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Research Article

Amino Acid Metabolism in Gilthead Seabream Is Affected by the Dietary Protein to Energy Ratios

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The dietary protein to energy ratio (P/E) has proven to influence protein utilization and/or growth in several fish species. This study intended to unravel the bioavailability and metabolic fate of lysine and methionine in gilthead seabream (*Sparus aurata*) juveniles fed plant diets with different P/E ratios. Seabream juveniles were fed two isonitrogenous diets (45% crude protein) differing in crude lipids (20 and 14%): LowP/E (P/E ratio = 20.0 mg protein kJ^{-1}) and HighP/E (P/E ratio = 21.4 mg protein kJ^{-1}). After three weeks, fish (11.6 ± 4.3 g) were tube-fed the respective diet labelled with ^{14}C-protein (L-amino acid mixture), ^{14}C-lysine, or ^{14}C-methionine. Protein, lysine, and methionine utilization were determined based on the proportion of ^{14}C-amino acid evacuated, retained in the free or protein-bound fraction of liver and muscle, or catabolized. This study revealed that a decrease in P/E ratio resulted in lower amino acid evacuation ($p < 0.05$), contributing to a more efficient amino acid uptake. Results indicate that amino acids are retained as protein in the liver and not only temporarily available in the free pool. The amount of free amino acids retained in the muscle of LowP/E fed fish was significantly higher than in HighP/E fish ($p < 0.05$) due to a simultaneous higher retention of lysine and methionine, without affecting the overall protein retention. Methionine catabolism was significantly lower than lysine or protein independently of the P/E ratio ($p < 0.05$), reinforcing that this amino acid is preferentially spared for metabolic functions and not used as energy source. In contrast, increasing the dietary P/E ratio decreased lysine catabolism and increased its availability for growth. The bioavailability and metabolism of individual amino acids should be considered when optimizing P/E ratios in diets for gilthead seabream juveniles. Formulating diets with optimum P/E ratios will improve diet utilization and fish performance.

1. Introduction

Fish growth performance is still one of the main concerns of the Aquaculture industry. This goal is pursued without disregarding the reduction of nutrient outputs to the aquatic environment and by the inclusion of more sustainable feed ingredients [1].

Protein utilization is influenced by numerous factors, including digestibility of ingredients, dietary protein and lipid content, amino acid profile, and protein to energy ratio (P/E) [2]. The optimization of the P/E ratios has proven to influence protein utilization and/or growth performance in

several fish species. For instance, a decrease in dietary P/E ratio by increasing lipid, and consequently energy content, has been shown to have a protein-sparing effect in salmonids [3–5]. In addition, it has led to improved growth performance and/or protein efficiency in species such as gilthead seabream, *Sparus aurata* [6–8]; common dentex, *Dentex dentex* [9]; Nile tilapia, *Oreochromis niloticus* [10]; Israeli carp, *Cyprinus carpio* [11]; red-spotted grouper, *Epinephelus akaara* [12]; and yellow croaker, *Larimichthys polyactis* [13]. Japanese seabass (*Lateolabrax japonicus*) fed diets with three levels of protein, at three lipid levels, showed that the protein-sparing effect of lipids was more pronounced in fish

fed the diet with the lowest protein content [14]. In addition to improved growth, an increase in dietary lipid content was shown to reduce the proportion of dietary protein catabolized by blunt snout bream, *Megalobrama amblycephala* [15, 16]. In contrast, in white seabream (*Diplodus sargus*) fed diets varying in protein (15% and 28%) and lipids (12% and 16%), growth performance was unaffected by the dietary P/E ratios but rather by the dietary protein content [17]. Moreover, Senegalese sole (*Solea senegalensis*) growth improved with increasing dietary P/E ratio, independently of the protein level, suggesting that this species has a low tolerance to high dietary lipid content [18, 19]. Consequently, the optimization of diet formulations needs to consider not only dietary P/E ratios but also the specificities of the species.

The bioavailability and metabolism of individual dietary amino acids need to be considered when pursuing the maximization of fish growth through protein utilization. Amino acids must be available in tissues at an optimum ratio since imbalances will increase amino acid catabolism, augmenting nitrogen outputs to the environment [20–22]. Feeding fish with plant-based diets has proven its feasibility, even in carnivorous fish species such as the gilthead seabream, as long as selected crystalline amino acids are added to overcome any deficiency or imbalance in the amino acid profile [23–26]. Lysine and methionine are considered the two most limiting amino acids in plant-based diets, and their dietary supplementation has been shown to improve growth performance in several fish species [20, 27–29]. Lysine, which is a ketogenic amino acid, is an indicator of protein synthesis and is involved in the formation of collagen, improving fish muscle integrity and fillet texture [30, 31]. Moreover, it is involved in lipid metabolism, although the precise mechanisms are poorly understood [32]. Methionine is a glucogenic amino acid and in addition to protein synthesis is involved in a multitude of physiological and metabolic functions such as the regulation of the expression of genes related to myogenesis [33], DNA, and protein methylation, as well as cell proliferation and differentiation [34, 35].

The current work intends to unravel the bioavailability and metabolic fate of ketogenic (lysine) and glucogenic (methionine) amino acids in gilthead seabream juveniles fed plant-based diets with different P/E ratios. Metabolic flux assays using ^{14}C-labelled diets were used to estimate evacuation, retention, and catabolism of protein (amino acid mixture), lysine, and methionine.

