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Stable Isotope Analysis Combined with Metabolic Indices Discriminates between Gilthead Sea Bream (*Sparus aurata*) Fingerlings Produced in Various Hatcheries

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ABSTRACT: There are few traceability systems other than genetic markers capable of distinguishing between sea products of different origin and quality. Here, we address the potential of stable isotopes combined with metabolic and growth parameters as a discriminatory tool for the selection of fish seeds with high growth capacity. For this purpose, sea bream fingerlings produced in three hatcheries (Spanish Mediterranean coast, MC; Cantabrian coast, CC; and South-Iberian Atlantic coast, AC) were subjected to isotopic analysis (δ^{15}N and δ^{13}C), and indices of growth (RNA and DNA) and energy metabolism [cytochrome-*c*-oxidase (COX) and citrate synthase (CS) activities] were calculated. These analyses were performed prior to and after a "homogenization" period of 35 days under identical rearing conditions. After this period, fingerlings were discriminated between hatcheries, with isotopic measures (especially δ^{15}N), metabolic parameters (COX and CS), and proximal composition (fat content) in muscle providing the highest discriminatory capacity. Therefore, particular rearing conditions and/or genetic divergence between hatcheries, affecting the growth capacity of fingerlings, are defined mainly by the isotopic imprint. Moreover, the muscle isotopic signature is a more suitable indicator than whole fish for discrimination purposes.

KEYWORDS: Sea bream (*Sparus aurata*), δ^{13}C/δ^{15}N, isotopic fractionation, RNA:DNA, citrate synthase, cytochrome-*c*-oxidase, growth capacity

INTRODUCTION

Gilthead sea bream (*Sparus aurata*) aquaculture was first introduced in the 1970s, and it is now the Mediterranean species most widely farmed in this way. Given the market relevance of this relatively new field, the traceability of fishery and aquaculture products is becoming increasingly important. Recently, intense efforts have been devoted to developing analytical tools to distinguish between farmed fish and those caught in the wild. In particular, stable isotope analysis (SIA), often combined with fatty acid composition, has been used successfully to discriminate between cultured and wild gilthead sea bream[1−3] and in studies on marine fish species like salmon,[4] sea bass,[5] and turbot.[6] The differences between farmed and wild fish presumably reflect variations in diet, which have a major influence on isotopic profiles because natural food and commercial diets differ in their isotopic ratios. In particular, the ratios of carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotopes, derived directly from the diet,[7,8] are extremely valuable when attempting to associate a specific animal with a particular food source, often being characteristics for production systems and feeding intensity. Furthermore, given their dependence on environmental water conditions, δ^{18}O and δ^{2}H are very useful for determining the geographical origin of fish.

Little information is available about the differentiation between fish from different farms on the basis of rearing systems or region. Muscle δ^{15}N is correlated with the geographical origin of farmed sea bream,[2] but this relationship seems to be more related to the feed ingredients available in the farming areas than to the geographical origin itself. Stable isotope ^{15}N analysis also allows discrimination between fish fed an artificial diet formulated with plant protein and those receiving fish protein.[9] It was recently reported that stable isotopes are the most informative variables in discriminating between fish from distinct farms.[10] In particular, measures of δ^{13}C or δ^{15}N in flesh can be linked with those of a specific commercial diet, while δ^{18}O can be used to associate fish with a specific water source. Thus, the combined information from these isotopes allows discrimination between fish origins. In addition, tissue fatty acid and proximate compositions, and several morphological parameters are useful to distinguish between farmed and wild fish;[1] however, these parameters are less informative for discriminating fish origin.[10]

The quality of fish produced by aquaculture varies greatly between farms and is influenced mainly by the quality of the rearing environment, the quantity, quality, and management of feed, and the culture methods used.[11,12] Similarly, variability in fish isotopic parameters is related to environmental factors and culture conditions, such as temperature, meal composition, and ration.[13,14] Therefore, while it is now possible to have a high degree of confidence in defining whether fish are wild or farmed, it is not as straightforward to determine their geographical origin or growth potential. There is an increasing need to develop

