

Response of European sea bass (*Dicentrarchus labrax*) to graded levels of methionine (total sulfur amino acids) in soya protein-based semi-purified diets

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The dietary methionine (Met) and total sulfur amino acid (TSAA) requirements of European sea bass (*Dicentrarchus labrax*) (initial body weight 13.4 (SD 0.2) g) were estimated in a 12-week dose–response experiment. Seven isonitrogenous (7.6% DM) and isoenergetic (gross energy, 21.2 MJ/kg DM) diets, based on soya protein and crystalline L-amino acids containing graded levels of L-Met (1.6–16.2 g/kg) at a constant cysteine (4 g/kg) level and a fish meal-based diet, were fed each to triplicate groups of fifty fish kept in 250 litre tanks in a thermoregulated ($23 \pm 0.5^\circ\text{C}$) seawater system. The Met and TSAA-deficient diet resulted in higher mortality, impaired feed intake and growth relative to the other treatments ($P < 0.01$). No signs of lens opacity due to limiting Met intake were observed and no feed intake or growth depression occurred at the highest level of dietary TSAA. Met and TSAA requirements for optimal N deposition or weight gain as fitted with the broken-line model resulted in estimated values of 8.0 and 12.0 g/kg diet (for example, 1.8 and 2.7% dietary protein) and 9.1 and 13.1 g/kg diet (for example, 2.0 and 3.0% dietary protein), respectively. Plasma levels of Met, homocysteine and cysteine increased in response to excess dietary TSAA, corroborating requirement estimates from growth data. N gain resulted in a linear function of TSAA consumption at marginal Met (TSAA) intake. The TSAA intake needed to maintain N balance resulted in a value of 20.0 mg TSAA/kg average body weight^{0.75} per d, which represents 23% of the total (maintenance + accretion) requirement.

European sea bass: Nutrition: Sulfur amino acids: Methionine requirement

Total sulfur amino acid (TSAA) nutrition of cultivated fish species currently deserves much attention from a practical standpoint due to a growing and extensive use of methionine (Met)-limiting plant protein sources such as soya and legume seed derivatives in aquafeeds.

Met is an indispensable amino acid (IAA) for normal growth of animals including fish⁽¹⁾ and plays a central role in protein synthesis and in transmethylation and trans-sulfuration to cysteine (Cys). Met is a precursor in the biosynthesis of important nutrients and bioactive compounds such as polyamine (spermine and spermidine), creatine, phosphatidylcholine, glutathione, taurine, coenzyme A and active sulfate⁽²⁾. Trans-sulfuration of Met to Cys has been shown to be 100% efficient on a sulfur basis⁽²⁾, hence the dietary requirement for TSAA in animals might be met by either Met alone or a combination of Met and Cys, where the latter amino acid has been shown to be able to spare approximately half of the dietary requirement for Met in fish^(1,3,4) as in poultry and mammals⁽²⁾.

Met deficiency has been shown for a long time to cause bilateral cataracts in salmonid species^(5,6) so that requirement values of Met (TSAA) have been set on maximum weight gain and absence of lens pathology in certain studies⁽¹⁾. In the last decade dietary allowances for Met or TSAA have been reported for several cultured fish species^(1,7–13). TSAA requirement estimates have been shown to vary within a quite broad

range of values (2.0–4.2% protein) either among or within fish species. As frequently questioned, at least part of the wide inter-intra specific disparity in dietary amino acid requirement estimates of fish could be due to various methodological approaches, experimental model design as well as to different diet composition, fish size, feeding levels and culture conditions adopted in single laboratory studies^(14–17). Moreover, as suggested by Twibell *et al.*⁽⁸⁾, Kasper *et al.*⁽¹⁸⁾ and recently reviewed in terrestrial animals⁽¹⁹⁾, even variable Met:Cys ratios and/or different levels of sulfur amino acid-related nutrients such as choline in test diets could explain some variability in Met or TSAA requirement values as assessed in previous experiments with fish.

In the case of European sea bass (*Dicentrarchus labrax*), a strictly carnivorous marine fish species of great economic interest in the Mediterranean area, Thebault *et al.*⁽²⁰⁾ provided a first estimate of the dietary Met requirement obtained in a dose–growth study which used just four levels of dietary Met in practical-like diets. The requirement value was found to be approximately 10 g/kg in a diet supplying 10 g Cys/kg, resulting in a TSAA allowance of approximately 4% protein. This latter value appears inconsistent with the corresponding values of 2.2 or 2.3% protein obtained by Tibaldi *et al.*⁽²¹⁾ and Kaushik⁽²²⁾ for the same fish species based on whole-body amino acid deposition⁽²³⁾ and the whole-body A/I ratio

Abbreviations: AMW, average metabolic weight; Cys, cysteine; Hcys, homocysteine; IAA, indispensable amino acids; Met, methionine; TSAA, total sulfur amino acids.

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(A/I = individual IAA/sum of IAA including Cys and tyrosine) relative to the lysine requirement, according to the ideal protein concept as outlined by Wilson⁽¹⁾, respectively. Discrepancies between amino acid requirement estimates from dose–response studies or from whole-body amino acid pattern and ratios to lysine requirement have frequently been noted^(17,24). There is growing evidence that such differences, in fish like in other animal models, may be primarily due to a different proportion or contribution of amino acids required for maintenance over the total dietary allowance^(25–28). Given the disparity of the currently available dietary allowance for Met or TSAA and the many factors that may affect amino acid requirement estimates, a dose–response study was undertaken to re-evaluate the Met and TSAA requirement of juvenile European sea bass at a constant level of dietary Cys, using seven levels of dietary Met in semi-purified diets not limiting in choline and where soyabean meal represented a major protein source. Besides growth and N gain, plasma concentrations of sulfur amino acids in response to varying levels of Met in the diet were also investigated.