2. Material and Methods

2.1. Diets. Two isonitrogenous diets (45% crude protein) were formulated differing in crude lipid content (20 and 14%), using fishmeal as well as plant meals and concentrates as protein sources (Table 1). Diets were designated LowP/E and HighP/E according to crude protein to gross energy (P/E) ratio: Diet LowP/E had a gross energy content of $22.3 \, \text{kJ g}^{-1}$ and presented a P/E ratio of 20.0 mg protein kJ^{-1}, while diet HighP/E contained $21.0 \, \text{kJ g}^{-1}$ of energy and had a P/E ratio of $21.4 \, \text{mg}$ protein kJ^{-1}. Diets were supplemented with selected crystalline indispensable amino acids and mono-calcium phosphate to avoid any indispensable

Table 1: Formulation and proximate composition of experimental diets.

Ingredients (%)	LowP/E	HighP/E
Fishmeal SP[a]	22.00	22.00
Fishmeal[b]	5.00	5.00
Soy protein concentrate[c]	6.40	6.00
Wheat gluten[d]	7.00	6.00
Corn gluten[e]	11.00	11.00
Soybean meal[f]	12.00	12.00
Rapeseed meal[g]	5.00	5.00
Sunflower meal[h]	4.00	5.00
Wheat meal[i]	2.90	8.90
Whole peas[j]	3.00	4.00
Fish oil[k]	10.60	6.50
Rapeseed oil[l]	6.60	4.10
Vitamin and mineral premix[m]	1.00	1.00
Vitamin E[n]	0.10	0.10
Choline chloride[o]	0.10	0.10
Betaine HCl[p]	0.50	0.50
Soy lecithin[q]	0.50	0.50
Guar gum[r]	0.50	0.50
Antioxidant powder[s]	0.20	0.20
Mono-calcium phosphate[t]	1.10	1.10
L-lysine[u]	0.30	0.30
L-threonine[v]	0.20	0.20
Proximate composition (% as fed)		
Dry matter	94.2	93.2
Ash	8.5	8.8
Crude protein	44.7	44.8
Crude lipids	20.3	13.8
Total phosphorus	1.2	1.2
Gross energy (kJ g^{-1})	22.3	21.0
P/E ratio (mg kJ[-1])∗	20.0	21.4

All values are reported as mean of duplicate analysis. ∗P/E, protein to energy ratio. [a]Super-Prime: 68% crude protein (CP), 8% crude fat (CF); Pesquera Diamante, Peru. [b]CONRESA 60: 65% CP, 10% CF; Conserveros Reunidos S. A., Spain. [c]Soycomil P: 63% CP, 8% CF; ADM, The Netherlands. [d]VITAL: 80% CP, 7.5% CF; Roquette Frères, France. [e]Corn gluten meal: 61% CP, 6% CF; COPAM, Portugal. [f]Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF; CARGILL, Spain. [g]Defatted rapeseed meal: 34% CP, 2% CF; Premix Lda., Portugal. [h]Solvent extracted dehulled sunflower meal: 43% CP, 3% CF; MAZZOLENI SPA, Italy. [i]Wheat meal: 10% CP, 1.2% CF; Casa Lanchinha, Portugal. [j]Yellow peas: 19.6% CP, 2.2% CF; Ribeiro e Sousa Lda., Portugal.[k] Sopropêche, France.[l] J.C. Coimbra Lda., Portugal. [m]PREMIX Lda., Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocoferol acetate 100 mg; sodium menadione bisulphate 25 mg; retinyl acetate 20 000 IU; DL-cholecalciferol 2 000 IU; thiamin 30 mg; riboflavin 30 mg; pyridoxine 20 mg; cyanocobalamine 0.1 mg; nicotinic acid 200 mg; folic acid 15 mg; ascorbic acid 1 000 mg; inositol 500 mg; biotin 3 mg; calcium panthotenate 100 mg; choline chloride 1 000 mg; and betaine 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate 0.65 mg; copper sulphate 9 mg; ferric sulphate 6 mg; potassium iodide 0.5 mg; manganese oxide 9.6 mg; sodium selenite 0.01 mg; zinc sulphate 7.5 mg; sodium chloride 400 mg; calcium carbonate 1.86 g; and excipient wheat middlings. [n]ROVIMIX E50, DSM Nutritional Products, Switzerland.[o] ORFFA, The Netherlands. [p]Beta-Key 95%, ORFFA, The Netherlands. [q]Lecico P700IPM, LECICO GmbH, Germany. [r]Guar gum, Seah International, France. [s]Paramega PX, KEMIN EUROPE NV, Belgium. [t]MCP: 22% P, 18% Ca; Fosfitalia, Italy. [u]L-Lysine HCl 99%; Ajinomoto Eurolysine SAS, France. [v]L-Threonine: 98%; EVONIK Nutrition & Care GmbH, Germany.

TABLE 2: Amino acid composition of experimental diets.

Amino acids (mg AA g⁻¹ as fed)	LowP/E	HighP/E
Arginine	45.1	42.7
Histidine	12.8	12.0
Lysine	43.0	41.1
Threonine	21.7	20.5
Isoleucine	24.1	23.2
Leucine	41.4	39.7
Valine	24.2	23.2
Methionine	13.0	12.2
Phenylalanine	27.7	26.7
Cystine	3.1	3.0
Tyrosine	23.1	22.0
Aspartic acid + asparagine	54.6	52.4
Glutamic acid + glutamine	104.5	100.0
Alanine	26.3	24.9
Glycine	29.0	28.0
Proline	32.2	30.6
Serine	26.4	25.2

All values are reported as mean of duplicate analysis.

amino acids or phosphorus imbalance. Formulation, proximate composition, and amino acid analysis of diets are presented in Tables 1 and 2.

