category C recommendations) and were conducted according to the European Economic Community animal experimentation guidelines directive of 24 November 1986 (86/609/EEC). Juvenile blackspot seabream were obtained from the Instituto Español de Oceanografía (IEO, Vigo, Spain) and transported to our facilities at CIIMAR, Porto, Portugal. After a quarantine and acclimatization period of 4 weeks, groups of 51 juveniles with an average body weight of 12 g (Table 4)

Table 3 Amino acid composition (g/16 g N) of the four diets

	Diets			
	45P	AS	AG	35P
Arginine	5.2	4.0	4.2	5.4
Histidine	2.9	2.1	2.1	2.9
Isoleucine	4.3	3.2	3.2	4.2
Leucine	7.1	5.4	5.3	7.2
Lysine	7.2	5.2	4.9	7.0
Methionine	3.1	2.3	2.4	3.1
Cystine	0.8	0.7	0.7	0.9
Phenylalanine	4.5	3.2	3.2	4.7
Tyrosine	3.1	2.0	2.4	3.0
Threonine	3.8	2.4	2.7	3.8
Valine	4.7	3.5	3.4	4.8
Alanine	5.7	17.7	4.1	5.9
Serine	3.2	10.2	2.4	3.3
Aspartic acid	8.4	6.1	21.5	8.4
Glutamic acid	13.9	11.9	35.5	15.4
Glycine	5.9	3.9	4.4	6.0
Proline	4.4	4.1	3.4	5.1
Total AA	88.2	87.9	105.6	91.0
IAA	46.7	33.9	34.3	46.9
DAA	41.5	54.0	71.3	44.1
IAA/DAA	1.1	0.6	0.5	1.1

Tryptophan was not possible to determine

were randomly distributed among 12 square fibre glass tanks (500 L) in a recirculating water system. Each tank was supplied with filtered, heated (19 ± 1°C) saltwater (33 g L^{-1}) with dissolved oxygen content above 8 mg L^{-1} at a flow-rate of 2 L min^{-1}. The pH, ammonia, nitrites and nitrates in the water were monitored during the entire trial and maintained at levels compatible with marine species. Fish were exposed to natural photoperiod. Triplicate groups of fish for each treatment were fed by hand to apparent satiety, three times a day (0930, 1330 and 1730 hours) for 80 days.

Fish were monthly bulk weighted under moderate anaesthesia (ethylene glycol monophenyl ether at 50 mg/L) and data on weight gain and distributed feed recorded. Prior to final tissue sampling, fish were fasted for 24 h and killed by a sharp blow on the head for enzyme determinations. At the beginning and at the end of the feeding trial, a pooled sample of 12 fish per treatment were taken and stored at −20°C for subsequent whole body composition analyses. Muscle and liver tissues (9 fish per treatment) were collected for analysis of total lipid and fatty acids (FAs), lipogenic enzymes, glycolytic, gluconeogenic and AA catabolism (9 fish per treatment) enzymes. All samples were frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

Blood was collected from 12 fish per treatment at 4 h post feeding, and in <3 min at each sampling time to avoid any plasma metabolite response induced by handling stress. Samples were taken from the caudal vein, with syringes and collection tubes containing 15–20 µL of sodium fluoride and potassium oxalate (4% each). Plasma was obtained after centrifugation (6,000×*g* for 10 min at 4°C) and stored at −80°C for glucose, cholesterol (CHOL), triacylglycerol (TAG) and free AA (FAA) profile analysis.

Feed and body composition analyses

Whole fish from each tank were pooled, ground and moisture content determined. Fish were subsequently freeze-dried before further analysis. Feed and whole body samples were analyzed for dry matter (105°C for 24 h), ash by combustion in a muffle furnace (550°C for 12 h), crude protein (micro-Kjeldahl; N x 6.25) after acid digestion, lipid content by petroleum ether extraction (at Soxhlet 40−60°C), and gross energy in an adiabatic bomb calorimeter (IKA, Werke C2000).

Total lipid and FA analyses

Total lipid was extracted and measured gravimetrically according to Folch et al. (1957) using dichloromethane instead of chloroform. FA methyl esters were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride methanol according to Santha and Ackman (1990) and analyzed as described in Figueiredo-Silva et al. (2009, 2010). FAs were identified by comparison with known standard mixtures (Sigma 189-19, St Louis, MO, USA) and quantified using a Star computer package (Varian).

AA analysis

AA analyses were carried out by a certificated laboratory (AGROBIO, Rennes, France) according to Moore and Stein (1951) and Stein and Moore (1954). Samples were hydrolyzed in 6 N HCL under vacuum at 110°C over 23 h. Total AA and FAA were measured by ion exchange chromatography in a Biochrom 30 amino acid analyzer using a sodium high resolution protein hydrolysate column or lithium high resolution protein hydrolysate column, respectively. Norleucine was used as an internal standard for chromatographic separation. Detection of AA were done through the colorimetric reaction with ninhydrin. Since tryptophan is destroyed by the hydrolysis process, it was therefore not part of the evaluation of the AA profile.

Table 4 Plasma-free amino acids (FAA) (μmol/ml), glucose (g/L), cholesterol and triacylglycerol (g/L) at 4 h postprandial in blackspot seabream fed the different diets