A further aim of the present study was to estimate the TSAA requirements for maintenance over total absolute requirement of juvenile European sea bass based on the response in N retention to graded intake of TSAA.

Materials and methods

Experimental diets

Seven semi-purified diets were formulated to be grossly isonitrogenous (7.7% DM), isolipidic (15.7% DM), isoenergetic (19.5 MJ/kg DM) and to contain graded levels of L-Met. They all included an enzyme-treated soyabean meal (H310; Hamlet Protein AS, Horsens, Denmark), low in trypsin inhibitor activity (<1 mg/g) as a major Met-limiting protein source. A first preparation (diet 1) was designed to be deficient in Met and TSAA (L-Cys, 1 g/kg; L-Met, 2 g/kg, as provided by dried fish soluble concentrate and soyabean meal) but otherwise resembling the amino acid profile of sea bass muscle protein through supplementation with mixtures of crystalline indispensable and dispensable L-amino acids (Sigma-Aldrich Chemicals, Milan, Italy). A second diet (diet 2) was formulated to minimise the level of TSAA (L-Cys, 4.2 g/kg; L-Met, 5 g/kg) as supplied by dried fish soluble concentrate (80 g/kg), wheat gluten meal (100 g/kg), enzyme-treated soyabean meal (250 g/kg) and gelatine (80 g/kg) of known amino acid composition. It also contained crystalline IAA and dispensable amino acid (DAA) mixtures to simulate, except Met and Cys, the amino acid profile of sea bass muscle protein. Five further preparations (diets 3–7) were obtained from diet 2 by adding 2.5, 5.0, 7.5, 10.0 or 12.5 g pure L-Met/kg, with reductions in equal amounts of N from the DAA mixture to compensate. Crystalline amino acid mixtures were coated with carboxymethylcellulose and/or gelatine before being added and mixed to the other feed ingredients. In addition, a fish meal-based complete feed was prepared to be used as a positive control (control diet).

All diets were pelleted through a 2.2 mm die, dried overnight at 35°C in a fluid bed drier, then stored at 3°C until used.

The composition, the analysed proximate composition, gross energy, and the Met and TSAA contents of the experimental diets are shown in Table 1.

Fish and experimental conditions

European sea bass (*D. labrax*) juveniles, obtained from a commercial hatchery (Panittica Pugliese, Torre Canne, Brindisi, Italy), selected to be uniform in size and free from grossly discernible anatomical malformations, were randomly divided among twenty-four groups. Fish groups were stocked in 250-litre fibreglass tanks being part of an indoor partially recirculating marine water system (14 m³ total volume, 5% daily water volume renewal rate, 12 h artificial daylength, 400 lux light intensity), provided with thermostatic control and regulation of water temperature, mechanical sand filter, bio-filter and UV lamp apparatus.

Fish were fed the control diet and adapted over 3 weeks to the experimental conditions. At the end of this preliminary period, under- or oversized specimens were removed to further limit size variation within a group. The twenty-four fish groups were then assigned to the eight diets according to a random design with triplicate units per treatment, with a stocking density of fifty fish per tank (average individual weight, 13.4 (SD 0.2) g).

The feeding trial lasted 12 weeks and the fish were hand-fed two meals daily to visual satiety (i.e. until the first feed item was refused). The actual feed intake and mortality per group were recorded on a daily basis. Fish were group-weighed every 3 weeks, after a 24 h fasting and under moderate anaesthesia. Water quality was monitored twice per week throughout the experiment and all parameters resulted in nearly constant and optimal values for sea bass (temperature, 23.0 (SD 0.5)°C, salinity, 35 (SD 1)‰, dissolved O₂, 6.2 (SD 0.3) mg/l, pH, 7.7 (SD 0.2), total ammonia N, <0.12 mg/l, nitrite N, <0.06 mg/l).

Fish and blood sampling

At the beginning of the trial, fifteen fish were sampled at random from the tanks, killed using an overdose of anaesthetic (Finquel®; Argent Laboratories, Redmond, WA, USA), then pooled, minced, freeze-dried and ground to be analysed for initial whole-body N content.

At the end of the trial, all fish were individually checked by visual inspection to eventually detect grossly discernible signs of Met deficiency and five fish were sampled at random from each unit. They were pooled and treated as previously described for the initial sample to be analysed for final whole-body N content.

At the end of the growth trial, after a 2-week adaptation period to a single meal per d, individual blood samples were withdrawn from three fish per each of the groups fed diets 1 to 7, at 6 or 24 h after the meal, to determine plasma concentrations of TSAA.

Blood samples, taken from caudal vessels using heparinised syringes, were immediately centrifuged (3000 g for 10 min at 4°C), deproteinised by ultrafiltration (9000 g for 10 min at 4°C; Waters-Millipore PLGC Membrane; Millipore, Billerica, MA, USA) and stored at –20°C until analysed.

