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Naturally Occurring Stable Isotopes Reflect Changes in Protein Turnover and Growth in Gilthead Sea Bream (*Sparus aurata*) Juveniles under Different Dietary Protein Levels

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ABSTRACT: Ideal nutritional conditions are crucial to sustainable aquaculture due to economic and environmental issues. Here we apply stable isotope analysis as an indicator of fish growth and feeding balance, to define the optimum diet for efficient growing conditions. Juveniles of gilthead sea bream were fed with six isoenergetic diets differing in protein to lipid proportion (from 41/26 to 57/20). As protein intake increased, δ^{15}N and $\Delta\delta^{15}$N of muscle and $\Delta\delta^{15}$N and $\Delta\delta^{13}$C of its protein fraction decreased, indicating lower protein turnover and higher protein deposition in muscle. This is reflected in the inverse relationship found between $\Delta\delta^{15}$N and growth rate, although no differences were observed in either parameter beyond the protein/lipid proportion 47/23. Principal component analysis (PCA) also signaled 47/23 diet as the pivotal point with the highest growing efficiency, with isotopic parameters having the highest discrimination load. Thus, muscle isotope composition, especially ^{15}N, can be used to evaluate nutritional status in farmed fish.

KEYWORDS: *stable isotope fractionation, muscle amino acid composition, protein turnover, dietary components, optimal growth*

INTRODUCTION

Fish growth is affected mainly by the dietary protein fraction, which represents the largest proportion of feed production costs.[1] It is commonly accepted that high-protein diets improve fish growth, especially in carnivorous species. However, such diets have several drawbacks with respect to the aquaculture industry. Protein is the most expensive component of fish feed, and the byproducts of protein catabolism are the most important source of nitrogen loading to waters. Moreover, there is an additional problem regarding the unreliability of material used for fish meal production. One approach to minimizing feed costs and achieving a reduction in feed and metabolic wastes is to improve protein sparing by dietary inclusion of energy sources like lipids or carbohydrates.

Gilthead sea bream (*Sparus aurata*) is a major finfish species farmed in the Mediterranean area and its production increases annually.[2] While the production of this species is a well-controlled process, knowledge of its nutritional requirements is still incomplete compared to other species like salmonids. Commercial diets for sparids like gilthead sea bream commonly include 20−23% lipids combined with 48−53% protein, although these proportions can vary during the life cycle. However, many studies have focused on determining the minimum percentage of dietary protein required to obtain satisfactory growth rates and thus reduce feed production costs for this species. The optimum level of dietary protein was first determined as being around 40% for sea bream juveniles.[3] Later, it was estimated to be 55% for fry[4] and 45−48% in larger fish,[5−8] denoting, as expected, a reduction of requirement with increased body size. The efficiency of protein utilization for growth can be improved in sea bream by replacing dietary protein with nonprotein energy sources, such as lipids[7−10] and carbohydrates,[11−14] thus producing a protein-sparing effect. This approach allows the protein content of diets to be reduced without seriously affecting growth rates.

Stable isotopes have been successful used in fishery sciences to discriminate between farmed and wild specimens of gilthead sea bream[15,16] and other fish species,[17,18] to determine the geographical origin and production systems of farmed fish,[19,20] to authenticate fish species[21] and to study the feeding ecology[22] and movement of wild stocks.[23] There is also growing use of stable isotopes in fish nutrition studies since the isotopic composition of animals reflects that of their diets plus a discrimination factor caused by the fractionation that occurs during chemical, physical, and biological processes.[24] Experimental studies have been performed mainly in larval nutrition, using stable isotopes as direct measures of feeding efficiency, nutrient incorporation, and turnover.[25] Few studies have been performed on larger fish, and these have mainly addressed ecological issues. Several studies analyzed the effect of feeding level on tilapia,[26,27] carp,[28] and sea bass,[29] reporting generally an inverse relationship between the δ^{15}N and δ^{13}C content of the fish and the amount of food ingested. Others, based on the quality of dietary sources, allowed isotopic discrimination between fish fed on animal or plant proteins.[26,30] Only two studies, both of tilapia, compared the effect of distinct dietary protein content on the stable isotope composition of fish. Gaye-

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Table 1. Formulation and Chemical Composition of Experimental Diets Fed to Gilthead Sea Bream[a]

	diet (% protein/% lipid)					
	A (41/26)	B (44/25)	C (47/23)	D (50/22)	E (54/21)	F (57/20)
			Raw Material, g/100 g			
wheat[b]	32.5	29.2	25.9	22.5	19.2	15.8
wheat gluten[c]	10.7	11.7	12.8	13.9	14.9	16.0
soybean concentrate[d]	10.7	11.7	12.8	13.9	14.9	16.0
fish meal[e]	26.7	29.4	32.0	34.7	37.3	40.0
fish oil, Nordic[f]	19.0	17.6	16.2	14.7	13.3	11.9
vitamin premix[g]	0.26	0.26	0.26	0.26	0.26	0.26
			Nutrient Analysis, g/100 g DM			
dry matter, g/100 g	92.1	92.2	92.3	92.3	92.4	92.5
crude protein	41.2	44.5	47.7	50.9	54.1	57.3
crude fat	26.2	25.0	23.8	22.6	21.3	20.1
NFE[h]	27.5	25.4	23.3	21.3	19.2	17.1
ash	5.1	5.5	5.9	6.3	6.7	7.1
crude energy, MJ/kg DM[i]	23.3	23.3	22.9	23.0	22.7	22.9
			Amino Acid Analysis, g/100 g of protein (g/100 g DM)			
arginine	5.5 (1.9)	5.4 (1.9)	5.4 (2.2)	5.4 (2.5)	5.5 (2.7)	5.6 (3.0)
cysteine	0.8 (0.3)	0.8 (0.3)	0.8 (0.3)	0.8 (0.4)	0.8 (0.4)	0.8 (0.4)
histidine	2.2 (0.7)	2.1 (0.8)	2.2 (0.9)	2.0 (0.9)	2.0 (1.0)	2.1 (1.1)
isoleucine	3.1 (1.0)	3.0 (1.1)	3.0 (1.2)	3.0 (1.4)	3.0 (1.5)	3.0 (1.6)
leucine	7.6 (2.7)	7.4 (2.6)	7.5 (3.0)	7.4 (3.4)	7.4 (3.7)	7.5 (4.1)
lysine	6.9 (2.4)	6.9 (2.5)	6.7 (2.7)	6.7 (3.1)	6.7 (3.3)	6.8 (3.7)
methionine	2.8 (0.9)	2.9 (1.0)	3.0 (1.2)	3.0 (1.4)	3.1 (1.5)	3.2 (1.7)
phenylalanine	4.7 (1.6)	4.7 (1.7)	4.7 (1.9)	4.7 (2.1)	4.6 (2.3)	4.6 (2.5)
threonine	3.6 (1.2)	3.6 (1.3)	3.6 (1.4)	3.6 (1.7)	3.8 (1.9)	3.8 (2.1)
tyrosine	2.6 (0.9)	2.6 (0.9)	2.7 (1.1)	2.7 (1.2)	2.7 (1.3)	2.7 (1.4)
valine	3.4 (1.2)	3.3 (1.1)	3.3 (1.3)	3.7 (1.7)	3.3 (1.7)	3.3 (1.8)
alanine	5.4 (1.8)	5.4 (1.9)	5.3 (2.1)	5.6 (2.6)	5.4 (2.6)	5.5 (3.0)
asparagine (Asn + Asp)	8.9 (3.1)	9.0 (3.2)	8.8 (3.5)	9.0 (4.1)	9.1 (4.5)	9.2 (5.0)
glutamine (Gln + Glu)	24.7 (8.5)	24.8 (8.8)	24.9 (9.9)	24.5 (11.2)	24.6 (12.2)	24.1 (13.1)
glycine	5.2 (1.8)	5.3 (1.9)	5.3 (2.1)	5.3 (2.5)	5.3 (2.6)	5.4 (2.9)
proline	7.4 (2.5)	7.5 (2.6)	7.5 (3.0)	7.3 (3.4)	7.3 (3.6)	7.1 (3.9)
serine	5.4 (1.8)	5.2 (1.8)	5.2 (2.1)	5.2 (2.4)	5.3 (2.6)	5.4 (3.0)
total IAA	43.1 (14.8)	42.8 (15.1)	42.9 (17.1)	43.1 (19.8)	43.0 (21.3)	43.2 (23.4)
total DAA	56.9 (19.5)	57.2 (20.2)	57.1 (22.8)	56.9 (26.1)	57.0 (28.2)	56.8 (30.8)
ratio IAA/DAA	0.76	0.75	0.75	0.76	0.75	0.76
			Isotopic Analysis,[j] ‰			
$\delta^{15}N$	6.42 ± 0.25	6.39 ± 0.48	6.69 ± 0.05	6.48 ± 0.39	6.48 ± 0.22	6.66 ± 0.05
$\delta^{15}N$ protein	6.43 ± 0.21	6.33 ± 0.08	6.71 ± 0.21	6.38 ± 0.12	6.30 ± 0.11	6.79 ± 0.19
$\delta^{13}C$	−25.86 ± 0.13 a	−25.50 ± 0.19 ab	−25.09 ± 0.18 bc	−24.95 ± 0.13 bc	−24.87 ± 0.27 c	−24.58 ± 0.29 c
$\delta^{13}C$ protein	−24.96 ± 0.01	−25.15 ± 0.07	−24.79 ± 0.21	−24.88 ± 0.30	−24.68 ± 0.09	−24.34 ± 0.04
$\delta^{13}C$ lipid	−26.95 ± 0.09	−27.00 ± 0.09	−26.99 ± 0.01	−27.18 ± 0.06	−27.22 ± 0.12	−27.31 ± 0.14