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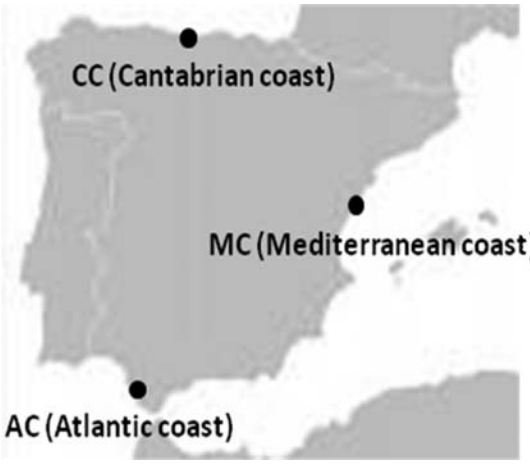

Figure 1. Schematic map of the Iberian Peninsula with the location of the three sea bream hatcheries.

analytical methods to discriminate between fish reared in distinct systems in different locations. Furthermore, the capacity to discriminate between fish "seeds" on the basis of growth performance is highly relevant for the aquaculture industry. Recent studies show that isotopic fractionation of ^{15}N varies inversely with growth in salmon[15] and that increases in ^{15}N are related to stress conditions, such as fasting[16] and low protein diets.[13] Therefore, SIA combined with other complementary physiological variables that are well established as metabolic indicators, such as nucleic acid content and enzyme activities, may be a useful approach to determine the quality of fish seeds. The RNA:DNA ratio (the amount of RNA per cell unit) is indicative of the status of the cell transcription rate, whereas the RNA:protein ratio is often used as an indicator of protein synthesis capacity.[17] The aerobic capacity of fish muscle is estimated by the activities of mitochondrial enzymes, such as cytochrome-*c*-oxidase (COX) and citrate synthase (CS), and both activities have been positively correlated with growth rate in many fish species.[18,19]

Most of the studies dealing with fish "origin" discrimination have focused on the isotopic analysis of fish and environmental factors (mainly food and water). However, no study has addressed the relationship between the isotopic composition of fish and metabolic and growth variables, an approach that goes beyond the discrimination of geographical origin.

Here, we sought to discriminate gilthead sea bream fingerlings from distinct hatcheries on the basis of SIA ($\delta^{15}N$ and $\delta^{13}C$) combined with metabolic (CS and COX activities) and growth parameter (RNA and DNA concentration) analyses to select the fish seed with the highest growth potential. To ensure that the rearing conditions (food composition and ration, temperature, etc.) did not interfere in the discrimination of the seeds with the highest growth potential, we analyzed these parameters before and after submitting fingerlings of the three farms to equal rearing conditions (homogenization period) for 35 days, a period long enough to circumvent the influence of these effects. To the best of our knowledge, this is the first study to address the use of SIA for this purpose. Muscle is one of the organs that reflect the largest isotopic variations;[20] therefore, it is one of the most suitable tissues in which to study differences between fish fed in a range of "isotopic-labeling" conditions. This tissue tends to integrate isotopic signatures of food assimilated over a longer period than faster metabolic tissues like liver.[21] However, we also analyzed whole fish to determine the contribution of muscle to the body isotopic composition.

Table 1. General Characteristics of the Three Sea Bream Hatcheries and Proximate (mg g^{-1}) and Isotopic (‰) Composition of the Commercial Diets Used

	hatchery		
	MC	CC	AC
water temperature (°C)	20.5	24	19
ration size[a]	4.5	6	3
diet digestible energy (MJ/kg)	21	20	19.6
diet proximate composition[b]			
protein	54	56	55
lipid	18	18	18
ash	11	10	11
NFE[c]	17	16	16
diet isotopic composition (‰)[d]			
$\delta^{15}N$ bulk diet	12.4 ± 0.2 a	9.4 ± 0.1 c	10.8 ± 0.3 b
$\delta^{15}N$ protein	12.8 ± 0.1 a	10.0 ± 0.1 c	10.8 ± 0.1 b
$\delta^{13}C$ bulk diet	−20.9 ± 0.1 a	−21.0 ± 0.1 a	−21.8 ± 0.2 b
$\delta^{13}C$ protein	−20.7 ± 0.1 a	−20.6 ± 0.1 a	−22.1 ± 0.2 b
$\delta^{13}C$ lipid	−23.8 ± 0.1 a	−24.6 ± 0.1 c	−24.1 ± 0.1 b

[a] g food ×100 g body weight^{-1} × day^{-1}. [b] g × 100 g^{-1} dry matter. [c] NFE, nitrogen free extract calculated by difference. [d] Values are reported as means ± SEMs (N = 3). Values with different letters in each row are significantly different (Tukey's posthoc test, $P < 0.05$).