Handling of the fish was done according to the European Union Directive (86/609EC) for the protection of animals

Table 1. Composition, proximate analysis, gross energy content and methionine (Met) and total sulfur amino acid (TSAA) levels of the experimental diets

	Diet							Control
	1	2	3	4	5	6	7	
Ingredients (g/kg)								
Danish fish meal	–	–	–	–	–	–	–	450.0
Fish protein concentrate*	80.0	80.0	80.0	80.0	80.0	80.0	80.0	100
Gelatine*	0.0	80.0	80.0	80.0	80.0	80.0	80.0	0.0
Wheat gluten*	0.0	100.0	100.0	100.0	100.0	100.0	100.0	50
Enzyme-treated soyabean*	80.0	250.0	250.0	250.0	250.0	250.0	250.0	0
Gelatinised wheat starch*	202.5	182.5	182.0	181.5	181.0	180.5	180.0	265.5
L-IAA mix†	144.0	53.0	53.0	53.0	53.0	53.0	53.0	0
L-DAA mix‡	290.0	79.0	77.0	75.0	73.0	71.0	69.0	0
L-Met	0.0	0.0	2.5	5.0	7.5	10.0	12.5	0
Cod liver oil*	140.0	126.0	126.0	126.0	126.0	126.0	126.0	85
Mineral mix§	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin mix	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Choline HCl	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Carboxymethylcellulose*	26.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Soya lecithin*	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Chemical analysis (%)								
Moisture	5.6	9.5	7.8	9.9	9.7	9.4	7.5	6.2
N	7.1	7.1	7.1	7.0	7.0	7.0	7.2	6.9
Crude lipid	14.8	14.1	14.6	14.1	14.2	14.1	14.5	14.5
Ash	2.9	3.9	3.9	3.8	3.8	3.8	3.9	9.0
Fibre	0.5	1.1	1.1	0.7	1.1	1.1	0.8	0.4
Gross energy (kJ/g)								
Met (g/kg)	1.6	4.9	7.3	10.6	12.0	14.4	16.2	12.0
TSAA¶ (g/kg)	2.6	8.9	11.3	14.6	16.0	18.4	20.2	16.2

IAA, indispensable amino acids; DAA, dispensable amino acids.

* Description of the ingredients: fish protein concentrate, CPSP 90, from Sopropêche (Boulogne-sur-Mer, France); gelatine from Sigma (Milan, Italy); wheat gluten and gelatinised wheat starch from Roquette Italia S.P.A. (Cassano Spinola, Italy); enzyme-treated soyabean, H310 from Hamlet Protein AS (Horsens, Denmark); cod liver oil, carboxymethylcellulose and soya lecithin from Laboratorio Dottori Piccioni S.r.l. (Gessate, Italy).

† Composition of the essential amino acid (Sigma Chemicals, Milan, Italy) mix (%): histidine, 5.8; lysine, 15.5; leucine, 30.7; isoleucine, 7.16; valine, 8.1; phenylalanine, 8.7; tyrosine, 10.7; threonine, 6.6; tryptophan, 6.7.

‡ Composition of the dispensable amino acid (Sigma Chemicals, Milan, Italy) mix (%): alanine, 13.7; aspartic acid, 24.3; glutamic acid, 35.2; glycine, 9.7; proline, 7.6; serine, 9.5.

§ Composition of the mineral mix (g/kg diet): MgO, 3.00; CaHPO₄, 1.00; KH₂PO₄, 5.00; NaCl, 0.59; FeCO₃, 0.10; KI, 0.15; ZnO, 0.05; MnO, 0.100; CuSO₄, 0.0; sodium selenite, 0.0004.

|| Composition of the vitamin mix according to National Research Council⁽⁶⁴⁾ (mg/kg diet): vitamin A (250 000 U/g), 2.5; vitamin D₃ (250 000 U/g), 4.8; ascorbate monophosphate (Stay-C[®]; Roche Vitamins, Inc. (Parsippany, NJ, USA) (35%), 100; tocopheryl acetate, 123; menadione, 10; thiamin HCl, 12; riboflavin, 12; pyridoxine HCl, 12; calcium pantothenate 40; vitamin B₁₂, 0.01; niacin, 15; biotin, 1.0; folic acid, 2; inositol, 430.

¶ (Met + cysteine).

used for experiments and other scientific purposes following the guidelines of the Commission Recommendation 2007/526/EC for fish.

Analytical methods

The proximate composition, the gross energy content (adiabatic calorimeter, IKA C7000; IKA Werker GmbH, Staufen, Germany) of the diets and the Kjeldhal-nitrogen content of pooled whole-body fish samples were determined according to AOAC⁽²⁹⁾. To verify the correspondence with the expected levels of TSAA, the diets were analysed by HPLC after derivatisation with AccQ-Tag (Waters Italia S.p.A., Milan, Italy) according to Cohen & De Antonis⁽³⁰⁾ using α -aminobutyric acid (2.5 mmol/l) as internal standard.

From each plasma sample, an aliquot (0.1 ml) was used to determine the free Met and amino acid levels by HPLC according to Waters Italia S.p.A., using α -aminobutyric acid (2.5 mmol/l) as internal standard. A further plasma sample

(0.2 ml), added with norleucine as internal standard was used to determine homocysteine (Hcys), Cys and glutathione levels according to Ubbink *et al.*⁽³¹⁾.

Statistical analysis

All the zootechnical responses to dietary treatments were analysed by one-way ANOVA. Plasma amino acid concentrations were subjected to a two-way ANOVA including the sampling time and dietary treatment as main factors. If appropriate, means were compared by Duncan's multiple-range test. Statistical comparison among means was carried out at the 5% significance level⁽³²⁾.