	Diets				ANOVA
	45P	AS	AG	35P	*P* value
FAA					
Arginine	0.38 ± 0.1^{a}	0.16 ± 0.04^{b}	0.18 ± 0.01^{b}	0.29 ± 0.04^{ab}	0.017
Histidine	0.27 ± 0.04^{ab}	0.33 ± 0.04^{a}	0.22 ± 0.04^{b}	0.24 ± 0.01^{ab}	0.021
Isoleucine	0.49 ± 0.02^{a}	0.41 ± 0.02^{b}	0.28 ± 0.03^{c}	0.36 ± 0.02^{b}	0.0004
Leucine	0.46 ± 0.01^{a}	0.40 ± 0.02^{b}	0.26 ± 0.03^{c}	0.34 ± 0.01^{b}	0.0002
Lysine	0.42 ± 0.02^{a}	0.24 ± 0.04^{b}	0.22 ± 0.02^{b}	0.32 ± 0.03^{a}	0.002
Methionine	0.26 ± 0.1^{a}	0.19 ± 0.004^{ab}	0.13 ± 0.03^{b}	0.20 ± 0.03^{ab}	0.009
Phenylalanine	0.24 ± 0.1^{a}	0.15 ± 0.01^{b}	0.16 ± 0.02^{b}	0.23 ± 0.01^{a}	0.005
Tyrosine	0.17 ± 0.03	0.10 ± 0.01	0.12 ± 0.02	0.17 ± 0.04	0.039*
Threonine	0.37 ± 0.1^{b}	0.41 ± 0.1^{a}	0.18 ± 0.03^{c}	0.28 ± 0.02^{bc}	0.001
Valine	0.53 ± 0.0^{a}	0.44 ± 0.03^{a}	0.30 ± 0.04^{b}	0.45 ± 0.05^{a}	0.001
Alanine	0.48 ± 0.03^{b}	1.58 ± 0.37^{a}	0.61 ± 0.30^{b}	0.53 ± 0.05^{b}	0.0003
Serine	0.16 ± 0.02^{b}	2.53 ± 0.55^{a}	0.12 ± 0.03^{b}	0.20 ± 0.03^{b}	0.0000
Aspartic acid	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.217
Glutamic acid	0.06 ± 0.01^{b}	0.09 ± 0.01^{a}	0.08 ± 0.01^{a}	0.07 ± 0.004^{b}	0.001
Glycine	0.30 ± 0.03^{b}	1.11 ± 0.15^{a}	0.28 ± 0.03^{b}	0.47 ± 0.1^{b}	0.0000
Proline	0.14 ± 0.03^{a}	0.09 ± 0.01^{c}	0.07 ± 0.01^{c}	0.11 ± 0.01^{bc}	0.002
Taurine	1.51 ± 0.42	1.77 ± 0.01	2.07 ± 0.51	2.94 ± 1.37	0.384
Glutamine	0.26 ± 0.1^{b}	0.41 ± 0.1^{a}	0.23 ± 0.05^{b}	0.22 ± 0.04^{b}	0.005
Asparagine	0.14 ± 0.02^{b}	0.28 ± 0.1^{a}	0.09 ± 0.02^{b}	0.15 ± 0.02^{b}	0.001
Hydroxyproline	0.04 ± 0.01^{b}	0.09 ± 0.02^{a}	0.05 ± 0.00^{b}	0.06 ± 0.01^{ab}	0.012
IAA	2.96 ± 0.79	2.83 ± 0.14	2.00 ± 0.34	2.45 ± 0.62	0.198
DAA	2.58 ± 0.80^{b}	7.42 ± 1.49^{a}	2.94 ± 1.24^{b}	3.80 ± 2.04^{ab}	0.013
Total FAA	5.54 ± 1.58^{b}	10.26 ± 1.54^{a}	4.94 ± 0.94^{b}	6.25 ± 2.58^{ab}	0.023
Glucose	0.94 ± 0.12^{a}	0.88 ± 0.08^{ab}	0.91 ± 0.15^{a}	0.73 ± 0.18^{b}	0.006
CHOL	1.8 ± 0.3^{a}	1.8 ± 0.3^{a}	1.3 ± 0.2^{b}	1.6 ± 0.2^{ab}	0.001
TAG	2.6 ± 0.9^{ab}	3.0 ± 0.9^{a}	2.2 ± 0.5^{ab}	2.1 ± 0.6^{b}	0.026

Mean values and standard deviations (±SD) ($n = 3$ for FAA and $n = 12$ for glucose, cholesterol and triacylglycerol)

Mean values within a row unlike superscript letters were significantly different ($P < 0.05$)

* Without significant differences with post hoc analysis

Plasma metabolite assays

Plasma glucose, CHOL and TAG were determined using commercial kits: Glucose RTU (no 61269), Cholesterol RTU (no 61218) and Triglycérides Enzymatique PAP 150 (no 61236) from Bio-Mérieux, Marcy-L'Etoile, France.

Enzyme activity measurements

Liver samples for lipogenic enzyme assays were homogenized in three volumes of ice-cold buffer (0.02 mol/L Tris–HCl, 0.25 mol/L sucrose, 2 mmol/L EDTA, 0.1 mol/L NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol/L β-mercaptoethanol, pH 7.4) and centrifuged at $30{,}000 \times g$, at 4°C for 20 min. Selected lipogenic enzyme activities were assayed in the supernatant as described in Figueiredo-Silva et al. (2009, 2010).

Liver samples for glycolytic enzymes assays were homogenized in five volumes of ice-cold buffer [80 mM Tris; 5 mM EDTA; 2 mM DTT; 1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, pH 7.6] and centrifuged at $900 \times g$ at 4°C for 10 min. Selected glycolytic enzyme activities were assayed in the supernatant as described in Figueiredo-Silva et al. (2009, 2010).

Liver samples for phosphoenolpyruvate carboxykinase activity (PEPCK, EC 4.1.1.32) assays were homogenized in ten volumes of ice-cold buffer (10 mmol/L HEPES; 0.25 mol/L Saccharose; 1 mmol/L DTT; pH 7.4). The

homogenates were treated by ultrasound for 1 min (Pulse 1 s, amplitude 70 W) and centrifuged at 900×*g* at 4°C for 10 min, and the resultant supernatant was centrifuged at 10,000×*g* at 4°C for 20 min. Total (cytosolic and mitochondrial) PEPCK enzyme activities were assayed at 37°C as described previously by Scholz et al. (1998).

Liver samples for assaying activities of enzymes of AA catabolism were homogenized in 10 volumes of ice-cold buffer (30 mM HEPES, 0.25 mM saccharose, 0.5 mM EDTA, 5 mM K_2HPO_4, 1 mM DTT, pH 7.4) and then separated in two fractions, one for alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) and one to measure glutamate dehydrogenase (GDH, EC 1.4.1.2) activities. Crude homogenate separated for GDH were firstly treated by ultrasound for 1 min (Pulse 1 s, amplitude 50 W) and then all homogenates (ALAT, ASAT and GDH) first centrifuged at 1,000×*g* at 4°C for 20 m and then at 15,000×*g* at 4°C for 20 m. ASAT and ALAT activities were assayed at 37°C on the respective recuperated supernatants using commercial kits from Enzyline (ALAT/GPT, ref. 63313; ASAT/GOT, ref. 63213). GDH activities were assayed at 37°C on the respective recuperated supernatants adding 10 mM of L-glutamic acid to the reaction mixture as described previously by Bergmeyer (1974).

For all enzymes, the activity (units IU), defined as μmoles of substrate converted to product, per min, at assay temperature, was expressed per mg of hepatic soluble protein (specific activity) or per g of tissue. Soluble protein content of tissues was determined on the supernatant fractions by the method of Bradford (1976) using bovine serum albumin as standard (Sigma, St Louis, MO, USA).