Upon grinding with a hammer (model SH1, Hosokawa-Alpine, Germany) and mixing in a double-helix mixer, all diets (pellet size 2 mm) were manufactured using a pilot-scale twin-screw extruder (CLEXTRAL BC45; Clextral, France) at SPAROS Lda. (Olhão, Portugal). Upon extrusion at 105 °C to 110 °C, diets were dried in a vibrating fluid bed dryer (model DR100; TGC Extrusion, France). Following drying, pellets were allowed to cool at room temperature, and subsequently the oil fraction was added under vacuum coating in a Pegasus vacuum mixer (PG-10VCLAB; DINNISEN, the Netherlands). Throughout the duration of the trial, experimental diets were stored at room temperature, in a cool and aerated storage room.

2.2. Fish Husbandry.

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Animal protocols were performed under Group-C licenses by the Direção Geral de Alimentação e Veterinária, Portugal.

Gilthead seabream (Sparus aurata) juveniles were kept at the Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Throughout the conditioning feeding period, fish were maintained in 40 L cylinder-conical tanks in a recirculating aquaculture system (water temperature: 20.1 ± 1.2°C; salinity: 32.2 ± 1.4 psu; and initial fish density (45 fish per tank): 4.8 kg m⁻³). Fish were assigned one of the two experimental diets and fed by automatic feeders, six times per day at a feeding ration of 3% body weight day⁻¹ for three weeks.

2.3. Metabolic Flux Assays.

After three weeks of feeding the experimental diets, fish from each dietary treatment (LowP/E and HighP/E) were transferred to the nutrient flux laboratory (CCMAR, Portugal) after 24 h of fasting. To determine the effect of P/E ratios on the overall metabolism of amino acids, fish were tube-fed with the respective diet labelled with ^{14}C-protein ([U-^{14}C]-protein hydrolysate (L-amino acid mixture); 1.85 MBq, American Radiolabeled Chemicals Inc., USA). The effect of the P/E ratios on the metabolism of lysine and methionine was assessed by tube-feeding fish with the correspondent diet labelled with ^{14}C-Lys ([U-^{14}C]-L-lysine; 1.85 MBq, Perkin Elmer, USA) or ^{14}C-Met ([1-^{14}C]-L-methionine; 1.85 MBq, American Radiolabeled Chemicals Inc.). Six fish per diet and tracer were subjected to this procedure (6 fish × 2 diets × 3 tracers, $n = 36$ fish in total). The average weight of analyzed fish was 11.6 ± 4.3 g (mean ± standard deviation).

2.3.1. Diet Labelling with ^{14}C-Tracers.

Radio-labelled ^{14}C-protein, ^{14}C-Lys, and ^{14}C-Met were diluted in Ringer solution for marine fish, and 3 μl of the tracer was dispensed with a micropipette on individual pellets ("hot" pellets) of the experimental diets (LowP/E and HighP/E). A non-radio-labelled solution (3 μl) of blue food colorant was dispensed in a second set of pellets ("cold" pellets) used to monitor possible pellet regurgitation. All pellets were dried at 50°C for 1 h. Two "hot" and one "cold" pellet per fish and diet were loaded into a hollow plastic tube of 1.5 mm inner diameter and stored for subsequent tube-feeding. The number of pellets per fish corresponded to a single meal (0.5% of fish body weight), based on the daily feed intake. Prior to tube feeding, the amount of radioactivity (disintegrations per minute; DPM) was determined in ten individual "hot" pellets from each experimental diet labelled with each tracer. The pellets were fully dissolved by adding an appropriate volume of Solvable™ (Perkin Elmer) at 50°C for 24 h, before DPM determination in a TriCarb 2910TR low activity liquid scintillation analyzer (Perkin Elmer) after adding the scintillation cocktail (Ultima Gold XR, Perkin Elmer).

2.3.2. Tube-Feeding Trial.

The in vivo methodology of tube-feeding was previously published by Teodósio et al. [20]. This method was adapted from Costas [36], which was modified for juvenile fish from the procedure described by Rønnestad et al. [37], a modification of the original method published by Rust et al. [38]. Anesthetized fish (50 μL L⁻¹ 2-phenoxyethanol, Sigma-Aldrich, Spain) were transferred onto a dry plastic tray. Fish were tube-fed the "hot" and "cold" pellets, in approximately 10 sec, taking the necessary measures to guarantee that the fish esophagus was not injured. The tube-fed tracer (^{14}C-protein, ^{14}C-Lys, or ^{14}C-Met) presented no nutritional value. Instead, the metabolic fate of the tracer was considered to represent the fate of the tracer of the diets [39].