[a]Skretting Aquaculture Co. designed the experimental diets and also performed nutrient analysis. DM, dry matter; IAA, indispensable amino acids; DAA, dispensable amino acids. [b]Statkorn, Norway. [c]Cerestar Scandinavia AS, Denmark. [d]Imcopa, Brazil. [e]Consortio, Peru. [f]Nordsildmel, Norway. [g]Proprietary formula of Skretting (Norway). Vitamin and mineral supplementation is estimated to cover requirements according to NRC. [h]NFE, nitrogen-free extract (calculated by difference). [i]Analyzed values from a bomb calorimeter. [j]Values are expressed as mean ± SEM (n = 3). Letters indicate significant differences ($p < 0.05$).

Siessegger et al.[31] showed a decrease in N and C isotope fractionation in fish with higher protein retention, whereas a recent study by Kelly and Martinez del Rio[32] proposed a positive correlation between ^{15}N fractionation in tissues and protein intake. The generality of these results, which are conflicting, awaits further experimental work. Therefore, given that changes in the proportion of dietary components affect fish isotopic composition, we propose that isotope determination be considered a useful tool to ensure adequate levels of dietary components for fish growth. To date, little attention has been devoted to this issue. Optimum fish production via an optimal dietary balance is especially important for the aquaculture sector because of the high economic and environmental costs of the high-protein diets used.

Here, we evaluate the use of stable isotopes as an indicator of feeding balance to assess the optimal nutritional conditions for growing fish. In order to test their usefulness, we examined the variations in the natural abundance of stable isotopes in

gilthead sea bream juveniles in response to changes in the protein-to-lipid ratio of their diet. White muscle was selected as the target tissue for isotopic analyses (δ^{15}N and δ^{13}C) since it reflects well physiological changes in isotopic composition and is less variable than whole body[20] or other tissues that are metabolically more active, such as liver.[33] In addition, we studied the muscle amino acid (AA) profile with the aim of ascertaining its potential implication in isotopic changes. Thus, relationships between diet and muscle indispensible amino acid (IAA) profiles were established in order to detect AA imbalances between the experimental groups.

MATERIALS AND METHODS

Animal Conditions and Sampling. Gilthead sea breams, with a weight of around 70 g and a length of 14 cm, were kept in IRTA installations (Sant Carles de la Ràpita, Tarragona, Spain) under natural conditions of temperature, oxygen level, salinity, and photoperiod throughout the study (May to July). The fish were initially adapted to the installation conditions over 2 weeks with a standard commercial fish feed. Following this period, they were randomly distributed in triplicate 400-L fiberglass tanks (30 fish per tank) for each experimental diet (6 diets × 3 tanks = 18 tanks in total). The experimental diets were formulated by Skretting Aquaculture to be isoenergetic (24.6 MJ/kg, calculated from gross composition: protein 24 MJ/kg, lipid 39 MJ/kg, and carbohydrate 17 MJ/kg) and to contain a gradual increase in the protein/lipid ratio from 41/26 (diet A) to 57/20 (diet F) in dry matter, as summarized in Table 1. The ratios of distinct protein sources were very similar among experimental diets (plant-to-animal protein ratio close to 1) since the proportions between major plant (wheat gluten and soybean concentrate) and animal (fish meal) protein ingredients were kept constant in the diets. Dietary pellets were extruded and the size (4 mm) was adequate for the corresponding fish weight. Fish were fed to satiation twice a day, 7 days a week for 12 weeks. Feed was automatically delivered for 1 h in each feeding session. Satiation was ensured by calculating estimated daily feed intake and allowing a ration 20% above this value. Feed delivery was recorded daily, and uneaten feed was collected daily and then dried and weighed to calculate true feed intake. Total biomass from triplicate tanks was recorded in order to evaluate weight gain during the experimental period and used to calculate feed conversion ratio (FCR = feed intake as dry matter/weight gain). To monitor growth, the fish from each tank were individually weighed and length was measured at the beginning, in the middle, and at the end of the trial. Specific growth rate (SGR) was calculated as follows: SGR (%·day^{-1}) = 100 × (ln W_2 − ln W_1)/t, where W_1 and W_2 are the initial and final weight of animals, respectively, after t days.