Statistical analysis

Statistical analyses followed methods outlined by Zar (1996) and were determined using the STATISTICS 7.0 package (StatSoft, Inc., Tulsa, OK, USA). All data were tested for normality and homogeneity of variances by Kolmogorov–Smirnov and Bartlett tests, and then submitted to a one-way ANOVA. When these tests showed significance ($P < 0.05$), individual means were compared using Tukey test. Tank average values for feed intake, growth, body composition and nutrient accretion analysis were used as experimental units for statistical analyses. Coefficients of determination (R^2) were obtained by a general regression model and considered significant when $P < 0.05$.

Results

In diets AS and AG, DAA content was increased by the incorporation of alanine and serine or aspartic and glutamic acids, respectively, changing the IAA/DAA ratio from 1.1 (diet 45P and 35P) to 0.6 (AS) or 0.5 (AG). Total alanine (8.2 g/100 g diet) and serine (4.7 g/100 g diet) contents in diet AS, and total aspartic (9.8 g/100 g diet) and glutamic (16.1 g/100 g diet) acid contents in diet AG comprise the amount supplemented and the basal content of those diets in these DAA.

Plasma FAA profile is presented in Table 4, and was positively correlated with the dietary AA profile ($R^2 = 0.23$; $P = 0.0001$) (Table 3). Dietary nitrogen reduction from 7.2 to 5.6 g/100 g DM (45P vs. 35P diet) resulted in diminished plasma isoleucine and leucine concentrations, but these differences were not sufficient to significantly affect total plasma-free IAA. The replacement of 1.6 g N/100 g DM by alanine and serine mixture significantly increased plasma alanine ($R^2 = 0.95$; $P = 0.025$) and serine levels ($R^2 = 0.99$; $P = 0.004$) and therefore the total free DAA concentrations. Plasma glycine, glutamine and asparagine levels were higher in fish fed AS diet than in fish fed 45P, AG or 35P diets. On the other hand, aspartic acid concentration in plasma did not vary significantly among diets ($P = 0.217$), whereas glutamic acid concentration although significantly different was only slightly higher in fish fed both DAA mixtures ($P = 0.001$). In fish fed AG diet, plasma histidine, isoleucine, leucine, threonine and valine levels were lower than in fish fed AS diet, but these differences were not sufficient to significantly affect total plasma-free IAA. Glucose, CHOL and plasma TAG levels varied significantly among dietary treatments (Table 4). Glucose levels were higher in fish either fed 45P or AG diets than in those fed the 35P diet. Fish fed AS presented significantly higher plasma CHOL levels than those fed AG. Moreover, TAG levels were also the highest in fish fed AS but just differed significantly from the 35P fed fish.

At the end of the growth trial (80 days), all groups of blackspot seabream tripled their initial body weight regardless of dietary treatment (Table 5). Fish fed the 45P diet (7.2 g N) presented the highest final body weight (FBW) and daily growth index (DGI).

Although fish fed 35P (5.6 g N/100 g dry matter) increased feed intake, this increase was not enough to reach the same daily nitrogen intake observed in fish fed the 45P diet, resulting in reduced FBW, low DGI and low dry feed/wet weight gain ratio (FGR). The replacement of 22.2% nitrogen from the fish meal by DAA led to reduced feed intake inducing lower DGI and FBW. Despite this reduction in FBW and DGI, fish fed AS or AG diets showed similar FGR and similar protein efficiency ratios when compared with the 45P fed group.

Despite the differences observed in feed intake and growth, blackspot seabream had similar whole body protein and energy contents (Table 6) irrespective of the

Table 5 Data on feed and nutrient intakes and growth performance of blackspot seabream fed the different diets over 80 feeding days

	Diets				ANOVA
	45P	AS	AG	35P	*P* value
Growth					
Initial body weight (g)	12.0 ± 0.1	12.0 ± 0.1	12.0 ± 0.1	12.0 ± 0.1	0.927
Final body weight (g)	41.0 ± 0.8^{a}	37.2 ± 1.1^{b}	35.1 ± 1.0^{bc}	34.5 ± 1.0^{c}	0.0006
FGR[A]	1.59 ± 0.06^{b}	1.60 ± 0.04^{b}	1.64 ± 0.02^{b}	1.88 ± 0.06^{a}	0.0002
DGI[B]	1.45 ± 0.03^{a}	1.31 ± 0.03^{b}	1.24 ± 0.03^{bc}	1.21 ± 0.04^{c}	0.0001
PER[C]	1.40 ± 0.05^{b}	1.36 ± 0.04^{ab}	1.35 ± 0.02^{b}	1.53 ± 0.05^{a}	0.005
Intake[D] (g or kJ/kg ABW[E]/day)					
Dry matter	21.7 ± 0.6^{b}	20.6 ± 0.3^{c}	20.2 ± 0.1^{c}	22.8 ± 0.1^{a}	0.0001
Nitrogen	1.57 ± 0.04^{a}	1.51 ± 0.02^{ab}	1.47 ± 0.01^{b}	1.27 ± 0.01^{c}	0.000002
Fat	2.27 ± 0.06^{b}	2.11 ± 0.03^{c}	1.97 ± 0.01^{d}	2.47 ± 0.01^{a}	0.000001
Gross energy	443.3 ± 12.0^{b}	417.3 ± 6.0^{c}	405.3 ± 1.9^{c}	472.0 ± 2.4^{a}	0.00001

Mean values and standard deviations (±SD) are presented for each parameter ($n = 3$)

Mean values within a row unlike superscript letters were significantly different ($P < 0.05$)

[A] Feed:Gain ratio (FGR) = dry feed intake/weight gain

[B] Daily growth index (DGI) = 100 × [(final body weight)$^{1/3}$ − (initial body weight)$^{1/3}$]/days

[C] Protein efficiency ratio = weight gain/crude protein intake

[D] Daily nutrient intake (g or kJ/kg ABW/day) = nutrient intake/[(initial body weight + final body weight)/2]/days

[E] Average body weight (ABW) = (final body weight + initial body weight)/2

dietary treatments. However, fish fed the AS diet had significantly higher whole body lipid content (12.9%) than the 35P fed group (10.7%). The reduced body lipid content observed in fish fed the low protein diet (diet 35P) correlates well with their reduced viscerosomatic index (VSI). The hepatosomatic index (HSI) was unaffected by dietary treatments, although a high liver lipid content was found in fish fed AS comparatively to those fed diet 45P. Muscle total lipid content ranged between 5 and 6% of wet weight irrespective of the dietary treatment.

Overall, nitrogen gain varied according to differences in daily nitrogen intake ($R^2 = 0.42$; $P = 0.02$), and consequently overall protein retention (% intake) was unaffected by dietary treatments so a different trend was observed with regard to lipid gain or retention, with fish fed 45P and AS diets showing higher lipid gain and retention than those fed the lowest protein (35P) diet. In fact, lipid gain was positively correlated with nitrogen intake ($R^2 = 0.53$; $P = 0.007$).