After tube feeding, each fish was placed in clean and aerated seawater to eliminate any residual anesthetic in skin and gills and monitored for eventual pellet regurgitation. Once recovered, the fish was transferred into individual
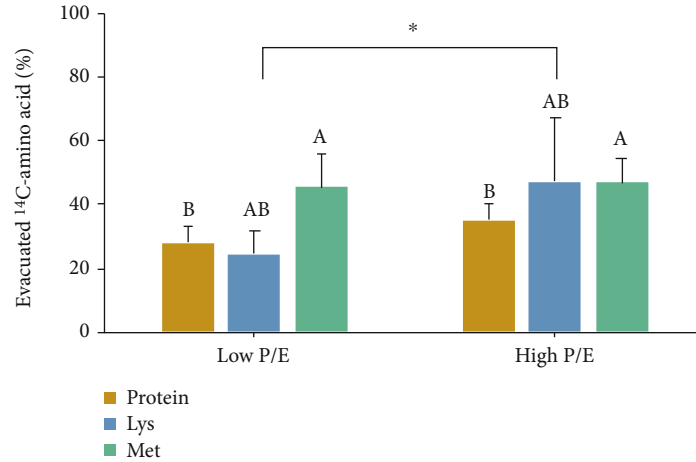
FIGURE 1: Proportion (%) of the total recovered ^{14}C-protein (protein), ^{14}C-lysine (Lys), and ^{14}C-methionine (Met) that were evacuated in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation ($n = 6$ fish for each diet and tracer). (A, B) Significant differences in the metabolic fate of the distinct amino acids at each dietary treatment; ∗denotes significant differences between dietary treatments ($p < 0.05$).

incubation chambers containing 2 L of seawater at 20 °C. Chambers were hermitically sealed and provided with a gentle oxygen flow. Each individual chamber was connected to a series of $^{14}CO_2$-metabolic traps, each containing 10 mL of 0.5 M KOH. After an incubation period of 18 h, the oxygen flow was stopped, and fish was euthanized inside the chamber by a lethal dose of anesthetic (1000 mg L^{-1} of MS-222 buffered with sodium bicarbonate). The incubation period was determined based on previous digestibility experiments with seabream juveniles fed at different temperatures (unpublished data). Gilthead seabream juveniles reared between 18 and 20 °C take approximately 10 h to fully digest one single meal. Therefore, a period of 18 h after ingestion ensures completion of the digestive process. After the removal of the fish for tissue sampling, chambers were resealed. Acidification of the incubation seawater was done gradually by adding 0.1 M HCl, leading to a decrease in pH and to the diffusion of any remaining $^{14}CO_2$ in the water to the $^{14}CO_2$-metabolic traps. The acidification procedure published by Rønnestad et al. [37] allows to distinguish between unabsorbed nutrients evacuated from the gut and molecules originating from catabolism of the absorbed nutrient, both of which are present in the incubation water.

2.3.3. Metabolic Budget Determination. After acidification of the incubation water, seawater samples ($n = 5$) from each individual chamber and individual samples from each CO_2-metabolic trap ($n = 3$) were collected for radioactivity determination. The amount of radioactivity present in the seawater resulted from evacuated (unabsorbed) ^{14}C-amino acids or from a negligible amount of other metabolites containing the ^{14}C-skeleton. The radioactivity present in the $^{14}CO_2$-metabolic traps resulted from ^{14}C-amino acid catabolism. To assess ^{14}C-amino acid retention, whole liver and whole left side skin-on fillet (muscle) from each fish were sampled. Tissues sampled in this trial represented approximately 40% of the total weight of fish. Amino acid retention in the liver and muscle of fish was evaluated in the free pool

and in protein-bound fractions (from hereafter designated as *Free* and *Bound*, respectively). To analyze the radioactivity present in the *Free* fractions, all tissues were incubated at 4 °C for 24 h with 6% (w/v) trichloroacetic acid (TCA) with periodical stirrings. After this period, tissues were transferred to a clean vial and the TCA samples were analyzed for radioactivity (DPM). Subsequently, all tissues were entirely dissolved by adding an appropriate volume of Solvable™ (Perkin Elmer) at 50 °C for 24 h. Liver was analyzed as whole, whereas two samples of dorsal muscle were used for DPM counting. Scintillation cocktail (Ultima Gold XR; Perkin Elmer) was added to all samples for radioactivity determination in a TriCarb 2910TR low activity liquid scintillation analyzer (Perkin Elmer). All samples were corrected for quench and lumex.

Amino acid metabolic fate in gilthead seabream juveniles as a function of the dietary P/E ratio was determined based on the percentage of ^{14}C-tracer evacuated, retained in *Free* or *Bound* fractions, or catabolized, as follows:

$$
\begin{aligned}
Evacuation\,(\%) &= (DPM_{SW}/DPM_{Total}) \times 100, \\[6pt]
Retention\,&in\,Liver\,Free\,fraction\,(\%) \\
&= (DPM_{Liver\,F}/DPM_{Total}) \times 100, \\[6pt]
Retention\,&in\,Liver\,Bound\,fraction\,(\%) \\
&= (DPM_{Liver\,B}/DPM_{Total}) \times 100, \\[6pt]
Retention\,&in\,Muscle\,Free\,fraction\,(\%) \\
&= (DPM_{Muscle\,F}/DPM_{Total}) \times 100, \\[6pt]
Retention\,&in\,Muscle\,Bound\,fraction\,(\%) \\
&= (DPM_{Muscle\,B}/DPM_{Total}) \times 100, \\[6pt]
Catabolism\,(\%) &= (DPM_{Traps}/DPM_{Total}) \times 100,
\end{aligned}
\tag{1}
$$