MATERIALS AND METHODS

Animal, Experimental Conditions, and Sampling Protocol. In May 2007, gilthead sea bream (*S. aurata*) fingerlings (3.5−4.5 g) were obtained from three farms in Spain specialized in sea bream fingerling production. Hatcheries were located far from each other (Mediterranean coast, MC; Cantabrian coast, CC; and South-Iberian Atlantic coast, AC) (Figure 1) and differed in rearing conditions (Table 1). Thirty fish from each farm were frozen in situ, transported in dry ice, and stored at −80 °C until required. Frozen fish were dissected on a tray set on ice, and muscle tissue samples consisting of the entire dorsal fillet above the lateral line were obtained. Because of the small size of the fish, 15 animals were pooled into three groups for further analyses in muscle and whole body samples to determine the conditions in the hatcheries.

Three hundred fingerlings from each hatchery were transported to our facilities (Faculty of Biology, University of Barcelona, Spain) and randomly distributed with a similar density (∼1 kg/m^3) into three 200 L fiberglass tanks per condition. Fish were reared with filtered seawater in a closed system with physical and biological filters, ozone skimmers, and continuous aeration under controlled temperature (22 ± 0.2 °C), salinity (31−38‰), oxygenation (<90% saturation), and photoperiod (12 L/12D). Before starting the experiment, fish were acclimated to the experimental facilities for 2 weeks, during which they were fed hatchery diets. During the 35 day homogenization period, they were fed to satiation (nearly to 3% of body weight) three times, and the food intake was measured daily. Although the compositions of the farm diets were isoproteic, isolipidic, and isoenergetic, the diet from the MC hatchery was selected for the experiment because of its higher quality (the highest values of $\delta^{15}N$, Table 1). After the experimental period, 18

animals from each hatchery (six fish per tank) were fasted for 24 h and then killed by severing the spinal cord. Entire dorsal fillet samples were taken from nine fish, while the other nine fish were kept intact. Muscle and whole fish samples were immediately frozen in liquid nitrogen and 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.

Growth Performance. Fish from each tank were bulk weighed weekly to readjust feed ration and to control growth. Food intake was recorded daily and specific growth rates (SGR) were calculated as follows:

$$\text{SGR}\ (\%\ \text{day}^{-1}) = 100 \times (\ln W_2 - \ln W_1)/t$$

where W_1 and W_2 are the initial and final weight of animals, respectively, after t days.

Principal Components of Tissue Samples and Diets. Diets and muscle and whole fish samples were homogenized in liquid nitrogen using a pestle and mortar to obtain a fine powder. The tissue water content was determined gravimetrically after drying the samples at 100 °C for 24 h. The total lipid content was purified from two methanol−chloroform (2:1) extractions. The washed lipid extracts were dried under N_2, and the lipid content was determined gravimetrically. Protein purification was carried out using defatted samples via precipitation with trifluoroacetic acid (100 mL L^{-1}) and after centrifugation at 1060*g* for 30 min. The protein content was calculated from the nitrogen obtained by elemental analysis (Elemental Analyzer Flesh 1112, ThermoFinnigan, Bremen, Germany) as follows:

$$\text{g protein}/100\ \text{g dry matter} = (\text{g N}/100\ \text{g dry matter}) \times 6.25$$

δ^{15}N and δ^{13}C Determination. Samples of diets and whole tissues were lyophilized and ground into a homogeneous powder for isotopic analysis. Aliquots (from 0.3 to 0.6 mg) from these samples, as well as their purified lipid and protein, were accurately weighed in small tin capsules (3.3 mm × 5 mm, Cromlab, Barcelona, Spain). Samples were analyzed to determine the carbon and nitrogen isotope composition using a Mat Delta C IRMS (Finnigan MAT, 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. δ 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 as follows: 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 compensate for machine drift and as a quality control measure. The same reference material examined over the analysis period was measured with ±0.2‰ precision. Nitrogen and carbon isotopic fractionation ($\Delta\delta^{15}N$ and $\Delta\delta^{13}C$) values were calculated as the difference between δ tissue and δ diet.