Muscle and liver fatty acid (FA) compositions of blackspot seabream are shown in Tables 7 and 8, respectively. The different dietary treatments significantly affected FA composition in both tissues. Muscle total saturated FAs were higher in fish fed both the 45P and the AS diet than in those fed the 35P, mainly due to an increase in 14:0 and 16:0 FAs. Liver 14:0 and 18:0 FA contents were higher in fish fed 45P, AS and AG than in those fed the low protein diet (35P). Nevertheless, these differences were not sufficient to significantly affect liver saturated FA fraction. Fish fed the 35P diets had the lowest monounsaturated fatty acid (MUFA) content in both muscle and liver mainly due to the low level of 18:1 (oleic acid) content, but this difference was only significant in muscle. Higher proportions of linoleic (18:2n-6) and then n-6 polyunsaturated fatty acid (PUFA) were found in both muscle and liver of fish fed diet 35P resulting from the higher dietary content of these FAs (Table 2). A trend to increased n-3 PUFA in liver and a significant increase in muscle was observed in fish fed diet 35P mainly due to the higher percentages of 22:6n-3. The higher muscle and liver n-6 FA content found in fish fed diet 35P consequently resulted in a lower n-3/n-6 FA ratio. Moreover, this ratio was lower in fish fed AS and AG diets than in those fed diet 45P, mainly due to the higher content of n-6 FAs in these diets (Table 2).

Data on the hepatic enzyme activities (lipogenic, glycolytic, gluconeogenic and AA catabolism) are presented in Table 9. Malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PD) and fatty acid synthase (FAS) activities were markedly depressed by the dietary nitrogen reduction (45P vs. 35P diet) but FAS-specific activity responded differently to the replacement of 22.2% fish meal nitrogen by the two different DAA mixtures. Regarding dietary nitrogen source, FAS-specific activities (mIU/mg protein) were reduced in fish fed AG diet compared to fish fed 45P and AS diets.

Neither the reduction nor the nitrogen replacement by the two different DAA mixtures had any effect on

Table 6 Whole body composition [% or kJ g^{-1} of wet weight (ww)], nutrient gain and energy retention in blackspot seabream fed the different diets

	Diets				ANOVA
	45P	AS	AG	35P	*P* value
Final body composition[A]					
Moisture %	65.3 ± 1.6	64.8 ± 1.3	66.5 ± 1.1	67.1 ± 0.9	0.172
Protein %	17.7 ± 0.7	17.9 ± 0.8	17.0 ± 0.6	17.1 ± 0.4	0.302
Lipid %	12.2 ± 0.4[ab]	12.9 ± 1.1[a]	11.5 ± 0.9[ab]	10.7 ± 0.7[b]	0.024
Energy kJ/g	8.4 ± 0.4	8.5 ± 0.5	8.2 ± 0.4	7.7 ± 0.1	0.136
HSI %[B]	1.4 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.2	0.596
VSI %[C]	6.4 ± 1.3	6.5 ± 1.1	6.5 ± 1.1	5.0 ± 1.3	0.034*
Nitrogen gain (mg/kg ABW[D]/d)	400.3 ± 25.6[a]	383.2 ± 23.1[ab]	343.8 ± 11.7[b]	340.3 ± 16.2[b]	0.015
Lipids gain (g/kg ABW[D]/d)	1.8 ± 0.1[a]	1.8 ± 0.2[a]	1.5 ± 0.1[ab]	1.3 ± 0.1[b]	0.007
Energy gain (kJ/kg ABW[D]/d)	123.0 ± 9.0[a]	117.9 ± 9.9[a]	108.0 ± 5.4[ab]	98.2 ± 1.2[b]	0.013
Retention (% of intake)					
Protein	25.5 ± 1.8	25.4 ± 1.9	23.4 ± 0.8	26.9 ± 1.3	0.126
Lipid	78.2 ± 5.9[a]	85.9 ± 11.9[a]	75.5 ± 3.7[ab]	53.3 ± 4.2[b]	0.003
Energy	27.8 ± 2.3[a]	28.3 ± 2.7[a]	26.7 ± 1.4[a]	20.8 ± 0.2[b]	0.005

Mean values and standard deviations (±SD) are presented for each parameter ($n = 3$)

Mean values within a row unlike superscript letters were significantly different ($P < 0.05$)

[A] Initial body composition was: moisture 68.4%, protein 16.3% ww, lipid 10.4% ww and energy 7.0 kJ/g

[B] Hepatosomatic index (HSI) = 100 × liver weight/body weight

[C] Viscerosomatic index (VSI) = 100 × weight of viscera/body weight

[D] Average body weight (ABW) = (final body weight + initial body weight)/2

* Without significant differences after post hoc analysis

hexokinase (HK) or glucokinase (GK) activities. The reduction of dietary nitrogen significantly increased pyruvate kinase (PK) activities. PEPCK-specific activities were significantly lower in fish fed diet 45P than in those fed AG or 35P diet.

Activities of AA catabolic enzyme (ALAT, ASAT, GDH) were higher in 45P than in AG or 35P fed groups, but when expressed as specific activities no significant differences ($P > 0.05$) were observed.

Discussion

The growth parameters and feed efficiencies obtained for blackspot seabream juveniles are well within the range of values for this species (Figueiredo-Silva et al. 2009, 2010). The reduction of dietary crude protein to levels below 45% level known to be optimal for this species (Silva et al. 2006), was previously shown to markedly depress growth rates (Figueiredo-Silva et al. 2009) due to the inability of fish fed with a 35% protein diet to reach the same daily nitrogen intake observed when fed a 45% protein diet.

The correspondence observed between dietary and postprandial FAA concentrations together with the three-fold increase in body weight attest the good acceptance of the different diets. Plasma alanine and serine concentrations were positively correlated with the respective dietary content, but the same was not true for aspartic and glutamic acids. The time-course of AA absorption is known to relate with the form of dietary supply (Murai et al. 1987; Cowey and Walton 1988), which to some extent may explain this discrepancy. In the present study, the eventual differences in the time-course of AA absorption does not fully explain the differences found in some particular plasma IAA and DAA levels between fish fed the AS and AG diets. Since AS and AG diets were fed at a similar ratio, a depletion on IAA absorption may be suggested as a possible explanation for the decreased plasma histidine, isoleucine, leucine, threonine and valine levels verified with AG diet when compared with AS diet. However, this hypothesis does not explain the higher plasma glycine, glutamine and asparagine levels found with AS diet that are probably result of an increased DAA interconversion and metabolism, as already reported in fish (Cowey and Walton 1989).