After the experimental period, the fish were fasted for 24 h before sampling. Nine fish were captured at random from each treatment (3 fish per tank × 3 tanks per condition × 6 dietary conditions = 54 fish in total) and anesthetized with 2-phenoxyethanol (100 ppm) diluted in seawater. Fish weight and size were measured. The animals were then killed by cutting the spinal column and eviscerated to measure mesenteric fat and liver weight. Epaxial white skeletal muscle (under the dorsal fin and above the lateral line) was dissected and frozen immediately in liquid nitrogen before being stored at −80 °C until analysis. The experiments complied with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005), and the University of Barcelona (Spain) for the use of laboratory animals.

Muscle Composition. Prior to analysis, muscle samples were ground in a mortar with liquid nitrogen. These samples were then separated in several fractions for composition (lipid, protein, glycogen, and water), isotopic, and AA measurements. Tissue water content was determined gravimetrically after the samples were dried at 100 °C for 24 h. Glycogen was evaluated by a spectrophotometer via the anthrone method following the procedures described elsewere.[34] Total lipid content was purified from two methanol−chloroform (2:1) extractions according to Folch et al.[35] The washed lipid extracts were dried under N_2 and the lipid content was determined gravimetrically. Protein was purified from defatted samples via precipitation with trifluoroacetic acid (100 mL·L^{-1}) and after centrifugation at 1060g for 30 min. The protein content was calculated from the nitrogen obtained by elemental analysis (Elemental Analyzer Flesh 1112, ThermoFinnigan, Bremen, Germany) using the coefficient 6.25.

δ^{15}N and δ^{13}C Determination. Samples of diets and muscle tissue were lyophilized and ground into a homogeneous powder for isotopic analysis. Appropriate aliquots of these samples and their purified fractions (lipid, glycogen, and protein), ranging from 0.3 to 0.6 mg in accordance with their theoretical C and N content, were accurately weighed in small tin capsules (3.3 × 5 mm, Cromlab, Barcelona, Spain). Samples were analyzed to determine the carbon and nitrogen isotope composition on a Mat Delta C IRMS (Thermo Fisher Scientific, Bremen, Germany) coupled to Flash 1112 elemental analyzer at the Serveis Científico-Tècnics at the University of Barcelona. Isotope ratios (^{15}N/^{14}N, ^{13}C/^{12}C) are given in ‰ (parts per thousand) on a δ-scale and refer to the deviation in measured ratio from the international accepted standards VPDB (Vienna Pee Dee Belemnite) for carbon and AIR for nitrogen. Delta values were determined as follows:

$$\delta = [(R_{sa}/R_{st}) - 1] \times 1000$$

where R_{sa} = ^{15}N/^{14}N or ^{13}C/^{12}C of samples and R_{st} = ^{15}N/^{14}N or ^{13}C/^{12}C of the international standards. The following standards certified by the International Atomic Energy Agency (IAEA, Vienna, Austria) were used: IAEA-N1 ($\delta^{15}N_{air}$ = 0.4‰), IAEA-NO-3 ($\delta^{15}N_{air}$ = 4.7‰), and IAEA-N2 ($\delta^{15}N_{air}$ = 20.3‰) for nitrogen and LSVEC ($\delta^{13}C_{VPDB}$ = −46.6‰), IAEA-CH-7 ($\delta^{13}C_{VPDB}$ = −32.15‰), USGS40 ($\delta^{13}C_{VPDB}$ = −26.4‰), and IAEA-CH-6 ($\delta^{13}C_{VPDB}$ = −10.45‰) for carbon. Every 10 measurements, three standard samples were analyzed to calibrate the system and compensate for machine drift and as a quality control measure. The precision of each isotopic measurement was 0.02‰, and the repeatability of each sample was smaller than 0.2‰. Nitrogen and carbon isotopic fractionation ($\Delta\delta^{15}$N and $\Delta\delta^{13}$C) values for muscle and its reserves (protein and lipid) were calculated as the difference between δ tissue and δ diet for each component.

Amino Acid Analysis. Three fish for each dietary condition were randomly selected and muscle samples were taken from the epaxial area (under the dorsal fin) for AA measurement. These samples were frozen in liquid nitrogen and then freeze-dried at −80 °C and weighed. Protein-bound AA samples were hydrolyzed in 6 M HCl at 108 °C over 24 h in nitrogen-flushed glass vials. We applied reversed-phase high-pressure liquid chromatography (HPLC) in a Waters Pico-Tag AA analysis system, using norleucine as an internal standard. The resulting chromatograms were analyzed with Breeze software (Waters). Results for tryptophan are not reported here since this AA is destroyed by acid hydrolysis. Glutamine is converted to glutamate during acid hydrolysis so these AAs are reported as their sum. The same occurs for asparagine and aspartate. IAA and dispensable amino acid (DAA) data are expressed in weight percentage of the total protein-bound AA pool, that is, (weight of one AA × 100)/weight of all AA, to allow comparisons between food and muscle AA profiles. Relationships between the two profiles were established for IAA.

Statistical Analysis. Statistical differences between treatments were analyzed by nested one-way analysis of variance (ANOVA) with tank as random factor to test possible tank effect. When tank effect was not found, an ANOVA followed by Tukey's or Dunnett's post hoc test was used when variances were uniform or not, respectively. Statistical differences were considered significant when p-values were less than 0.05. The Shapiro−Wilk test was previously used to ensure normal distribution of data, and the uniformity of variances was determined by the statistical Levene's test. The relationships between muscle isotopic parameters and dietary protein content or SGR were tested by linear regression. Linear correlations between parameters were accepted as significant when the p-value was <0.05. The Pearson correlation index was calculated for all significant correlations found; significant correlations are indicated in all cases when found. Principal component analysis (PCA) was performed to study the structure of

Table 2. Body, Feed, and Growth Parameters of Gilthead Sea Bream Juveniles Fed Experimental Diets for 12 Weeks[a]