where DPM_{Total} is the sum of the radioactivity (DPM) determined in the incubation seawater (DPM_{SW}), *Free* and *Bound*
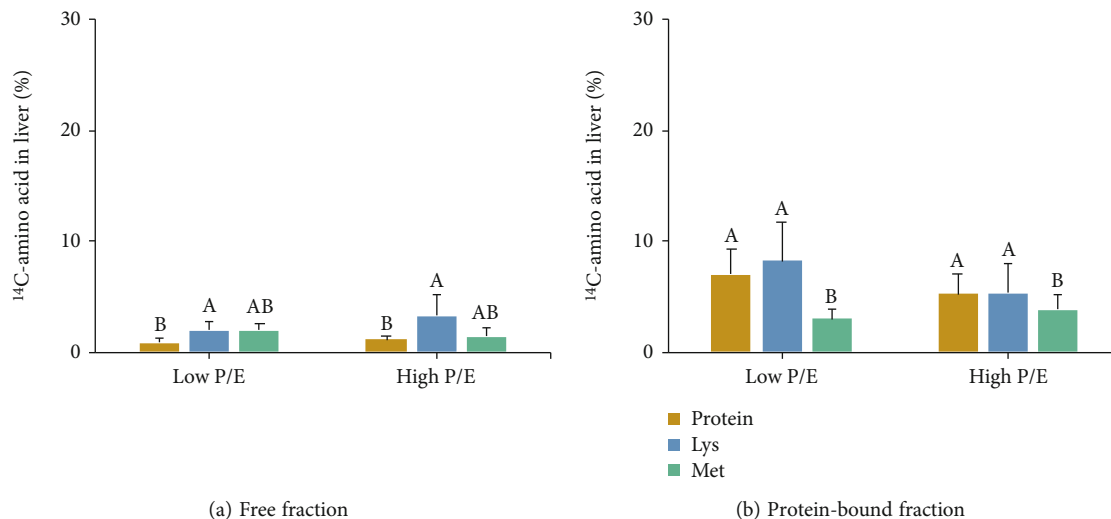
(a) Free fraction

(b) Protein-bound fraction

FIGURE 2: Proportion (%) of the total recovered ^{14}C-protein (protein), ^{14}C-lysine (Lys), and ^{14}C-methionine (Met) that were retained in the *Free* (a) and *Bound* (b) fractions of liver in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean $\pm$ standard deviation ($n = 6$ fish for each diet and tracer). Significant differences ($p < 0.05$) in the metabolic fate of the distinct amino acids at each dietary treatment are represented by (a, b).

liver ($DPM_{Liver\,F}$ and $DPM_{Liver\,B}$) and muscle ($DPM_{Muscle\,F}$ and $DPM_{Muscle\,B}$) fractions, and $^{14}CO_2$-metabolic traps (DPM_{Traps}).

2.4. Chemical Analysis. Dry matter, ash, crude protein (N x 6.25), crude lipid, gross energy and phosphorus contents in diets were analyzed following standard procedures of the Association of Official Analytical Chemists [40] and were run in duplicates. Diets total amino acid profile was determined by ultra-high-performance liquid chromatography (UPLC), after acid hydrolysis. All analyses were performed as described by Teodósio et al. [41].

2.5. Data Analysis. Data from the metabolic flux assays are presented as mean $\pm$ standard deviation. Data expressed as a percentage were arcsine square root transformed previously to the statistical analysis [42]. All data was checked for normal distribution and homogeneity of variances. The experimental design was randomized in a 2×3 factorial design with two levels of dietary P/E ratios (LowP/E = 20.0 mg protein kJ^{-1} and HighP/E = 21.4 mg protein kJ^{-1}) and three dietary components (protein, Lys, and Met). The main and interaction effects were identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test at $p < 0.05$ level of significance. Statistical analyses were performed using the IBM SPSS Statistics 26 software.

Additionally, metabolic flux assays data were subjected to a principal component analysis (PCA) to verify differences between amino acids and diet formulations and find potential clusters of observations. PCA was carried out using the standard prcomp R function in the auto-scaled matrices. Score plots were generated for the two first principal components (PC1 and PC2) using the ggbiplot package for R. Loadings for PC1 and PC2 were calculated to determine the weight of each original variable in the corresponding PCs. Analyses were carried out using the open-source software R version 4.0.4.

3. Results

The proportion of the ^{14}C-amino acids evacuated was influenced by the P/E ratios (Figure 1, $p < 0.05$). Feeding gilthead seabream juveniles with the HighP/E diet significantly increased the evacuation of amino acids when compared with LowP/E fed fish ($p < 0.05$). Evacuation of protein and lysine in fish fed the HighP/E diet increased from 28% to 35% and from 24% to 47%, respectively, compared with LowP/E fed fish. Independently of the diet, methionine was significantly more evacuated ($\sim$ 46%) than protein ($p < 0.05$), while lysine presented intermediate results (see Supplementary Table 1 for more details).

In the *Free* and *Bound* fractions of the liver, amino acid retention was not affected by the dietary treatment (Figure 2, $p > 0.05$). Lysine was significantly more retained in the *Free* fraction than protein ($p < 0.05$), while methionine presented intermediate values (Figure 2(a)). However, in the hepatic *Bound* fraction, methionine was significantly less retained than protein or lysine ($p < 0.05$), which presented similar retention levels (Figure 2(b)).