Table 7 Muscle total lipid content (% wet weight) and fatty acid composition (g/100 g total fatty acids) of blackspot seabream fed the different diets

	Diets				ANOVA
	45P	AS	AG	35P	*P* value
14:0	3.1 ± 0.3^{a}	2.9 ± 0.3^{ab}	2.7 ± 0.2^{b}	2.7 ± 0.2^{b}	0.009
15:0	0.9 ± 0.5	0.7 ± 0.4	0.9 ± 0.5	0.8 ± 0.5	0.822
16:0	18.0 ± 0.6^{a}	17.3 ± 0.5^{a}	16.4 ± 1.0^{b}	16.1 ± 0.4^{b}	0.000
17:0	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.02	0.4 ± 0.03	0.063
18:0	6.6 ± 0.2^{a}	6.6 ± 0.4^{a}	6.7 ± 0.4^{a}	5.9 ± 0.3^{b}	0.000
20:0	0.2 ± 0.01^{a}	0.2 ± 0.01^{ab}	0.2 ± 0.01^{a}	0.2 ± 0.01^{b}	0.013
Saturates	29.2 ± 0.8^{a}	28.3 ± 0.5^{ab}	27.3 ± 1.7^{bc}	26.5 ± 0.9^{c}	0.0001
16:1	4.8 ± 0.4^{a}	4.5 ± 0.3^{ab}	4.4 ± 0.3^{b}	4.4 ± 0.3^{b}	0.019
17:1	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02	0.742
18:1	21.1 ± 1.5^{a}	20.3 ± 0.7^{a}	21.0 ± 1.2^{a}	18.2 ± 1.0^{b}	0.000
20:1	2.3 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	0.089
22:1	1.9 ± 0.2^{b}	2.0 ± 0.1^{ab}	2.0 ± 0.1^{ab}	2.1 ± 0.1^{a}	0.011
MUFA	30.3 ± 1.9^{a}	29.4 ± 0.6^{a}	30.0 ± 1.6^{a}	27.4 ± 1.4^{b}	0.001
16:2 n-4	0.4 ± 0.04^{a}	0.3 ± 0.04^{b}	0.3 ± 0.03^{b}	0.4 ± 0.04^{ab}	0.005
16:3 n-4	0.4 ± 0.1^{a}	0.4 ± 0.04^{b}	0.4 ± 0.04^{b}	0.4 ± 0.04^{b}	0.004
16:4 n-1	0.5 ± 0.1^{a}	0.4 ± 0.1^{b}	0.4 ± 0.04^{b}	0.4 ± 0.1^{b}	0.002
18:2 n-6	4.4 ± 0.2^{c}	5.3 ± 0.2^{b}	4.7 ± 0.2^{c}	6.6 ± 0.5^{a}	0.000
18:3 n-6	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.01	0.185
20:2 n-6	0.3 ± 0.02^{b}	0.3 ± 0.02^{b}	0.3 ± 0.03^{b}	0.3 ± 0.02^{a}	0.000
20:3 n-6	0.2 ± 0.01^{b}	0.2 ± 0.02^{a}	0.2 ± 0.01^{a}	0.2 ± 0.02^{b}	0.000
20:4 n-6	0.8 ± 0.1^{ab}	0.8 ± 0.1^{ab}	0.8 ± 0.02^{b}	0.9 ± 0.1^{a}	0.006
n-6	5.9 ± 0.3^{c}	6.9 ± 0.2^{b}	6.2 ± 0.3^{c}	8.2 ± 0.5^{a}	0.000
18:3 n-3	0.7 ± 0.04^{b}	0.7 ± 0.02^{b}	0.7 ± 0.1^{b}	0.9 ± 0.1^{a}	0.000
18:4 n-3	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.04	0.8 ± 0.1	0.121
20:3 n-3	0.1 ± 0.01^{b}	0.1 ± 0.01^{b}	0.1 ± 0.01^{ab}	0.1 ± 0.02^{a}	0.005
20:4 n-3	0.8 ± 0.04	0.8 ± 0.04	0.8 ± 0.05	0.8 ± 0.06	0.318
20:5 n-3	9.0 ± 0.4^{a}	8.4 ± 0.3^{b}	7.8 ± 0.2^{c}	8.5 ± 0.4^{b}	0.000
21:5 n-3	0.4 ± 0.02^{a}	0.3 ± 0.04^{b}	0.4 ± 0.02^{b}	0.4 ± 0.03^{b}	0.0002
22:5 n-3	3.4 ± 0.3	3.5 ± 0.3	3.5 ± 0.3	3.5 ± 0.2	0.769
22:6 n-3	11.6 ± 1.3^{b}	12.2 ± 0.7^{ab}	11.1 ± 0.4^{b}	13.2 ± 1.1^{a}	0.0004
n-3	26.7 ± 1.9^{ab}	26.8 ± 1.3^{ab}	25.2 ± 0.8^{b}	28.1 ± 1.3^{a}	0.001
PUFA	34.0 ± 2.1^{bc}	34.8 ± 1.4^{b}	32.5 ± 1.0^{c}	37.5 ± 1.5^{a}	0.000
Sat/PUFA	0.9 ± 0.1^{a}	0.8 ± 0.04^{b}	0.8 ± 0.1^{b}	0.7 ± 0.04^{b}	0.000
n-3/n-6	4.5 ± 0.2^{a}	3.9 ± 0.1^{b}	4.1 ± 0.2^{b}	3.4 ± 0.2^{c}	0.000
Muscle total lipid %(wet weight)	5.6 ± 0.7	4.9 ± 0.7	5.5 ± 0.5	5.4 ± 0.8	0.096

Mean values and standard deviations (±SD) are presented for each parameter ($n = 9$)

Mean values within a row unlike superscript letters were significantly different ($P < 0.05$)

The time-course of changes in plasma DAA concentrations do not always demonstrate a direct influence of the DAA composition of the test meal (Kaushik 1977). Dietary glutamic acid does not lead to an increase in plasma glutamic acid levels also in higher animals (Rérat et al. 1992; Moundras et al. 1993) given its metabolic utilization at the splanchnic bed. Whether similar mechanisms are active also in fish is worth further investigation.