Different regression models were preliminary tested in fitting the relationship between weight gain or N gain and Met (TSAA) dietary level or intake (i.e. broken-line⁽³³⁾, exponential⁽²⁵⁾, four-parameter logistics⁽³⁴⁾ or saturation kinetic models⁽³⁵⁾) but none of the non-linear ones outperformed the broken-line model for the goodness of fit after being analysed

in terms of root mean square error (RMSE) and probability plots of standardised residuals⁽¹⁶⁾. Hence, Met (TSAA) requirement values were estimated using the broken-line model as outlined below:

$$y = L + U(R - x),$$

where y is the requirement value corresponding to the break-point x value, U is the slope of the first segment, L is the asymptote of the first segment, $R - x$ is defined as zero when $x > R$, Met (TSAA) requirement for maintenance = $-L/U$, and Met (TSAA) requirement for 1 g N deposition = $1/U$.

All data were processed by SPSS/PC for Windows release 16.0.0 (SPSS Inc., Chicago, IL, USA).

Results

Feed, N, TSAA intakes and mortality of juvenile sea bass as affected by dietary treatments are shown in Table 2. Although the test diets were readily accepted since the first administration, after 3 weeks from the beginning of the trial feed consumption started to be progressively reduced in fish groups fed the Met-deficient diet, which resulted in negligible feed intake during the last week of the trial. As a consequence, diet 1 resulted in impaired feed and N intakes relative to the other semi-purified diets supplying increasing levels of Met (TSAA) (12.8 v. 16.2 mg/kg average body weight per d; $P < 0.001$) which did not differ from each other or from the control diet, except in the case of diet 7 where a slightly higher N intake was noted ($P < 0.001$). As a result, the calculated Met (TSAA) intake in fish fed graded levels of Met increased in a stepwise fashion ($P < 0.001$).

In addition to reduced feed intake, fish given the TSAA-deficient preparation suffered severe mortality from week 8 onwards. Some minor losses occurred also in fish groups fed diet 2, while mortality was absent or negligible in those fish subjected to the other dietary treatments. At visual inspection, no grossly discernible evidence of lens opacity was detected in fish fed diets varying in Met (TSAA) content.

The growth performance, feed conversion ratio and gross N retention efficiency in fish fed the test diets are shown in Table 3. At the end of the experiment, sea bass fed diet 1 resulted in significantly lower weight gain (30.9%), severely impaired feed conversion rate and negligible N retention, when compared with the other dietary treatments

($P < 0.001$). As shown in Table 3, specific growth rate and gross N retention improved in response to graded levels of Met (TSAA) in the diet without any further significant change over a dietary Met and TSAA content of 10.6 and 14.6 g/kg, respectively (diet 4). All diets supplying more than this Met (TSAA) level resulted in a growth rate and N retention efficiency which did not differ from those attained by fish given the control preparation.

The concentrations of Met, Hcys and Cys measured in plasma of fish fed graded levels of Met (TSAA) 6 and 24 h after a single meal are shown in Table 4. No significant interaction between dietary treatment and sampling time was found after subjecting the data to a two-way ANOVA, so plasma amino acid levels are presented according to main factors. As expected and regardless of the dietary treatment, the 6 h post-feeding plasma Met concentration was found to be substantially higher than the levels measured under post-absorptive conditions ($P < 0.001$). On the other hand, blood sampling time little affected plasma levels of Hcys ($P = 0.337$) and Cys ($P = 0.456$). Irrespective of the sampling time, plasma TSAA levels increased in response to graded levels in the diet, with a sharp increase in fish fed excessive Met as indicated by growth parameters ($P < 0.001$). A similar trend towards increasing plasma levels was also observed for Hcys and Cys.

The dietary Met (TSAA) requirement was estimated by fitting the broken-line model to the dose-response relationship using either body-weight gain or N accretion as the dependent variable (for parameter estimates, see Table 5). Break points indicated estimates of dietary Met relative requirement values to be 9.1 (SE 0.03) g/kg diet (2.0% dietary protein) and 8.0 (SE 0.01) g/kg diet (1.8% dietary protein) using weight gain and N deposition, respectively (Fig. 1). Given an approximately constant level of Cys in diets 2-7 (4.0 g/kg), the corresponding TSAA requirement values were calculated to be 13.1 g/kg (3.0% dietary protein) and 12.0 g/kg (2.7% dietary protein) when weight gain and body N accretion were used as the response criterion, respectively.

When the relationship between N gain and TSAA intake (mg/kg average metabolic weight (AMW) per d) was fitted by the broken-line model, the break point indicated that 87.4 (SE 0.02) mg TSAA/kg AMW per d were required to optimise fish response (Fig. 2).