Measurements of RNA/DNA Ratios. Bulk RNA and DNA contents were determined in muscle using a modification of the Schimdt−Thannhauser UV-based method,[22] following the procedures for fish samples described Buckley and Bulow.[23] Muscle samples were removed from liquid nitrogen and homogenized with a polytron (Kinematica, Luzern, Switzerland) in cold 0.2 N $HClO_4$ to precipitate nucleic acids. Centrifugation was done at 12000*g* for 15 min at 4 °C, and the supernatant was discarded. The process was repeated to effectively remove free nucleotides, amino acids, and other acid-soluble compounds. The resulting sediment was resuspended in dilute alkali (0.3 N NaOH), which also achieved hydrolysis of RNA and its chemical partitioning from protein and DNA. The hydrolysate was acidized with cold 1.32 N $HClO_4$ to remove RNA from DNA and protein. DNA was then hydrolyzed and separated from the remaining protein by addition of hot 0.6 N $HClO_4$. RNA and DNA were estimated from the absorbance of the appropriate hydrolysate at 260 nm using the following extinction coefficient: A_{260} of a 1 μg mL^{-1} solution of hydrolyzed RNA or DNA is 0.3. The absorbance was measured using a using a spectrophotometer (Spectronic Genesys 2/Milton Roy Co., Rochester, NY). Nucleic acid contents were recorded as μg RNA or DNA per mg wet tissue.

Table 2. Weight, Feed Intake, Feed Efficiency, and Growth Rate of Fingerlings from the Three Hatcheries during the 35 Days of the Experiment[a]

	hatchery		
	MC	CC	AC
initial weight (g)	4.63 ± 0.03 a	3.40 ± 0.05 b	3.45 ± 0.20 b
final weight (g)	9.10 ± 0.51 a	6.50 ± 0.33 b	6.03 ± 0.29 b
feed intake[b]	2.54 ± 0.02 b	2.68 ± 0.04 a	2.69 ± 0.02 a
feed efficiency[c]	0.87 ± 0.05	0.73 ± 0.06	0.58 ± 0.01
SGR[d]	1.96 ± 0.10	1.80 ± 0.10	1.37 ± 0.04

[a] Values are reported as means ± SEMs (N = 3 tanks). Values with a different letter in each row are significantly different (Tukey's posthoc test, $P < 0.05$). Results without any letter indicate no significant differences. [b] Feed intake: g 100 × g body weight^{-1} × day^{-1}. [c] Feed efficiency: g fish weight gain × g feed offered^{-1}. [d] SGR = 100 × [(Ln final weight − Ln initial weight) × days^{-1} of experiment].

Muscle Enzyme Assays. Enzyme activities were assayed from crude extracts of muscle obtained by homogenizing frozen tissue (50 mg/mL) in detergent solution (1.24 mM TRITON X-100, 1 mM EDTA, and 1 mM $NaHCO_3$) and stabilizing solution (0.27 M EDTA and 5 mM 2-β-mercaptoethanol), 1:1 v/v. Homogenates for measuring CS (EC 2.3.3.1) and COX (EC 1.9.3.1) activity were centrifuged at 700*g* at 4 °C for 10 min. The CS activity was determined from absorbance increases at 412 nm of DTNB reagent, using oxalacetic acid as the substrate, following the method described by Srere.[24] The COX activity was obtained by adapting a commercial kit (CYTOC-OX1, Sigma-Aldrich Inc., St. Louis, MO). This colorimetric assay measures the decrease in ferrocytochrome *c* absorbance caused by oxidation of the latter by COX.