Although fish growth has been shown to depend on the nature of DAA (Hughes 1985; Fauconneau 1988) and its

Table 8 Liver total lipid content (% wet weight WW) and fatty acid composition (g/100 g total fatty acids) of blackspot seabream fed the different diets

	Diets				ANOVA
	45P	AS	AG	35P	*P* value
14:0	2.3 ± 0.3[a]	2.5 ± 0.1[a]	2.3 ± 0.1[a]	1.6 ± 0.004[b]	0.003
15:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.02	0.2 ± 0.01	0.806
16:0	17.4 ± 1.9	16.2 ± 1.3	16.0 ± 0.6	14.2 ± 1.0	0.138
17:0	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.02	0.4 ± 0.02	0.063
18:0	11.1 ± 0.3	10.2 ± 0.6	11.4 ± 0.1	10.2 ± 0.6	0.036*
20:0	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.02	0.369
Saturates	31.7 ± 1.9	29.8 ± 1.8	30.6 ± 0.5	26.9 ± 1.5	0.052
16:1	3.9 ± 0.2	4.1 ± 0.2	3.8 ± 0.1	3.4 ± 0.1	0.022*
17:1	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01	0.255
18:1	32.3 ± 1.7	31.3 ± 1.4	31.0 ± 1.2	28.2 ± 1.1	0.076
20:1	3.0 ± 0.4	3.0 ± 0.1	3.2 ± 0.1	3.7 ± 0.2	0.071
22:1	1.4 ± 0.2[b]	1.6 ± 0.1[b]	1.6 ± 0.1[ab]	2.0 ± 0.03[a]	0.011
MUFA	40.9 ± 1.6	40.3 ± 1.1	40.0 ± 1.0	37.5 ± 1.2	0.102
14 PUFA	0.05 ± 0.002	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.474
16:2 n-4	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.02	0.495
16:3 n-4	0.2 ± 0.02	0.2 ± 0.04	0.2 ± 0.04	0.1 ± 0.03	0.139
16:4 n-1	0.1 ± 0.01	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.02	0.240
18:2 n-6	3.1 ± 0.5[c]	4.4 ± 0.5[ab]	3.6 ± 0.3[bc]	5.8 ± 0.5[a]	0.002
18:3 n-6	0.1 ± 0.003	0.2 ± 0.07	0.2 ± 0.02	0.2 ± 0.02	0.072
20:2 n-6	0.5 ± 0.2[b]	0.5 ± 0.1[b]	0.6 ± 0.05[b]	0.9 ± 0.1[a]	0.004
20:3 n-6	0.3 ± 0.05	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.02	0.056
20:4 n-6	0.6 ± 0.1	0.5 ± 0.03	0.50 ± 0.01	0.6 ± 0.03	0.067
n-6	4.6 ± 0.7[c]	6.2 ± 0.7[b]	5.4 ± 0.3[bc]	8.1 ± 0.5[a]	0.002
18:3 n-3	0.4 ± 0.1[b]	0.5 ± 0.1[ab]	0.5 ± 0.1[ab]	0.7 ± 0.1[a]	0.046
18:4 n-3	0.2 ± 0.02	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.134
20:3 n-3	0.1 ± 0.1[b]	0.2 ± 0.02[ab]	0.2 ± 0.02[ab]	0.2 ± 0.02[a]	0.030
20:4 n-3	0.9 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.04	0.220
20:5 n-3	3.9 ± 0.2	3.6 ± 0.3	3.3 ± 0.3	3.7 ± 0.7	0.242
21:5 n-3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.04	0.4 ± 0.02	0.292
22:5 n-3	4.1 ± 1.3	4.2 ± 0.6	4.4 ± 0.5	6.0 ± 0.1	0.123
22:6 n-3	6.5 ± 0.6[b]	6.4 ± 0.4[b]	6.4 ± 0.3[b]	8.0 ± 0.5[a]	0.026
n-3	16.5 ± 1.7	16.6 ± 1.6	16.3 ± 1.3	20.5 ± 1.3	0.060
PUFA	21.5 ± 2.4[b]	23.4 ± 2.4[ab]	22.4 ± 1.7[b]	29.0 ± 1.8[a]	0.029
Sat/PUFA	1.5 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	0.9 ± 0.1	0.058
n-3/n-6	3.6 ± 0.3[a]	2.7 ± 0.1[b]	3.0 ± 0.1[b]	2.5 ± 0.01[b]	0.000
Liver total lipid %(WW)	24.2 ± 2.3[b]	33.0 ± 2.8[a]	26.8 ± 2.8[ab]	31.3 ± 4.0[ab]	0.027

Mean values and standard deviations (±SD) are presented for each parameter (3 pools of 3 fish each, $n = 3$)

Mean values within a row unlike superscript letters were significantly different ($P < 0.05$)

* Without significant differences after post hoc analysis

level (Mambrini and Kaushik 1994), the results are quite controversial. In rainbow trout, Fauconneau (1988) reported better growth rates with diets supplemented with aspartic acid than with alanine or glutamic acid. However, no effect of the DAA nature on fish growth performance was found when nitrogen was partially replaced by a single

Table 9 Effects of different dietary treatments on blackspot seabream key hepatic enzymes activities

	Diets				ANOVA
	45P	AS	AG	35P	*P* value
Lipogenic enzymes					
ME					
IU/g liver	3.6 ± 1.0 [a]	2.6 ± 0.7 [a]	2.6 ± 0.1[a]	0.7 ± 0.2[b]	0.000001
mIU/mg protein	49.5 ± 12.2[a]	45.9 ± 13.2[a]	39.9 ± 17.0[a]	13.9 ± 5.5[b]	0.00001
G6PD					
IU/g liver	9.7 ± 2.2[a]	9.2 ± 2.2[a]	9.0 ± 1.2[a]	5.4 ± 1.9[b]	0.0001
mIU/mg protein	96.6 ± 19.3[a]	108.6 ± 15.1[a]	94.6 ± 14.8[a]	57.4 ± 11.7[b]	0.000001
FAS					
IU/g liver	1.6 ± 0.6[a]	1.1 ± 0.5[ab]	0.9 ± 0.6[ab]	0.4 ± 0.2[b]	0.005
mIU/mg protein	15.2 ± 5.3[a]	13.6 ± 5.9[ab]	8.2 ± 4.5[bc]	4.5 ± 2.7[c]	0.001
Glycolytic enzymes					
HK					
IU/g liver	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.102
mIU/mg protein	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.064
GK					
IU/g liver	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.02	0.929
mIU/mg protein	0.7 ± 0.4	0.8 ± 0.5	0.9 ± 0.3	0.7 ± 0.3	0.897
PK					
IU/g liver	15.9 ± 4.8[b]	12.9 ± 2.7[b]	15.3 ± 4.9[b]	26.2 ± 6.6[a]	0.0001
mIU/mg protein	181.9 ± 69.1[b]	159.4 ± 35.1[b]	201.6 ± 63.5[b]	332.2 ± 81.6[a]	0.0001
Gluconeogenic enzymes					
PEPCK					
IU/g liver	1.7 ± 0.3	2.1 ± 0.7	2.4 ± 0.4	2.1 ± 0.5	0.071
mIU/mg protein	18.4 ± 3.0[b]	23.5 ± 6.9[ab]	30.0 ± 5.9[a]	26.4 ± 7.2[a]	0.011
Amino acid catabolism					
ALAT					
IU/g liver	116.9 ± 35.0[a]	87.6 ± 18.9[ab]	77.7 ± 27.4[b]	80.4 ± 17.4[b]	0.016
mIU/mg protein	1252.4 ± 429.0	1205.0 ± 279.0	1147.3 ± 340.3	1016.8 ± 278.0	0.528
ASAT					
IU/g liver	147.5 ± 33.7[a]	110.3 ± 21.0[b]	100.4 ± 23.1[b]	93.1 ± 22.5[b]	0.0006
mIU/mg protein	1587.1 ± 406.5	1530.1 ± 388.2	1413.5 ± 318.0	1264.0 ± 344.0	0.270
GDH					
IU/g liver	8.3 ± 1.0[a]	7.1 ± 0.9[ab]	5.9 ± 0.9[bc]	5.3 ± 1.4[c]	0.00001
mIU/mg protein	82.5 ± 28.3	92.8 ± 17.9	77.2 ± 18.6	69.9 ± 16.1	0.165