The dietary P/E ratio influenced the retention of amino acids in the *Free* fraction of the muscle (Figure 3(a)). A significantly higher proportion of ^{14}C-amino acids was found in the *Free* muscle fraction of fish fed the LowP/E diet compared with the HighP/E fed fish ($p < 0.05$). A significantly higher percentage of the tube-fed ^{14}C-Met was found in this fraction compared with ^{14}C-protein or ^{14}C-Lys, with average values of 19%, 6%, and 5%, respectively ($p < 0.05$). No significant differences among dietary treatments or amino acids were found in the *Bound* muscle fraction of fish (Figure 3 (b), $p > 0.05$).
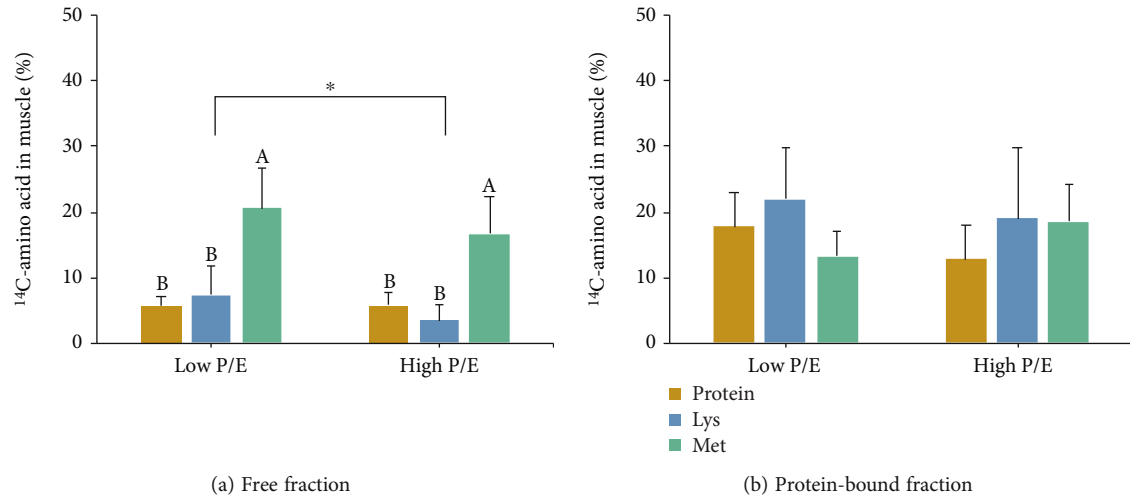
(a) Free fraction

(b) Protein-bound fraction

FIGURE 3: Proportion (%) of the total recovered ^{14}C-protein (protein), ^{14}C-lysine (Lys), and ^{14}C-methionine (Met) that were retained in the *Free* (A) and *Bound* (B) fractions of muscle in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation ($n = 6$ fish for each diet and tracer). (A, B) Significant differences in the metabolic fate of the distinct amino acids at each dietary treatment;∗denotes significant differences between dietary treatments ($p < 0.05$). Absence of letters denotes no significant differences.
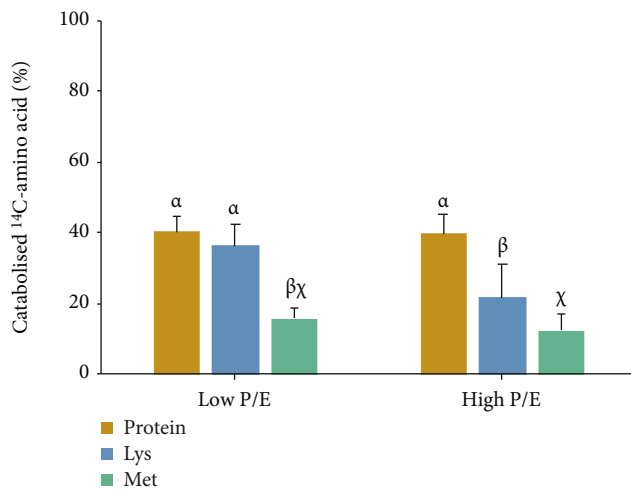

FIGURE 4: Proportion (%) of the total recovered ^{14}C-protein (protein), ^{14}C-lysine (Lys), and ^{14}C-methionine (Met) that were catabolized in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation ($n = 6$ fish for each diet and tracer). Interacting effects ($p < 0.05$) between the metabolic fate of the distinct amino acids and dietary treatments are represented by (a, b, c).

Interacting effects between the metabolic fate of the distinct amino acids and the dietary treatments were found for amino acid catabolism (Figure 4, $p < 0.05$). Catabolism of ^{14}C-protein remained unaltered in fish fed the LowP/E and HighP/E diets. However, lysine catabolism significantly decreased from 36% in fish fed the LowP/E diet to 22% in HighP/E fed fish ($p < 0.05$). ^{14}C-Met was significantly less catabolized than ^{14}C-protein and ^{14}C-Lys in fish from both dietary treatments ($p < 0.05$). Additionally, methionine catabolism did not differ significantly between dietary treatments ($p > 0.05$).

Principal component analysis (PCA) was used to reduce the complexity of the data from the metabolic flux assays (Figure 5). PC1 and PC2 accounted for 45% and 25% of the total variability of the data. The analysis of the score plots indicated that methionine and protein data were separated along the PC1 as well as the PC2 axis. Evacuation and retention in the *Bound* fraction of liver were the loadings that contributed the most for the dissimilarities observed between fish fed ^{14}C-protein labelled diets and fish fed diets labelled with ^{14}C-Met along the PC1 axis. Along the PC2 axis, catabolism and retention in the *Free* fraction of liver and muscle were responsible for the clustering of fish fed diets labelled with ^{14}C-protein and ^{14}C-Met in different groups.