	diet (% protein/% lipid)					
	A (41/26)	B (44/25)	C (47/23)	D (50/22)	E (54/21)	F (57/20)
Body Parameters, n = 9 fish						
initial body weight, g	71.75 ± 2.28	71.98 ± 1.03	72.29 ± 0.85	72.54 ± 0.99	72.46 ± 1.19	71.38 ± 0.66
final body weight, g	188.99 ± 1.44 ab	181.14 ± 6.25 a	200.28 ± 2.48 abc	204.41 ± 5.66 bc	216.83 ± 3.30 c	208.56 ± 3.23 c
final body length, cm	21.91 ± 0.22 a	21.76 ± 0.17 a	22.49 ± 0.04 ab	22.73 ± 0.19 b	22.90 ± 0.13 b	22.89 ± 0.17 b
CF,[b] %	1.00 ± 0.03	1.02 ± 0.01	0.98 ± 0.04	0.93 ± 0.02	0.97 ± 0.02	0.95 ± 0.01
HSI,[c] %	1.79 ± 0.07 a	1.54 ± 0.07 ab	1.52 ± 0.09 ab	1.36 ± 0.07 b	1.41 ± 0.08 b	1.29 ± 0.03 b
MFI,[d] %	1.43 ± 0.17	1.34 ± 0.17	1.46 ± 0.12	1.44 ± 0.14	1.62 ± 0.04	1.20 ± 0.14
Growth Performance, n = 3 tanks						
weight gain, kg biomass	3.45 ± 0.29 ab	3.20 ± 0.39 a	3.84 ± 0.13 abc	3.83 ± 0.25 abc	4.23 ± 0.16 c	4.07 ± 0.23 bc
SGR,[e] %	1.14 ± 0.03 ab	1.08 ± 0.03 a	1.20 ± 0.02 bc	1.22 ± 0.03 bc	1.29 ± 0.02 c	1.26 ± 0.02 c
FCR[f]	1.25 ± 0.04	1.26 ± 0.05	1.16 ± 0.01	1.16 ± 0.03	1.14 ± 0.01	1.12 ± 0.01
PER[g]	1.95 ± 0.06 a	1.79 ± 0.07 ab	1.81 ± 0.02 ab	1.70 ± 0.04 bc	1.62 ± 0.01 bc	1.56 ± 0.02 c
Feed Intake,[h] n = 3 tanks						
DM, g·kg^{-1}·day^{-1}	13.02 ± 0.04 ab	12.56 ± 0.22 a	12.80 ± 0.06 ab	12.72 ± 0.07 ab	13.27 ± 0.05 b	12.84 ± 0.21 ab
protein, g·kg^{-1}·day^{-1}	5.37 ± 0.02 a	5.59 ± 0.10 a	6.10 ± 0.03 b	6.47 ± 0.04 c	7.18 ± 0.03 d	7.36 ± 0.12 d
lipid, g·kg^{-1}·day^{-1}	3.42 ± 0.01 a	3.14 ± 0.05 b	3.04 ± 0.01 b	2.87 ± 0.02 c	2.83 ± 0.01 c	2.59 ± 0.04 d
NFE, g·kg^{-1}·day^{-1}	3.56 ± 0.01 a	3.19 ± 0.06 b	3.01 ± 0.01 c	2.74 ± 0.02 d	2.60 ± 0.01 d	2.27 ± 0.04 e
energy, MJ·kg^{-1}·day^{-1}	0.30 ± 0.00	0.29 ± 0.01	0.29 ± 0.00	0.29 ± 0.00	0.30 ± 0.00	0.29 ± 0.01

[a]Values are expressed as mean ± SEM. Letters indicate significant differences ($p < 0.05$). DM, dry matter. [b]CF (condition factor) = (weight/size^3) × 100. [c]HSI (hepatosomatic index) = (liver weight/animal weight) × 100. [d]MFI (mesenteric fat index) = (fat weight/animal weight) × 100. [e]SGR (specific growth rate) = [(ln initial biomass − ln final biomass) × 100]/days. [f]FCR (feed conversion ratio) = dry feed intake/weight gain. [g]PER (protein efficiency ratio) = weight gain/crude protein intake. [h]Nutrient intake = total nutrient intake/(ABW × days); ABW (average body weight) = (initial body weight + final body weight)/2.

the whole data set. Score plots from the PCA explore the main trends in the data, and their respective loading reveals variables with a significant loading. All statistical analyses were done with commercial software (IBM SPSS Statistics v20.0, IBM Corp., Armonk, NY).

RESULTS

Animal Growth and Body Parameters. Final body weight and growth rate (SGR) of gilthead sea bream juveniles rose as the dietary protein-to-lipid ratio increased, although no differences were found between groups fed above 47% dietary protein (Table 2). Changes in body length followed the same pattern as body weight, so the condition factor did not differ between groups. The mesenteric fat index (MFI) did not change either, although the group fed the highest lipid diet (diet A, 41% protein/26% lipid) had the highest hepatosomatic index (HSI).

All experimental groups were fed ad libitum on the different experimental diets (ranging from A, 41/26, to F, 57/20) to avoid dietary restrictions, with the total feed intake very similar for all of them (Table 2). The only difference was observed between groups B and E, since the former group showed less hunger during the trial period. The amount of energy ingested was almost the same in the experimental groups, since experimental diets were formulated to be isoenergetic, even though this energy was distributed differently according to the dietary composition. Thus, the amount of ingested protein increased from the group fed diet A to that fed diet F, whereas the ingested lipid followed the opposite trend, as did the nitrogen-free extract (NFE). Although the feed conversion ratio (FCR) did not change significantly between the experimental groups, fish on low-protein diets showed a higher protein efficiency ratio (PER).

Proximal Composition and Amino Acid Profile of Muscle. The shift in the dietary protein-to-lipid ratio did not affect the proximal composition of white muscle between groups (Table 3). The AA balance in muscle did not vary either, as shown by the similar relationships between muscle and diet amino acid composition observed between groups (Figure 1). This is attributed to the small variation in the AA profiles of the experimental diets (Table 1), which show the similar proportionality between the different dietary protein sources. Muscle amino acid profiles were also quite similar, showing only slight differences in valine and glycine (Table 3). Valine levels were lower in group B than in E and F, whereas the groups fed low-protein diets (A, B, and C) had higher glycine values than the groups on high-protein diets (D, E, and F). Figure 1 also indicates that lysine and valine were the most limiting IAAs in all groups because of the greatest difference between the AA content in muscle and in diet.

Muscle Isotopic Composition. The isotopic compositions of muscle tissue and muscle fractions (protein, lipid, and glycogen) are shown in Table 3. Fish fed high-protein diets had lower δ^{15}N values in muscle tissue and in the muscle protein fraction, both parameters showing an inverse relationship with the dietary protein content ($r = 0.803$ and $p < 0.05$ for muscle tissue; $r = 0.762$ and $p < 0.05$ for muscle protein). In contrast, δ^{13}C values did not change either in muscle tissue or in the protein, lipid, or glycogen fractions of muscle.

The N isotopic composition of experimental diets did not differ, since the proportion of different protein sources was practically equal among diets, which is also shown by the similar AA profile. Thus, the inverse relationship observed between the isotopic fractionation of N ($\Delta\delta^{15}$N), that is, the isotopic difference between δ values of diet and tissue ($\Delta\delta = \delta_{muscle} - \delta_{diet}$), and dietary protein content reflected the changes observed in δ^{15}N of muscle tissue and its protein fraction (Figure 2A). This indicates that muscle δ^{15}N values are closer to those of its diet as protein intake increases. Furthermore, the muscle $\Delta\delta^{15}$N correlated inversely with growth (SGR), with those fish with lower muscle $\Delta\delta^{15}$N showing better growth