Fitting the linear change in N gain at marginal TSAA intake ($y = 1.92x - 38.47$; $n = 9$) to $y = 0$, resulted in a daily intake of

Table 2. Feed, nitrogen, methionine (Met) and total sulfur amino acid (TSAA) intakes and mortality in sea bass (*Dicentrarchus labrax*) fed the test diets over 84 d

(Mean values and mean square errors)

	Diet							Control	MSE
	1	2	3	4	5	6	7		
Met (g/kg diet)	1.6	4.9	7.3	10.6	12.0	14.0	16.2		
TSAA (g/kg diet)	2.6	8.9	11.3	14.6	16.0	18.4	20.2		
Feed intake (g/kg ABW per d)	12.8 ^b	16.0 ^a	16.2 ^a	16.1 ^a	16.4 ^a	16.4 ^a	16.8 ^a	16.3 ^a	0.96
N intake (mg/kg ABW per d)	907 ^c	1141 ^b	1132 ^b	1127 ^b	1151 ^b	1153 ^b	1205 ^a	1120 ^b	26.6
Met intake (mg/kg ABW per d)	26 ^g	80 ^f	121 ^e	161 ^d	205 ^c	245 ^b	293 ^a	195 ^c	4.78
TSAA intake (mg/kg ABW per d)	40 ^g	147 ^f	189 ^e	229 ^d	274 ^c	314 ^b	364 ^a	264 ^c	6.20
Mortality (%) [*]	68.0 ^c	4.0 ^b	0.0 ^a	0.7 ^a	0.7 ^a	0.7 ^a	0.0 ^a	0.0 ^a	4.45

ABW, average body weight = (initial biomass + final biomass)/2.

^{a-g} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

^{*} ANOVA on arcsin-transformed data.

Table 3. Growth performance, feed conversion ratio (FCR) and gross nitrogen retention (GNR) efficiency in sea bass (*Dicentrarchus labrax*) fed the test diets over 84 d (Mean values and mean square errors)

	Diet							Control	MSE
	1	2	3	4	5	6	7		
Met (g/kg diet)	1.6	4.9	7.3	10.6	12.0	14.0	16.2		
TSAA (g/kg diet)	2.6	8.9	11.3	14.6	16.0	18.4	20.2		
Initial weight (g)	13.5	13.5	13.2	13.4	13.4	13.4	13.1	13.5	0.18
Final weight (g)	17.6 ^e	26.9 ^d	34.7 ^c	40.2 ^b	40.6 ^b	40.6 ^b	42.7 ^{a,b}	43.9 ^a	1.60
SGR*	0.32 ^d	0.82 ^c	1.15 ^b	1.31 ^{a,b}	1.32 ^a	1.32 ^a	1.40 ^a	1.40 ^a	0.059
FCR†	9.62 ^c	2.11 ^b	1.55 ^{a,b}	1.39 ^{a,b}	1.40 ^{a,b}	1.39 ^{a,b}	1.35 ^{a,b}	1.33 ^a	0.154
GNR‡	0.04 ^d	15.69 ^c	23.71 ^b	27.52 ^a	26.68 ^a	26.64 ^a	26.44 ^a	27.36 ^a	0.884

Met, methionine; TSAA, total sulfur amino acids; SGR, specific growth rate.

^{a–e} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

*SGR = $100 \times (\ln \text{ final body weight} - \ln \text{ initial body weight})/d$.

†FCR = feed intake per group/weight gain per group.

‡GNR = $100 \times (\text{whole-body N gain per group}/\text{N intake per group})$.

20 mg TSAA/kg AMW per d corresponding to the amount needed to maintain N balance. The inverse value of the slope of the linear fit at marginal TSAA intake indicated 520 mg as the estimated TSAA intake required for 1 g N deposition in juvenile sea bass.

Discussion

Depressed feed intake, growth retardation and the development of nutritional pathologies are known to occur in response to diets containing disproportionate levels of Met (TSAA) or certain IAA in several animal models⁽³⁶⁾, including fish^(1,17,37). In the present study, juvenile sea bass exhibited a progressive anorectic behaviour as a primary response to a diet severely deficient in Met (TSAA) resulting in negligible growth and high mortality at the end of the trial. Loss of appetite or reduced feed intake in fish fed purified or semi-purified diets deficient or limiting in Met (TSAA) have been reported in other dose–response studies carried out with the same⁽²⁰⁾ or other fish species^(7,10,37–39). This was sometimes associated with some mortality, particularly in experiments that, like the present one, had quite a long duration (more than 8 weeks), used juvenile stages and when fish were fed diets containing less than 65% of the minimal Met (TSAA) level ensuring optimal growth (i.e. grouper⁽¹⁰⁾, Japanese flounder⁽⁷⁾, yellowtail⁽⁴⁰⁾, rainbow trout^(37,39,41–43), red drum⁽⁴⁴⁾, striped bass⁽⁴⁵⁾).

In fish, the development of bilateral cataracts is a pathological sign often associated with a dietary deficiency of Met or TSAA, as they appear among the most susceptible amino acids to be modified after oxidation of proteins and glutathione in the lens; the latter protects lens proteins and cell membranes from oxidative damage by detoxification of H_2O_2 and other organoperoxides⁽⁴⁶⁾. Lens opacity induced by a dietary deficiency of TSAA has been observed in different fish species such as rainbow trout, Arctic charr, striped bass and Japanese sea bream^(5,6,37,45,47) but not in all the fish species studied to date. At the end of the present trial sea bass fed the TSAA-deficient diet (diet 1) did not show, at gross visual inspection, any lens opacity and their plasma glutathione levels were not significantly reduced 6 or 24 h after a meal (data not shown). Likewise, Thebault *et al.*⁽²⁰⁾ and Hidalgo *et al.*⁽³⁾ in their studies on sulfur amino acid nutrition of sea bass did not observe lens alteration in fish fed diets deficient or limiting in TSAA. So European sea bass similarly to other fish species studied to date appears less susceptible to this pathology related to Met dietary deficiencies.