Mean values and standard deviations (±SD) are presented for each parameter ($n = 9$)

ME malic enzyme, *G6PD* glucose-6-phosphate dehydrogenase, *FAS* fatty acid synthase, *HK* hexokinase, *GK* glucokinase, *PK* pyruvate kinase, *PEPCK* phosphoenolpyruvate carboxykinase, *ALAT* alanine aminotransferase, *ASAT* aspartate aminotransferase, *GDH* glutamate dehydrogenase

Mean values within a row unlike superscript letters were significantly different ($P < 0.05$)

DAA (alanine, aspartic or glutamic acid) (Kirchner et al. 2003a) or by either a single (alanine, glutamate or glycine) or a mixture of DAA (alanine, aspartic and glutamic acid, glycine, proline and serine) (Mambrini and Kaushik 1994), as also observed here. It is generally accepted that reduced utilization of synthetic AA relative to protein-bound AA for anabolic purposes results from their rapid absorption (Cowey and Walton 1988; Ambardekar et al. 2009). Thus, the inferior FBW obtained with AG (<20%) than with AS diet (<13%) when compared with the 45P, may resulted from a faster or poorer absorption of aspartic and glutamic acids than of alanine and serine. When supplying high levels of crystalline amino acids in the feeds for fish, coating with agar has been shown to be efficient and

beneficial (Mambrini and Kaushik 1994). Such a treatment would have possibly improved the responses in the present study.

A point worth mentioning is also that of the low plasma-free threonine level in fish fed the diet AG. Threonine imbalance due to supply of DAA has been reported since long (Tews et al. 1980) mainly due to competitive inhibition of threonine transport by DAA, although the role of aspartic or glutamic acids in this inhibition was not shown by these authors. In pigs, irreversible oxidation of threonine into glycine, mainly at the level of the digestive tract, is another factor affecting threonine metabolism (Le Floc'h and Seve 1996). Whether similar mechanisms are active in fish and whether the possible involvement of threonine also in feed intake regulation (Feurte et al. 1999) in fish is not known.

Fish body fat was shown to associate with dietary DAA nature pointing out the different individual DAA contribution as metabolic intermediate precursors (Mambrini and Kaushik 1994). The similarity of the data concerning lipid gain and body lipid content between 45P and AS or AG fed blackspot seabream showed the ability of this species to use these two different mixtures of DAA as precursors for FA synthesis, with lipid gain being positively correlated with nitrogen intake. Although not statistically different, fish fed the diet AS had a higher daily lipid gain and body lipid content compared to those fed AG, possibly explaining the increased somatic weight gain found in fish fed AS diet. Compared with the low protein diet (35P), fish fed with diet AS or AG showed a higher or similar lipid gain and whole body lipid content, respectively. Considering the absence of significant differences in muscle lipid content, irrespective of the dietary treatment, and the low contribution of liver lipid content for whole body lipid content, this accretion seems to mainly result from a higher visceral fat deposition evidenced by the higher VSI found in fish fed both AS or AG diets when compared with fish fed the 35P diet. An increased HSI has been reported in rainbow trout fed with DAA supplemented diets (Kirchner et al. 2003a); our data do not show any such increase in liver size, although fish fed the DAA had higher liver total lipid levels when compared with the 45P diet. The still elevated liver lipid contents of blackspot seabream, fed the 35P diet, probably results from a higher liver uptake of circulating TAG to fuel β-oxidation. The decreased muscle MUFA content together with the reduced VSI of fish fed the 35P diet suggests that FA are being mobilized to fuel β-oxidation, a process well established in fish in order to meet energy demands (Sargent et al. 2002). Although dietary treatments have to some extent altered some of the tissue FA composition, both muscle and liver FA profile were generally well within those previously found for this species fed a diet with similar FA composition (Figueiredo-Silva et al. 2009, 2010). Higher linoleic acid (18:2n-6) and subsequently n-6 PUFA percentages were found in muscle and liver of fish fed the low protein diet reflecting the higher content of these FAs in diet 35P, due to the increase in wheat bran content (30%).

In the present work, the reduction of dietary nitrogen supply from 7.2 g to 5.6/100 g dry matter g led to a reduction in all the lipogenic enzyme activities (ME, G6PD and FAS). Dietary protein supply is recognized as a potent regulator of lipid biosynthesis in higher vertebrates (Herzberg and Rogerson 1981; Rosebrough et al. 1996) as well as in fish (Henderson and Sargent 1981; Shikata and Shimeno 1997; Alvarez et al. 1999; Dias et al. 1998, 2003, 2005), particularly in blackspot seabream (Figueiredo-Silva et al. 2009). Moreover, the nitrogen source has also been shown to modify body lipid content (Kaushik et al. 2004; Dias et al. 2005) and lipogenesis (Gómez-Requeni et al. 2003; Dias et al. 2005) in other fish species and in higher vertebrates (Iritani et al. 1986, 1996; Kayashita et al. 1996; Padmakumarannair et al. 1998). The replacement of fish meal by high inclusions of corn gluten meal (Dias et al. 2005) or by corn and wheat gluten (Kaushik et al. 2004) appears to up-regulate FAS activities and increase body lipid content, but the effects of soy protein concentrates in hepatic lipogenesis showed to be highly variable (Dias 1999). A significant interaction between dietary protein/lipid level and protein source (fish meal vs. wheat gluten) was previously found to affect blackspot seabream lipid retention and lipogenesis (Figueiredo-Silva et al. 2010). Accordingly, in the present study, a major role of dietary nitrogen source on blackspot seabream lipogenic pathways was evidenced by the ability of fish either fed 45P, AS or AG to show similar lipogenic enzyme activity levels and similar levels of 14:0 and 18:0 FAs in the liver. These FAs together with the 16:0 are the main newly synthesized FAs in fish (Corraze 2001; Sargent et al. 2002). Contrary to fish fed AG, fish fed AS exhibited specific FAS activities similar to that in 45P fed fish, suggesting a further role on this lipogenic pathways. Hepatic lipogenic enzyme activities have been found to be down-regulated in gilthead seabream fed high dietary glutamic acid (Gómez-Requeni et al. 2003). Similarly, it was here demonstrated that the partial replacement of dietary fish meal nitrogen by a mixture of aspartic and glutamic acids mixture has in fact the ability to reduce the specific FAS.