Table 3. White Muscle Proximal, Amino Acid, and Isotopic Composition of Gilthead Sea Bream Fed Experimental Diets for 12 Weeks[a]

	diet (% protein/% lipid)					
	A (41/26)	B (44/25)	C (47/23)	D (50/22)	E (54/21)	F (57/20)
Proximal Composition, g/100 g of muscle; n = 9 fish						
moisture	76.31 ± 0.29	76.44 ± 0.31	76.59 ± 0.27	76.40 ± 0.13	76.03 ± 0.16	76.75 ± 0.28
protein	20.80 ± 0.28	20.84 ± 0.25	20.85 ± 0.18	21.01 ± 0.20	21.16 ± 0.20	20.87 ± 0.30
lipid	1.93 ± 0.15	1.84 ± 0.06	1.87 ± 0.13	1.82 ± 0.12	1.76 ± 0.17	1.67 ± 0.07
glycogen	0.35 ± 0.04	0.42 ± 0.03	0.41 ± 0.04	0.39 ± 0.03	0.48 ± 0.03	0.46 ± 0.02
Amino Acid Composition, g/100 g of protein; n = 3 fish						
arginine	6.87 ± 0.03	6.84 ± 0.07	6.79 ± 0.10	6.92 ± 0.05	6.81 ± 0.03	6.74 ± 0.01
cysteine	0.43 ± 0.01	0.41 ± 0.01	0.56 ± 0.14	0.55 ± 0.06	0.50 ± 0.06	0.58 ± 0.06
histidine	2.98 ± 0.02	2.96 ± 0.01	2.90 ± 0.06	2.98 ± 0.04	2.98 ± 0.02	2.98 ± 0.03
isoleucine	4.60 ± 0.03	4.59 ± 0.05	4.48 ± 0.09	4.59 ± 0.01	4.66 ± 0.04	4.60 ± 0.00
leucine	8.21 ± 0.05	8.22 ± 0.07	8.25 ± 0.04	8.37 ± 0.02	8.29 ± 0.03	8.32 ± 0.04
lysine	10.28 ± 0.04	10.26 ± 0.09	10.28 ± 0.11	10.59 ± 0.13	10.57 ± 0.02	10.50 ± 0.04
methionine	3.40 ± 0.05	3.36 ± 0.06	3.35 ± 0.08	3.40 ± 0.02	3.34 ± 0.03	3.30 ± 0.09
phenylalanine	4.49 ± 0.02	4.47 ± 0.03	4.45 ± 0.05	4.49 ± 0.01	4.54 ± 0.02	4.50 ± 0.02
threonine	4.99 ± 0.03	4.94 ± 0.06	4.91 ± 0.07	4.98 ± 0.06	4.93 ± 0.05	4.95 ± 0.02
tyrosine	3.10 ± 0.01	3.11 ± 0.01	3.13 ± 0.06	3.19 ± 0.05	3.14 ± 0.02	3.12 ± 0.04
valine	5.30 ± 0.03 ab	5.24 ± 0.03 a	5.27 ± 0.02 ab	5.27 ± 0.04 ab	5.40 ± 0.03 b	5.38 ± 0.02 b
alanine	5.95 ± 0.09	5.96 ± 0.02	5.99 ± 0.04	5.94 ± 0.00	5.94 ± 0.03	6.03 ± 0.04
asparagine (Asn + Asp)	10.42 ± 0.26	10.55 ± 0.27	10.47 ± 0.22	10.73 ± 0.29	10.62 ± 0.19	10.69 ± 0.16
glutamine (Gln + Glu)	15.62 ± 0.04	15.79 ± 0.08	15.81 ± 0.05	15.81 ± 0.03	15.59 ± 0.06	15.60 ± 0.04
glycine	5.16 ± 0.13 a	5.20 ± 0.02 a	5.21 ± 0.06 a	4.78 ± 0.11 b	4.61 ± 0.03 b	4.66 ± 0.05 b
proline	3.46 ± 0.02	3.44 ± 0.08	3.50 ± 0.04	3.51 ± 0.07	3.51 ± 0.09	3.47 ± 0.07
serine	4.74 ± 0.03	4.65 ± 0.05	4.64 ± 0.07	4.70 ± 0.08	4.58 ± 0.01	4.68 ± 0.01
total IAA	54.65 ± 0.07	54.40 ± 0.12	54.38 ± 0.32	55.05 ± 0.14	55.16 ± 0.17	54.88 ± 0.15
total DAA	45.35 ± 0.07	45.60 ± 0.12	45.62 ± 0.32	44.95 ± 0.14	44.84 ± 0.17	45.12 ± 0.15
ratio IAA/DAA	1.21 ± 0.01	1.19 ± 0.01	1.19 ± 0.02	1.22 ± 0.01	1.23 ± 0.01	1.22 ± 0.01
Isotopic Compositio, ‰; n = 9 fish						
δ^{15}N	10.57 ± 0.09 a	10.36 ± 0.08 ab	10.50 ± 0.06 ab	10.22 ± 0.08 bc	10.02 ± 0.06 c	10.22 ± 0.04 bc
δ^{15}N protein	11.24 ± 0.06 a	10.97 ± 0.07 ab	10.94 ± 0.05 ab	10.83 ± 0.10 bc	10.66 ± 0.05 c	10.91 ± 0.05 bc
δ^{13}C	−21.39 ± 0.05	−21.45 ± 0.11	−21.37 ± 0.07	−21.45 ± 0.05	−21.61 ± 0.10	−21.30 ± 0.06
δ^{13}C protein	−21.94 ± 0.02	−22.15 ± 0.10	−22.17 ± 0.06	−22.16 ± 0.09	−22.26 ± 0.12	−22.13 ± 0.05
δ^{13}C glycogen	−21.62 ± 0.18	−21.27 ± 0.13	−21.44 ± 0.08	−21.42 ± 0.19	−21.33 ± 0.13	−21.02 ± 0.16
δ^{13}C lipid	−27.38 ± 0.05	−27.50 ± 0.03	−27.32 ± 0.07	−27.45 ± 0.08	−27.62 ± 0.05	−27.52 ± 0.05

[a]Values are expressed as mean ± SEM. Letters indicate significant differences ($p < 0.05$). IAA, indispensable amino acids; DAA, dispensable amino acids.

performance (Figure 3). It is noteworthy that no significant changes in either $\Delta\delta^{15}$N or SGR were observed between experimental groups fed more than 47% protein in diet.

We also found an inverse relationship between the isotopic fractionation of C ($\Delta\delta^{13}$C) and dietary protein content (Figure 2B) for both muscle tissue and muscle protein samples, but with some differences. For protein $\Delta\delta^{13}$C, the correlation was almost equal to that of the protein N fractionation, whereas for muscle $\Delta\delta^{13}$C, the correlation found was more pronounced with a slope more than 2 times higher. This is due to the differences in δ^{13}C values of raw diets, which decline when dietary lipid content increases (Table 1).

Principal Component Analysis. PCA analysis of the whole data set (growth, feed, and body parameters together with muscle proximal, amino acid, and isotopic composition) provided good discrimination between experimental groups (Figure 4) on the basis of the dietary protein-to-lipid ratio. Factor 1 provided the greatest discrimination, accounting for over a third of total data variability (Table 4). The variables with the highest load on the first factor (Table 5) were related mainly to muscle isotopic fractionation (muscle $\Delta\delta^{13}$C, protein $\Delta\delta^{15}$N, muscle $\Delta\delta^{15}$N, and protein $\Delta\delta^{13}$C), growth parameters (SGR, PER, and FCR), and N isotopic composition of muscle (protein δ^{15}N and muscle δ^{15}N).