By virtue of their particular structural or metabolic features, excessive intake of sulfur amino acids results in impaired growth and toxicity in various animal models^(36,48), with Met being recognised as the most growth-depressing amino acid when fed in huge excess to farm livestock⁽²⁾. Data on piscine literature appear contradictory in this respect. A marked

Table 4. Effects of test diets and sampling time on the sea bass (*Dicentrarchus labrax*) plasma levels of methionine (Met), homocysteine (Hcys) and cysteine (Cys) 6 and 24 h after a single meal (Mean values and mean square errors)

	Sampling time (h after meal)		Diet							MSE
	6	24	1	2	3	4	5	6	7	
Met (g/kg diet)			1.6	4.9	7.3	10.6	12.0	14.0	16.2	
TSAA (g/kg diet)			2.6	8.9	11.3	14.6	16.0	18.4	20.2	
Plasma amino acid ($\mu\text{mol/l}$)										
Met	27.8 ^a	7.5 ^b	1.3 ^e	5.7 ^{d,e}	13.5 ^{c,d}	15.8 ^c	15.9 ^c	25.6 ^b	45.9 ^a	33.06
Hcys	4.8	5.1	1.8 ^e	2.9 ^{d,e}	4.1 ^{c,d}	5.1 ^{b,c}	5.8 ^{b,c}	6.7 ^b	8.8 ^a	7.02
Cys	29.5	32.1	13.5 ^d	16.1 ^d	20.3 ^d	33.8 ^c	33.8 ^c	43.9 ^a	53.8 ^a	92.39

TSAA, total sulfur amino acids.

^{a–e} Mean values within a row, for sampling time or diet, with unlike superscript letters were significantly different ($P < 0.05$).

Table 5. Parameters estimated by fitting the broken-line model to the experimental data using weight gain (percentage initial body weight) and nitrogen gain (mg/kg average body weight per d) as dependent variables against dietary levels of methionine

	L	R	U	M	RMSE
Weight gain	205.7	9.1	27.04	1.49	0.13
N gain	309.0	8.0	49.95	1.81	0.10

L, plateau value; R, requirement; U, slope; M, $x -$ intercept; RMSE, root mean square error.

decline in growth and feed conversion efficiency due to a slight excess of Met or TSAA relative to the requirement level has been observed either in certain carnivorous fish species (yellow croaker, cobia, European sea bass, rainbow trout)^(11,12,20,49) and in omnivorous or herbivorous ones (Indian major carps, tilapia, catla, milkfish)^(50–53). To what extent growth depression observed in the above-mentioned studies could be simply a consequence of reduced feed palatability or consumption, as suggested by Griffin *et al.*⁽⁵⁴⁾, seems questionable, since data on actual feed intake had not always been reported. On the other hand, the results of the present trial revealed no adverse effect on feed consumption or growth impairment in European sea bass juveniles fed diets supplying up to 60% excess Met and even larger surpluses of dietary Met or TSAA did not result in impaired growth performance in other fish species or studies. Hence, there is no apparent distinction in food habits, growth stage, size or environmental needs which may give a ready explanation for the inconsistent response to excessive Met or TSAA intake among or within fish species. This warrants the need for comparative studies to ascertain if variable response to excess dietary Met or TSAA reflects real species differences or are mostly due to changes in basal diet composition (major ingredients, source and bioavailability of sulfur amino acids, overall amino acid profile, Met:Cys ratio).

Consistent with most studies aimed at estimating amino acid requirements of fish, even in the present experiment weight gain was the primary response criterion to evaluate the Met (TSAA) requirement of European sea bass based on the dose–response relationship. Nearly optimal growth performance is a prerequisite to avoid biased estimates of the requirement to be made and this condition had not always been met in certain amino acid studies with fish, particularly when test diets containing large amounts of crystalline amino acids were used without precautions⁽¹⁷⁾. Under these circumstances growth rates could be lower than those ensured by diets where dietary protein is supplied as all-bound amino acids, possibly reflecting impaired protein synthesis and deposition or increased amino acid catabolism due to a much faster absorption of pure amino acids relative to protein-bound ones⁽¹⁵⁾. Increasing feeding frequency or protecting dietary pure amino acid to retard their absorption has been shown to be effective in minimising growth-depressive effects^(26,55–57). Even in the present study, coating crystalline amino acid mixtures with gelatine or carboxymethylcellulose in diets not limiting in Met (TSAA) resulted in growth and N retention efficiency which were similar to those of fish fed an intact-protein control diet, thus leading to an unbiased estimate of the requirement to be made.

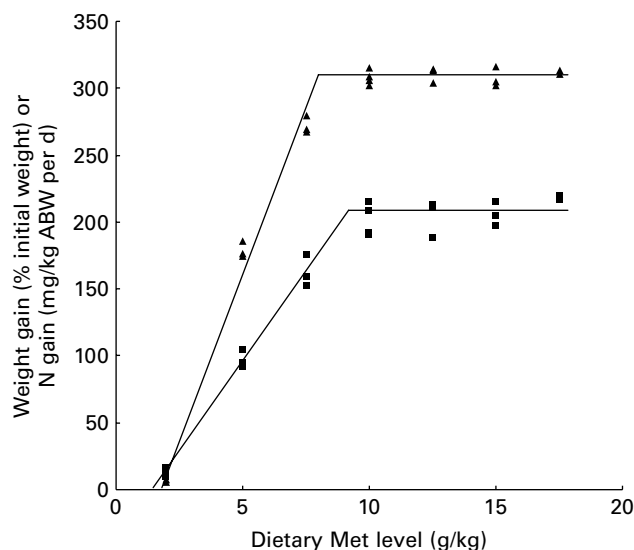


Fig. 1. Weight gain (percentage initial weight; ■) and N gain (mg/kg average body weight (ABW) per d; ▲) of juvenile sea bass (*Dicentrarchus labrax*) fed graded levels of dietary methionine (Met) as fitted by the broken-line model.