4. Discussion

Metabolic flux assays were used to unravel the effect of dietary P/E ratios in the bioavailability and metabolic fate of protein, lysine, and methionine in gilthead seabream juveniles. In the present study, amino acid evacuation was influenced by the amino acids and dietary P/E ratios. Methionine was significantly more evacuated than protein, independently of the P/E ratios. Methionine evacuation (~ 46%) was similar to data observed in a previous study where metabolic flux assays were performed in gilthead seabream juveniles fed four different diets with distinct protein (44 and 40%) and/or lipid (21 and 18%) levels [43]. Evacuation of ^{14}C-protein (~ 30%) was in line with a previous study that determined that the nutritional background (naïve or larvae exposed to a dietary glucose stimuli) did not influence protein utilization in seabream juveniles [44]. PCA results confirmed that methionine and protein were clustered in different groups along the PC1 axis mainly due to evacuation data, suggesting that methionine absorption is less efficient compared with protein-bound amino acids. In the current
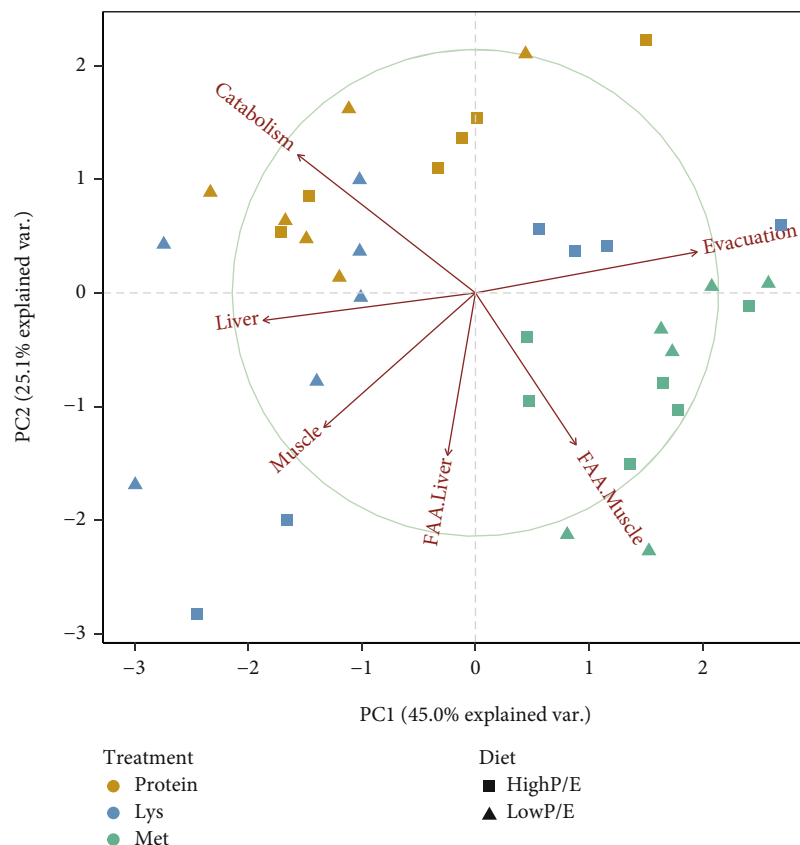
FIGURE 5: Principal component analysis (PCA) of the metabolic flux assays data generated by gilthead seabream juveniles fed LowP/E and HighP/E diets labelled with ^{14}C-protein (Protein), ^{14}C-lysine (Lys), and ^{14}C-methionine (Met). Each point represents the projection of an individual sample in the PC1 and PC2 axis. Each dietary treatment is identified as a unique shape and each ^{14}C-amino acid as a unique color, as indicated in the figure.

trial, the dietary formulation significantly influenced the evacuation of the distinct amino acids. In contrast, in the abovementioned study in seabream fed diets with different protein and lipid levels, evacuation of lysine, tryptophan, and methionine was not affected by the dietary formulations [43]. On the other hand, the intestinal uptake of certain amino acids such as methionine was shown to increase with a higher inclusion of dietary lipids in European seabass fingerlings [45]. Accordingly, in the present study, a lower evacuation was observed with the decrease of dietary P/E ratio. The increase in dietary lipid content may overload the digestive capacity of fish and consequently may delay gastric evacuation giving more time for the uptake of dietary lipids [46] and amino acids. A slower intestinal transit in fish fed high lipid diets (low dietary P/E ratio) may be one of the factors contributing to a more efficient amino acid uptake that could be translated into improved protein utilization and consequently growth, not measured in the present study.

In the current study, ^{14}C-amino acid retention in the hepatic *Free* and *Bound* fractions differed significantly among amino acids. Lysine was significantly more retained than protein in the former and than methionine in the latter. As previously suggested [43], the relatively high retention in liver, 18 h after feeding, may be related to amino acids essen-

tial role in energy homeostasis. However, the current study went further since the proportion of amino acids retained in the tissues was determined in the *Free* and *Bound* fractions and not as total. The protein-bound amino acids retained in the liver were higher than in the free pool in fish fed both diets. These findings reveal that protein synthesis is occurring in the liver, since the amino acids are being mostly incorporated into hepatic proteins within 18 h after feeding and not only temporarily available in the free pool.