DISCUSSION

All experimental groups were fed ad libitum to avoid dietary restrictions. However, fish fed on low-protein/high-lipid diets grew less. The negative effect of high-lipid diets on growth has been previously reported in gilthead sea bream. It was related to the ingestion of excessive energy, which reduces food consumption.[36] This is not the case in our study, since no excess energy intake was recorded between groups. However, the increase in HSI observed in fish on high-lipid diets would indicate fat deposition in liver. In this regard, steatosis in liver has been described in gilthead sea bream that were fed diets containing 22% lipid or higher,[37] with the negative physiological consequences involved.[38] This could explain the lower feed intake observed in group B (44% protein/25% lipid) since the total lipid content of the diet negatively affects food intake in rainbow trout juveniles,[39] possibly because of the negative feedback on appetite of the accumulation of lipids in depots (lipostatic control). On the other hand, the feed conversion ratio (FCR) suggests that all our experimental diets had a

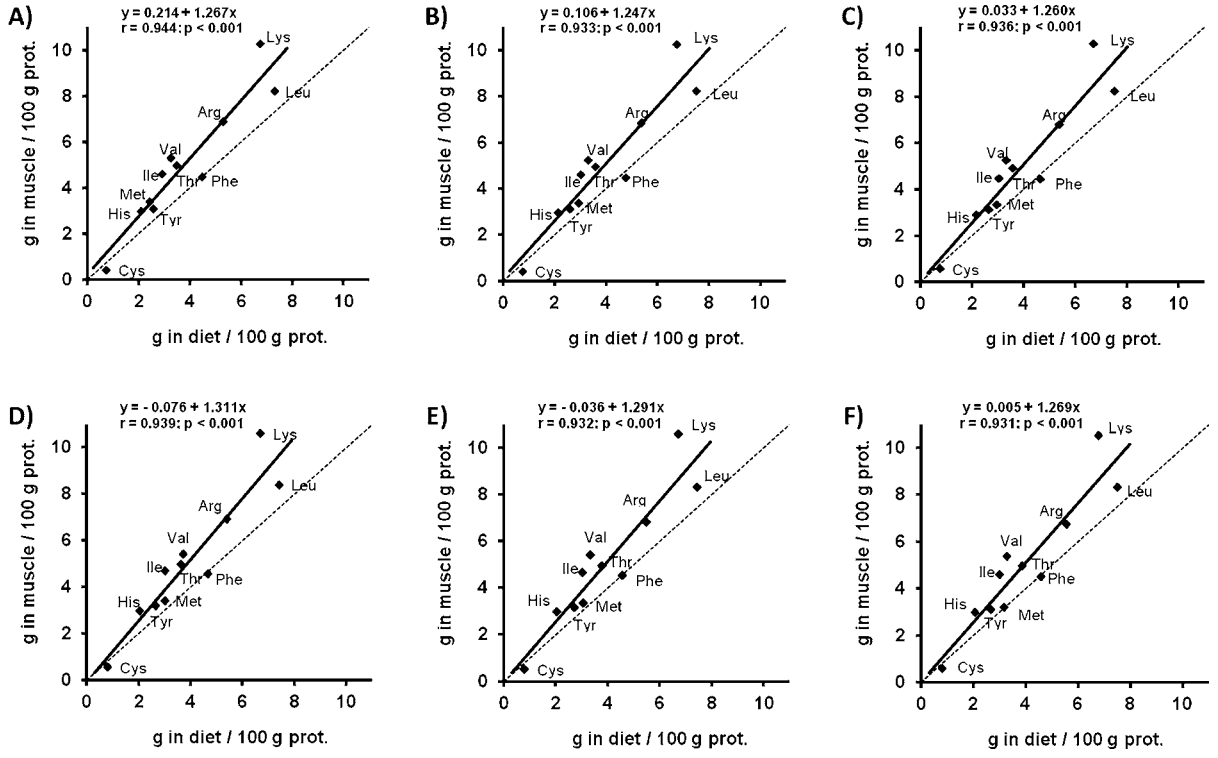

Figure 1. Comparison between indispensable amino acid (IAA) profiles of muscle from gilthead sea bream juveniles and those of the experimental diets (panel A, diet A, to panel F, diet F). Linear regression (bold line) corresponds to the observed relationship, which can be compared with the line of equality (dotted line). Points above the line of equality suggest restrictions for that amino acid in the food. Each point represents a mean of three fish.

similar degree of digestibility, even though no proper analysis was performed. This is supported by several studies of gilthead sea bream[8] and other fish species[40] reporting that increased dietary protein or dietary lipid does not affect digestibility. Indeed, feed efficiency was not altered by dietary protein in sea bream juveniles when diets contained at least 21% lipid level, with the digestibility of both components high (between 92% and 95%).[8] The proportionality kept between different protein sources also suggests similar digestibility, since previous studies in gilthead sea bream have demonstrated that the two plant ingredients used in our experimental diets show high digestibility in their protein fraction (82% for wheat flour and 87% for soybean meal), similar to that of fish meal (83%).[41] In the same study, the digestibility of carbohydrates was high (87−90%), similar to that of lipid (93−95%) and protein (81−88%) when wheat was the main carbohydrate source in diets, as in the present study. Furthermore, the inclusion in diet of high levels of wheat does not reduce the high digestibility of this cereal or affect the growth of this species.[11] The AA profiles of experimental diets also show similar digestibility, since they were formulated to give an AA composition comparable to the optimum requirements calculated for gilthead sea bream juveniles.[42] This is reflected in the similar AA balance between muscle and diets of experimental groups. Glutamine, asparagine, and lysine were the most abundant AA found in muscle, as observed in other studies analyzing muscle[43] and whole body[44] in gilthead sea bream. Furthermore, it has been stated that the main essential amino acids in marine aquatic organisms were lysine, leucine, and arginine,[45] as in the present study, indicating the high quality of the aquaculture products as protein sources.

In terms of protein efficiency, PER values of our study indicate that low-protein/high-lipid diets perform significantly better. This is consistent with results reported by Vergara et al.,[4] who observed an increase in PER for increasing dietary lipid (from 9% to 15%) and decreasing dietary protein contents (from 58% to 46%), but weight gain was improved with the higher protein diets for gilthead sea bream fingerlings. The same pattern was observed by Santinha et al.,[6] who found that 55% dietary protein gave the best growth in gilthead sea bream juveniles, but 40% protein diet showed the highest protein efficiency. The explanation is that the highest protein retention efficiency is generally achieved under restricted protein feeding, as previously shown in gilthead sea bream.[9] A protein-sparing effect of lipids was also observed in further studies carried out in gilthead sea bream.[7−10] The increasing amount of carbohydrate content in low-protein diets in our study also contributed to spare protein, as reported in other studies carried out in the same species.[11−14]

Differences in the dietary protein-to-lipid ratio between experimental groups modified the muscle isotope composition, especially δ^{15}N, which is directly linked to protein changes, since almost all the N in an organism derives from the protein fraction. Changes in muscle δ^{15}N mirrored those in protein δ^{15}N, with the latter values being higher since lipid extraction during protein purification led to <1‰ isotope shift in δ^{15}N.[46] The inverse relationship found between muscle δ^{15}N and dietary protein content showed differences in nitrogen balance between experimental groups. Tissue enrichment in δ^{15}N has been observed in fish subjected to conditions of changes in protein turnover, such as fasting or restricted protein intake,[26−29] since ^{14}N is preferentially excreted during deamination and transamination whereas ^{15}N is retained in