In the present study, the dose–response Met (TSAA) requirement was also estimated using N gain as a more conservative measure of growth, protein deposition being the main determinant of amino acid requirements⁽⁵⁸⁾. This resulted in a slightly lower Met requirement estimate than that obtained using weight gain as the dependent variable (8.0 (SE 0.01) v. 9.1 (SE 0.03) g/kg; 1.8 v. 2.1% dietary protein), whereas no such differences were observed in previous studies on Met requirements of carp⁽⁵⁹⁾, threonine requirements in salmonids fry⁽⁶⁰⁾, lysine in European sea bass⁽⁵⁶⁾, while even higher values were estimated from N gain than weight gain in the case of Met, lysine and histidine requirements of rainbow trout^(25,58,61). Discrepancies between weight and N gain as response criteria in amino acid

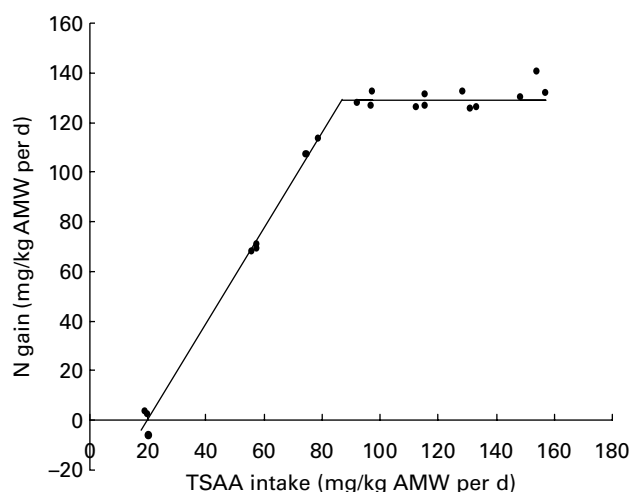


Fig. 2. Relationship between N gain and total sulfur amino acid (TSAA) intake in juvenile sea bass (*Dicentrarchus labrax*) as fitted by the broken-line method ($y = 129.21 + 1.92 \times (87.4 - x)$ root mean square error (RMSE) = 0.08). AMW, average metabolic weight ((initial body weight^{0.75} + final body weight^{0.75})/2).

requirement experiments have frequently been ascribed to changes in whole body composition, particularly protein content, due to varying levels of the test amino acid in the diet⁽⁶²⁾. This, however, barely applies to the present study where, except in fish fed the diet lowest in Met, N content of the whole body was little affected by dietary Met (TSAA) level (data not shown). Hence there is no ready explanation for such a discrepancy, suggesting a further role for Met (TSAA) metabolism in fish beyond its role in protein deposition.

Besides growth and protein accretion, plasma or serum Met levels in response to graded TSAA levels in the diet have proven useful to reinforce requirement estimates in previous studies with fish^(54,63). In the present research, irrespective of the blood sampling time, plasma concentrations of Met, as well as Hcys and Cys, were in response to graded dietary sulfur amino acid levels, making it difficult to meaningfully determine a requirement estimate. However, a sharp increase in plasma Cys concentration in sea bass fed dietary TSAA levels beyond 7.3 g/kg (diet 3) was consistent with the estimated requirement based on N gain data.

According to the most widely used mode of expressing IAA requirements in piscine literature (i.e. g/kg diet or percentage dietary protein), the TSAA requirement value of the European sea bass juveniles estimated in the present study (12.0–13.1 g/kg, 2.7–3.0 % protein, in the presence of 4 g Cys/kg, depending on the response criterion adopted) was lower than the value estimated for the same species by Thebault *et al.*⁽²⁰⁾ (20 g/kg, 4 % protein, in the presence of 10 g Cys/kg) with practical-like diets containing 50 % crude protein. Our estimates fall in the lower range of TSAA requirement values reported for other fish species: channel catfish (5.6 g/kg; 2.3 % protein, in the absence of Cys)⁽⁶³⁾; rainbow trout (5.2–8.0 g/kg; 2.0–2.3 % protein, in diets varying from 3 to 6 g Cys/kg)^(25,39); Nile tilapia (9.0 g/kg; 3.2 % of dietary protein, in the presence of 1.5 g Cys/kg)⁽¹³⁾; hybrid striped bass (10.0 g/kg; 3.03 % of dietary protein, in the presence of 1.3 g Cys/kg)⁽⁴⁵⁾; yellow perch (10.0 g/kg; 3.0 % dietary protein, in the presence of 1.3 g Cys/kg)⁽⁸⁾; red drum (10.6 g/kg; 3.03 % of dietary protein, in the presence of 1.2 g Cys/kg)⁽⁴⁴⁾; carp (12.7 g/kg; 3.2 % of dietary protein, in the presence of 4.2 g Cys/kg)⁽⁵⁹⁾; Japanese eel (12.0 g/kg; 3.2 % of dietary protein, in the absence of Cys)⁽⁶⁴⁾; Indian major carp (12.9 g/kg; 3.2 % of dietary protein, in the presence of 10 g Cys/kg)⁽⁹⁾; gilthead seabream (14 g/kg; 4.0 % of dietary protein)⁽⁶⁵⁾; yellowtail (14.2 g/kg; 3.3 % of dietary protein, in the presence of 3.1 g Cys/kg)⁽⁴⁰⁾; Japanese flounder (15.5 g/kg; 3.1 % of dietary protein, in the presence of 0.6 g Cys/kg)⁽⁷⁾; juvenile grouper (15.7 g/kg; 3.3 % of dietary protein, in the presence of 2.6 g Cys/kg)⁽¹⁰⁾; chinook salmon (16 g/kg; 4.0 % of dietary protein, in the presence of 1.0 g Cys/kg)⁽⁶⁶⁾; Arctic charr (17.0 g/kg; 4.2 % of dietary protein, in the presence of 10 g Cys/kg)⁽⁶⁾. From the list above, food habits or environmental needs of the different fish species studied to date do not seem able to explain such a wide spread in their Met or TSAA requirements. It should be also noted that, although reduced, major disparities still exist even when requirements are expressed as percentage dietary protein. As frequently questioned, this disparity could reflect a variety of experimental conditions, diet composition, response criteria and methodologies adopted in separate