The proportion of methionine found in the fish muscle, *i.e.*, the sum of *Free* and *Bound* fractions, was higher than lysine or protein. In seabream fed diets with distinctive protein and/or lipid levels, total retention for methionine and lysine in the muscle was similar to the present results. Methionine was preferentially retained in the muscle, being more available for growth or other metabolic purposes [43]. Similarly, in Senegalese sole larvae and juveniles and in white seabream larvae, most of the methionine was recovered in the muscle [36, 47–49]. However, the results from the current study showed that in fish fed both diets, no significant differences were found in the retention of amino acids in the muscle *Bound* fraction. Conversely, in the *Free* fraction of the muscle, methionine was significantly more retained than protein or lysine. PCA results corroborate these findings since fish fed both diets labelled with [14]C-

Met were grouped in a cluster according to data from the retention in the muscle *Free* fraction. These findings demonstrate that the higher retention of methionine in the muscle was due to its presence in the free pool and not as protein-bound, contrarily to what was observed for lysine. This reinforces the pivotal role of methionine in several metabolic functions beyond protein synthesis. Apart from protein synthesis, methionine participates in numerous metabolic reactions as a precursor of S-adenosylmethionine, cysteine, glutathione, taurine, or polyamines [50, 51]. Moreover, this indispensable amino acid has been shown to be involved in the methylation of DNA and protein, cell proliferation, and differentiation [34, 35]. Lysine retention in the muscle was similar to protein, independently of the diet, confirming that lysine is a good indicator of muscle accretion. The amount of free amino acids retained in the muscle of fish fed the LowP/E diet was significantly higher than in the HighP/E fed fish. This change was due to a simultaneous higher retention of lysine and methionine since the retention of protein remained relatively unaltered. These results imply that a decrease in the dietary P/E ratios affects amino acid utilization and may favour the retention of indispensable over dispensable amino acids in the muscle.

A fraction of the absorbed amino acids is unavoidably catabolized and used for energy rather than protein synthesis [22]. In a work in gilthead seabream fed diets with distinctive levels of protein and/or lipids, catabolism was found to be predominantly ketogenic considering that lysine was the preferred amino acid to be used for energy even when deficient in the diet [43]. The current work corroborates these findings since, irrespectively of the diet, lysine was significantly more catabolized than methionine. Catabolism was influenced by the interaction between the metabolic fate of the distinct amino acids and the P/E ratios, with the catabolism of protein and methionine remaining reasonably unaltered in fish fed both diets, but with significant differences in the case of lysine. Based on data from the catabolism of fish fed both diets labelled with ^{14}C-protein and ^{14}C-Met, PCA results demonstrate that protein and methionine were clustered in two different groups along the PC2 axis. Determination of the metabolic flux of protein revealed that the proportion of amino acids catabolized was approximately 40% of the ^{14}C-protein, independently of the diet. The increase of methionine availability in the muscle *Free* fraction with a decrease in dietary P/E ratio was not translated into a higher catabolism. Methionine was the least catabolized (13-16%) amino acid, indicating that it is preferentially spared for other metabolic purposes instead of being used for energy. In contrast, it appears that a higher availability of free lysine resulted in increased catabolism in fish fed the LowP/E diet. While fish fed the LowP/E diet presented values as high as the ones for protein, fish fed the HighP/E diet drastically reduced lysine catabolism from 36 to 22%. Fish are able to selectively retain or catabolize distinct amino acids [43, 49, 52, 53]. For instance, Senegalese sole postlarvae were found to use a significant higher proportion of the dispensable amino acids glutamate and alanine for energy, rather than the indispensable amino acids lysine and arginine, sparing the latter for growth [54]. The current findings suggest that although fish fed both diets were equally catabolizing amino acids for energy, the dietary P/E ratios influenced amino acids' fate. An increase in the P/E ratio in seabream diets reduces lysine catabolism and may channel other amino acids (*e.g.*, dispensable amino acids) for energy, sparing lysine for growth.

5. Conclusion

Maximization of fish growth while reducing nitrogen losses to the environment is strongly dependent on tunning amino acid utilization. This study revealed that a decrease in the P/E ratio resulted in lower amino acid evacuation. Methionine was more available in the muscle free pool, demonstrating its importance for metabolic functions other than protein synthesis. Moreover, increasing the dietary P/E ratio may spare lysine for growth. An optimization of P/E ratios in diets for gilthead seabream juveniles will improve diet utilization and fish performance.

Abbreviations

P/E: Protein to energy ratio
[14]C-protein: [U-^{14}C]-protein hydrolysate (L-amino acid mixture)
[14]C-Lys: [U-^{14}C]-L-lysine
[14]C-Met: [1-^{14}C]-L-methionine.

Data Availability

Data is available on request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Cláudia Aragão and Sofia Engrola were joint senior authors.

Acknowledgments

Supplementary Materials

Supplementary Table 1 contains data on the proportion (%) of the total recovered ^{14}C-protein (Protein), ^{14}C-lysine (Lys), and ^{14}C-methionine (Met) that were evacuated, retained in the liver and muscle as *Free* and *Bound* fractions, and catabolized in gilthead seabream juveniles fed LowP/E or HighP/E diets. *(Supplementary Materials)*

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