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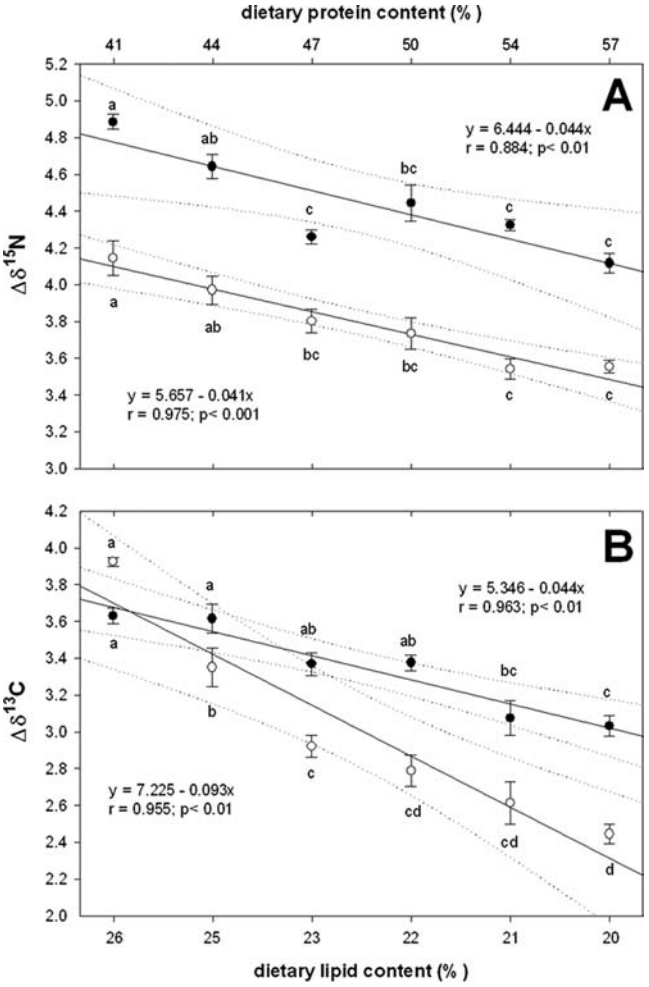

Figure 2. Relationship between dietary protein and lipid content and muscle isotopic fractionation (A, $\Delta\delta^{15}N$; B, $\Delta\delta^{13}C$). Values are represented as the mean and SEM (error bars) of nine fish (letters indicate significant differences, $p < 0.05$). (○) Tissue values; (●) protein values. Linear regressions (—) are indicated together with 95% confidence interval (···).

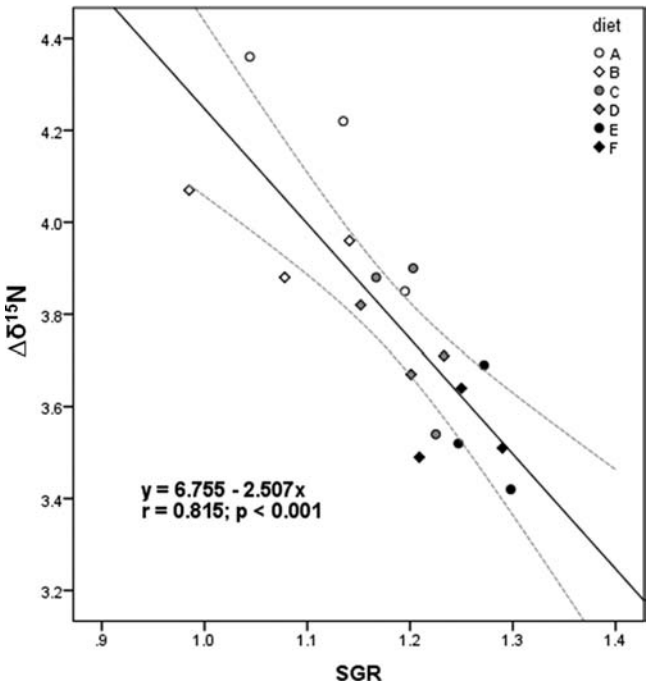

Figure 3. Relationship between SGR and $\Delta\delta^{15}N_{muscle}$. Each point represents the SGR and $\Delta\delta^{15}N$ values of triplicate tanks from each experimental group ($\Delta\delta^{15}N$ values are the mean of 3 individuals analyzed per tank). Linear regression (—) is indicated, together with 95% confidence interval (···).

newly synthesized AAs and proteins.[47,48] Therefore, we posit that the higher muscle $\delta^{15}N$ values observed in fish fed low-protein diets in our study were caused by higher protein turnover in muscle induced by a restriction in the balanced input of AAs in muscle. Indeed, fish fed low-protein diets showed more dispensable glycine and less indispensable valine in muscle. These differences in muscle AA profile would also indicate that fish on low-protein diets have higher AA turnover, as a result of the lower dietary availability of AAs, than fish on high-protein diets do.

The C isotopic composition of muscle, unlike $\delta^{15}N$, showed no changes despite the isotopic differences in $\delta^{13}C$ observed in experimental diets. The increasing $\delta^{13}C$ values of high-protein/low-lipid diets respond to changes in fat content, since lipids are ^{13}C-depleted compared to other components.[49] The lack of differences in $\delta^{13}C$ values of each fraction (protein, lipid, and glycogen) in muscle and in diet among experimental groups suggests a similar transformation of the dietary components through their incorporation into muscle reserves. This finding could be explained by a low differential allocation of isotopically distinct dietary components (i.e., "isotopic routing").[50] Unchanged values in $\delta^{13}C$ of muscle energy reserves (i.e., glycogen and lipid) also point to a similar catabolic use of them since their enhanced use leads to isotopic enrichment of tissue reserves as observed in the white muscle of gilthead sea bream under exercise training.[51]

Isotopic fractionation ($\Delta\delta = \delta_{tissue} - \delta_{diet}$), like the effect of all physiological processes that lead to differences between an animal and its diet, varies in function of changes in tissue composition and physiological state of individuals. The N and C isotope fractionation values found in our experiment were consistent with previously reported values in gilthead sea bream.[30] However, in the case of ^{15}N, fractionation values ($\Delta\delta^{15}N_{muscle\text{-}diet}$) were relatively high due to the low $\delta^{15}N$ values of experimental diets. This is explained by the high levels of protein (about 50%) from plant-derived ingredients, which are depleted in ^{15}N relative to fish meal protein.[30] Martinez del Rio and Wolf[52] predicted that, on one hand, $\Delta\delta^{15}N$ should increase with dietary protein content and, on the other, $\Delta\delta^{15}N$ should decrease with the increase of N deposition efficiency (measured as the ratio between protein assimilation and protein loss). The same authors indicated later that the former prediction had not been empirically supported in a consistent way since the variation in magnitude of $\Delta^{15}N$ depends not only on protein intake but also on protein quality and thus protein use efficiency.[24] So the inverse relationship between $\Delta\delta^{15}N$ and dietary protein content observed in the present study, in which dietary protein quality remained constant, indicates a higher deposition of dietary protein in muscle of fish fed high-protein diets. This implies that protein accretion increases as ^{15}N fractionation decreases, because more dietary AAs are used directly for protein synthesis. Thus, the $\delta^{15}N$ values of the fish approach the isotopic values of the diet, as observed in carp[28] and tilapia.[26,27,31] In support of this assumption, ^{15}N and ^{13}C fractionation of the protein fraction showed similar behavior to