trials and laboratories^(15,16,39,62). In particular, with reference to diet composition, there is evidence in fish that some of the among-study variability in TSAA requirement values could be ascribed to variable levels of Cys and sulfur amino acid-related nutrients such as choline in test diets^(8,18,24). Recent studies also suggest possible underestimation of IAA requirements, expressed as percentage dietary protein, when test diets are formulated to contain excess protein and their IAA profile, excluding the test amino acid, is not ideal or nearly so^(62,67,68). Hence, besides different experimental conditions and diet composition, even different Cys and protein levels in the test diets could explain the disparity between TSAA requirements of European sea bass estimated in the present study and those reported by Thebault *et al.*⁽²⁰⁾.

Partitioning the total IAA requirement into the two components, maintenance and growth, is a recent challenge in fish nutrition^(26,58,69–71) but it is of great interest since there is growing evidence that in fish, like in other animal models, maintenance requirement over total dietary needs for a given IAA can be different depending on the specific amino acid as well as fish species^(17,26,58). As the absolute intake of the limiting amino acid is a function of both actual feed consumption and its dietary concentration⁽⁷²⁾, the relationship between absolute TSAA intake and N gain was considered here to estimate TSAA requirements for maintenance and for 1 g N gain in sea bass. Similarly to other studies with other IAA^(69,70,73) in salmonid species, also in the present experiment N deposition was linearly related to TSAA consumption at marginal Met intakes. This constant efficiency of utilisation of dietary TSAA allowed us to estimate that the TSAA consumption required to obtain 1 g N deposition was 520 mg TSAA. The maintenance TSAA requirement value calculated in the present study (20 mg/kg AMW per d) under culture and dietary conditions ensuring nearly optimal growth for the European sea bass is similar to the value already estimated for rainbow trout (16 mg/kg average body weight per d)⁽⁵⁸⁾ or the rat (21 mg/kg AMW per d)⁽³⁴⁾, but somewhat lower than the fivefold value reported by Mambrini & Kaushik⁽⁷¹⁾ for rainbow trout, Atlantic salmon and European catfish. Unfortunately, very few data are actually available for the Met and TSAA maintenance requirements in fish species and this variation can only partially be explained by differences in experimental conditions or mathematical models adopted to estimate requirements. Anyhow, differences among species in the utilisation of a specific amino acid cannot be excluded as it was the case of arginine maintenance requirement reported for sea bass, sea bream, turbot and rainbow trout⁽²⁶⁾.

The TSAA requirement for maintenance estimated in the present trial represents 23 % of the total requirement for TSAA (maintenance + accretion), which is similar to the relative scope for maintenance observed in mammals as well as in the fish species studied by Mambrini & Kaushik⁽⁷¹⁾. Recent studies aimed at evaluating the maintenance requirement of individual amino acids in fish species, as well as the proportion of ingested amino acids used to cover the maintenance requirement, suggest marked differences among individual amino acids⁽⁵⁸⁾, making previous assumptions on a negligible proportion of maintenance requirement over the total amino acid requirement in fish questionable⁽²³⁾.

The relatively high intake and proportion of TSAA required for maintenance in European sea bass as in other animals could possibly reflect their complex metabolism and multiple roles beyond protein synthesis⁽²⁾. This could also explain the differences in the TSAA requirement value obtained in the present study and previous estimates based on alternative approaches such as whole-body amino acid deposition or pattern relative to lysine requirement^(21,22) which assume that IAA are utilised with similar efficiency and that maintenance requirements for all IAA are similar.

In conclusion, based on N and weight gain, the requirement of sea bass juveniles for Met was estimated to be 8.0 and 9.1 g/kg diet (1.8–2.0% of dietary protein) in the presence of 4 g dietary Cys/kg in a diet supplying 45% protein. The corresponding TSAA requirement resulted to be 12.0–13.1 g/kg diet (2.7–3.0% of dietary protein). This estimate was corroborated by the response of plasma free sulfur amino acid levels to increasing dietary Met (TSAA). Besides, the response of N gain to increasing TSAA intake allowed us to estimate a TSAA intake for maintenance of 20 mg TSAA/kg AMW per d, which represents 23% of the total (maintenance + accretion) requirement for the European sea bass juveniles.

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E. T. and F. T. developed the initial idea and designed the study; M. C. was responsible for the fish trial; M. M. carried out the analytical work and analysed the data. F. T. performed the statistical analysis and drafted the manuscript.

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