Glycolytic pathways were unaffected by the high dietary DAA content as previously found on rainbow trout (Kirchner et al. 2003a) or gilthead seabream (Gómez-Requeni et al. 2003). The up-regulation of PK and reduction of plasma TAG levels with the reduction in dietary nitrogen levels attest the important role of dietary protein level on glycolytic pathways regulation in blackspot seabream (Figueiredo-Silva et al. 2009) as in rainbow trout (Kirchner

et al. 2003b). The PK activation associated to a rapid uptake by the liver of circulating TAG seems to result from a metabolic adaptation of fish fed 35P to a low dietary energy supply status.

An excess of different individual DAA (alanine, aspartic or glutamic acid) failed to modify gluconeogenesis in the rainbow trout (Kirchner et al. 2003a), but as suggested by the authors, fish were fed protein-rich diets (53%), which in fact could possibly mask potential effects of dietary DAA. Although, no distinction between cytosolic and mitochondrial PEPCK forms was made in our study, PEPCK total activities were well within values found for rainbow trout (Kirchner et al. 2003b), with the surplus dietary content on aspartic and glutamic acids shown to up-regulate PEPCK activities when compared with 45P fed group. However, this up-regulation did not result in a concomitant increase of plasma glucose levels. Moreover, at similar PEPCK activities, reduced glycaemia was verified on blackspot seabream fed 35P than in group fed the diet AG. Several works carried out in mammals suggests that PEPCK flux may interact with energy generation in the hepatic TCA (She et al. 2000; Burgess et al. 2004, 2007; Hakimi et al. 2005). PEPCK was indeed identified as a critical enzyme for the synthesis of glycerol-3-phosphate required to re-esterify part of the FA coming from adipocytes hydrolyze, back to TAG (Forest et al. 2003; Chakravarty et al. 2005). Therefore, it seems that under unfavourable energy state and the concomitant FA β-oxidation activation, blackspot seabream up-regulate both PEPCK and PK. PEPCK produces phosphoenolpyruvate and the PK converts it into pyruvate to uphold the pyruvate flux between mitochondria and cytosol in an attempt to generate energy through the TCA cycle, and not to produce glucose (gluconeogenesis). Furthermore, the increased plasma cortisol levels previously found in blackspot seabream fed a similar low protein diet (Figueiredo-Silva et al. 2009) favour this hypothesis, given that amplified PEPCK activity and expression are found at high glucocorticoids circulating levels (Gunn et al. 1975; Hanson and Reshef 1997). Hence, it would be of interest to study the PEPCK activity and expression responsiveness to different fish energy states.

The measurement of key hepatic enzymes activity involved in AA catabolism (ALAT, ASAT, GDH) has been pointed as an useful indicator of the metabolic utilization of dietary components by fish. However, the effect of dietary factors on the activity of these enzymes is often relatively contradictory within fish species (Cowey and Walton 1989). In blackspot seabream, the AA catabolic enzymes were generally within the activities found in species with alike protein requirements like gilthead seabream (Metón et al. 1999; Gómez-Requeni et al. 2003, 2004; Enes et al. 2008) or sea bass (Enes et al. 2006). Nevertheless, in blackspot seabream, the three main enzymes involved in AA catabolism displayed different responses to dietary nitrogen level and nature. Hepatic aminotransferases ALAT and ASAT and GDH deaminating enzyme showed higher activities in fish fed high protein diet (45P) than in those fed the low protein diet (35P), standing for the hypotheses that fish fed with higher levels of dietary protein use considerable amount of dietary AA for FA synthesis or energy production. The up-regulation of ALAT by dietary protein level was also found in rainbow trout (Lupiañez et al. 1989), gilthead seabream (Metón et al. 1999) or in Nile tilapia (Gaye-Siessegger et al. 2006). Minor response of hepatic aminotranferases to dietary AA profile was noticed in gilthead seabream (Gómez-Requeni et al. 2003) or Nile tilapia (Gaye-Siessegger et al. 2007) while a reduction on the specific activity of GDH was observed in gilthead seabream fed high dietary glutamic acid content (Gómez-Requeni et al. 2003). Conversely, high dietary levels of glutamic acid induced increased GDH activity in rainbow trout (Moyano et al. 1991). In blackspot seabream, AA catabolic enzymes have responded (IU/g liver) or showed a tendency (mIU/mg protein) to respond accordingly to dietary nitrogen nature, particularly to nitrogen provided by aspartic and glutamic acids mixture (AG). As mentioned above, the role of threonine and specifically the activity of threonine dehydrogenase are also worth assessment. Further studies are needed to elucidate the role of DAA on these important AA catabolic enzymes.

Conclusion

Blackspot seabream juveniles responded well to dietary crystalline supplementation showing the different role of DAA, alanine and serine (pyruvate precursors) and aspartic and glutamic acids (TCA intermediates), in terms of growth response and intermediary metabolic pathways. Compared to fish fed a low protein diet (35P), blackspot seabream juveniles appear to make a more efficient use of the nitrogen provided by alanine and serine than that provided by aspartic and glutamic acid mixture in terms of growth. Although not statistically different, fish fed the diet AS showed a tendency to increased daily lipid gain and body lipid content when compared with those fed AG diet, which probably has contributed to the superior gain of weight verified in those fish. Contrary to fish fed AG, fish fed AS attained similar specific FAS activities to 45P fed fish, suggesting a further role of alanine and serine on this lipogenic pathway. Both, dietary nitrogen reduction (45P vs. 35P) or its replacement by aspartic and glutamic acids mixture (diet AG) appear to up-regulate PEPCK activity but without increasing plasma glucose levels. Dietary nitrogen level and nature seems to exert a complex

regulation on energetic pathways through the gluconeogenesis/TCA interaction. Future work on fish nutrition should give due consideration also to the DAA, given the specific roles of these amino acids in different pathways of intermediary metabolism.

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