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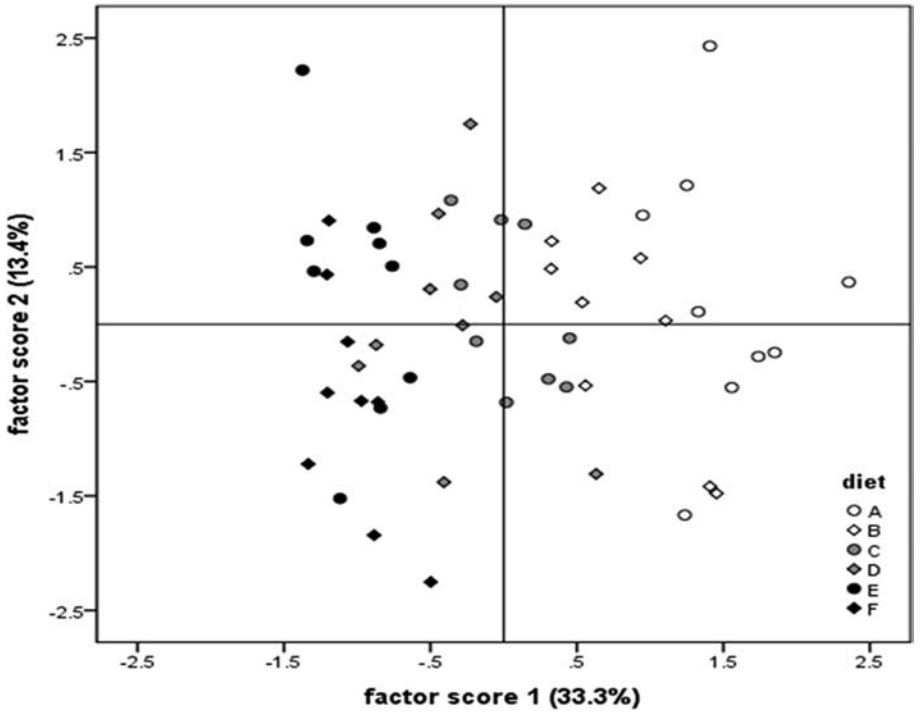

Figure 4. Principal component analysis (PCA) plot of feeding, growth, and muscle proximal, amino acid, and isotopic composition data from gilthead sea bream juveniles fed experimental diets. Factors 1 and 2 represent the first and second principal components, with the percentage of explained variance indicated in parentheses.

Table 4. Eigenvalues for PCA of Muscle Composition, Feed, and Growth Parameters from Gilthead Sea Bream Juveniles under Different Dietary Conditions

	eigenvalue	variability, %	cumulative eigenvalue	cumulative variability, %
F1	6.988	33.276	6.988	33.276
F2	2.813	13.397	9.801	46.674
F3	2.208	10.513	12.009	57.187
F4	1.798	8.564	13.807	65.750
F5	1.340	6.383	15.147	72.133
F6	1.042	4.964	16.189	77.097

Table 5. PCA Factor Loadings of Variables Measured in Gilthead Sea Bream Juveniles under Different Dietary Conditions[a]

variable	F1	F2
$\Delta\delta^{13}$C muscle	**0.906**	0.004
$\Delta\delta^{15}$N protein	**0.853**	−0.038
$\Delta\delta^{15}$N muscle	**0.838**	0.157
$\Delta\delta^{13}$C protein	**0.808**	−0.140
SGR	**−0.778**	−0.038
PER	**0.766**	0.145
δ^{15}N protein	**0.764**	−0.289
FCR	**0.739**	0.140
δ^{15}N muscle	**0.695**	0.103
HSI	0.571	0.445
CF	0.481	0.084
glycogen	−0.397	0.305
moisture	0.077	**−0.784**
δ^{13}C protein	0.267	**−0.642**
lipid	0.152	0.541
δ^{13}C glycogen	−0.196	−0.478
δ^{13}C lipid	0.298	0.448
δ^{13}C muscle	0.443	−0.310
protein	−0.171	0.560
MFI	0.002	0.285

[a]Numbers in boldface type indicate loadings >0.6.

muscle $\Delta\delta^{15}$N. Moreover, we found that the fish growth rate (as an indirect calculation of muscle growth and hence protein gain) was inversely related to muscle $\Delta\delta^{15}$N, which strengthens this conclusion. This has also been found in blue crabs[53] and salmon.[54] Recently, in tilapia fingerlings, Kelly and Martinez del Rio[32] found a positive asymptotic relationship between muscle $\Delta\delta^{15}$N and dietary protein content when protein quality remained constant. In their experiment, the dietary protein content of the groups (3.75%, 7.5%, 15%, and 30%) was below the optimum protein level (35%) for tilapia fry.[55] In contrast, the protein content of our experimental diets was around the optimum values for gilthead sea bream juveniles.[3−8] Unlike muscle $\Delta\delta^{15}$N, the $\Delta\delta^{13}$C values of muscle tissue are due not only to the protein fraction but also to the increase in δ^{13}C of raw diets as dietary fat content decreased, as mentioned above.

The PCA plot shows a clear distinction among groups according to the gradation of the protein-to-lipid content of experimental diets. Groups with lower protein intake and growth are clearly separated from the rest, protein-related isotopic variables being those with the highest load in the main discrimination factor. These results indicate C (47% protein/23% lipid) as the optimal diet for aquaculture purposes, as it combines high SGR with high PER, resulting in high protein deposition efficiency in fish muscle and moderate protein turnover. From this proportion on, no differences were observed in either fish growth or ^{15}N fractionation of muscle, as explained above. Therefore, $\Delta\delta^{15}$N may indicate the state where catabolism and recycling of protein increase significantly in muscle due to the decrease in the amount of dietary protein.

Beyond this threshold, the dietary protein-to-lipid ratio is not enough to maintain maximal growth. The optimal dietary proportions deduced from this study are consistent with established optimum values of these components in gilthead sea bream.[5−10,36,37] Therefore, our results indicate that the protein-sparing effect of other dietary energy sources, such as lipids and carbohydrates, could be primed by a slight decrease in the dietary protein-to-energy ratio. In summary, the present data show that stable isotope analysis (especially $\Delta\delta^{15}$N, which responds to changes in protein metabolism) is a reliable indicator of nutritional status in fish. Therefore, it may be a valuable and complementary tool to take into account when determining the optimal nutritional conditions for farmed fish.

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Notes

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