Statistical Analysis. One-way analysis of variance (ANOVA) was used to test significant differences among fish from the three origins, and Tukey's posthoc test was used when significant differences were found. Initial and final data for each group were compared by an independent two-sample *t* test. The Shapiro−Wilk test was previously used to ensure the normal distribution of data, and the equality of variances was determined by statistical Levene's test. The relationships between lipid content and $\delta^{13}C$ of whole body and muscle tissue were tested using linear regression. Principal component analysis (PCA) was performed to study the structure of the data. 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 using commercial software (SPSS 16.0, SPSS Inc., Chicago, IL).

Table 3. Initial (Hatchery) and Final (After the Homogenization Period) Whole Fish and Muscle Composition (g 100 g^{-1}) of Sea Bream Fingerlings from the Three Hatcheries[a]

		whole fish		muscle	
		initial	final	initial	final
protein	MC	14.1 ± 0.5	15.4 ± 0.3	16.0 ± 0.6	17.3 ± 0.4 ab
	CC	13.7 ± 1.2	15.1 ± 0.4	17.3 ± 0.1	18.3 ± 0.3 a
	AC	14.9 ± 0.6	16.2 ± 0.4	15.2 ± 0.8	16.5 ± 0.5 b
lipid	MC	8.1 ± 0.3 a	9.2 ± 0.6 a	4.9 ± 0.3 a	5.2 ± 0.4 a
	CC	5.5 ± 0.5 b	8.3 ± 0.5 ab,*	3.4 ± 0.1 b	4.2 ± 0.2 a,*
	AC	3.8 ± 0.3 c	6.9 ± 0.4 b,*	2.3 ± 0.2 c	3.1 ± 0.1 b,*
moisture	MC	73.6 ± 0.1 b	71.3 ± 0.3 b,*	76.0 ± 0.6 b	74.8 ± 0.4 b
	CC	76.6 ± 0.1 a	72.3 ± 0.6 ab,*	77.4 ± 0.3 ab	75.1 ± 0.3 b,*
	AC	77.4 ± 0.5 a	73.3 ± 0.4 a,*	78.4 ± 0.6 a	77.0 ± 0.2 a,*

[a] Values are reported as means ± SEMs (initial $N = 3$ pools of five fish; final $N = 9$ fish). Values with a different letter in each row are significantly different (Tukey's posthoc test; $P < 0.05$), and an asterisk denotes significant differences (t test; $P < 0.05$) after (initial) and before (final) the homogenization period. Results without any letter or symbol indicate no significant differences.

RESULTS

Growth Parameters and Changes in the Proximate Composition. Although no significant differences in SGR or feed conversion efficiency were observed between groups, AC fingerlings showed the lowest values of these two variables (Table 2). Consistent with the greater body mass at the beginning and end of the experiment, the MC group showed the highest lipid content in whole fish and muscle throughout the homogenization period. In contrast, the smallest AC fingerlings also showed the lowest lipid content (Table 3). During the homogenization period, the lipid content of whole fish and muscle samples from the AC and CC groups increased significantly. At the end of this period, the CC group registered the highest muscle protein content, while the AC group showed the lowest.

Isotopic Composition and Fractionation. The stable isotope composition of the commercial diets used by the three hatcheries varied because of the dietary ingredients used in the formulation (Table 1). Differences in δ^{15}N of bulk diet reflected those of the dietary protein. These differed as much as 3‰ between the MC (12.4‰) and the CC (9.4‰) diets. δ^{13}C varied between diets, with the AC diet showing the lowest values (−21.8‰). These changes also paralleled the variation found in protein δ^{13}C because protein was the major dietary component. However, variations in lipid δ^{13}C differed between the three diets. Initial δ^{15}N of whole fish samples did not differ significantly among the three groups (Figure 2). However, the MC group showed the highest muscle δ^{15}N, while the AC group showed the lowest. After submitting all three groups to identical rearing conditions for 35 days, the δ^{15}N of whole fish and muscle samples increased in a similar manner (∼2‰); thus, the initial differences between the groups were maintained. However, no differences in δ^{13}C of whole fish or muscle samples were found at the beginning of the experiment. After the homogenization period, the δ^{13}C of whole fish and muscle also increased in all groups but was significant only in the latter (∼0.7‰). Nevertheless, whole MC fish continued to show lower δ^{13}C of bulk tissue than AC fish ($p < 0.05$) because of the lower lipid content of the latter.

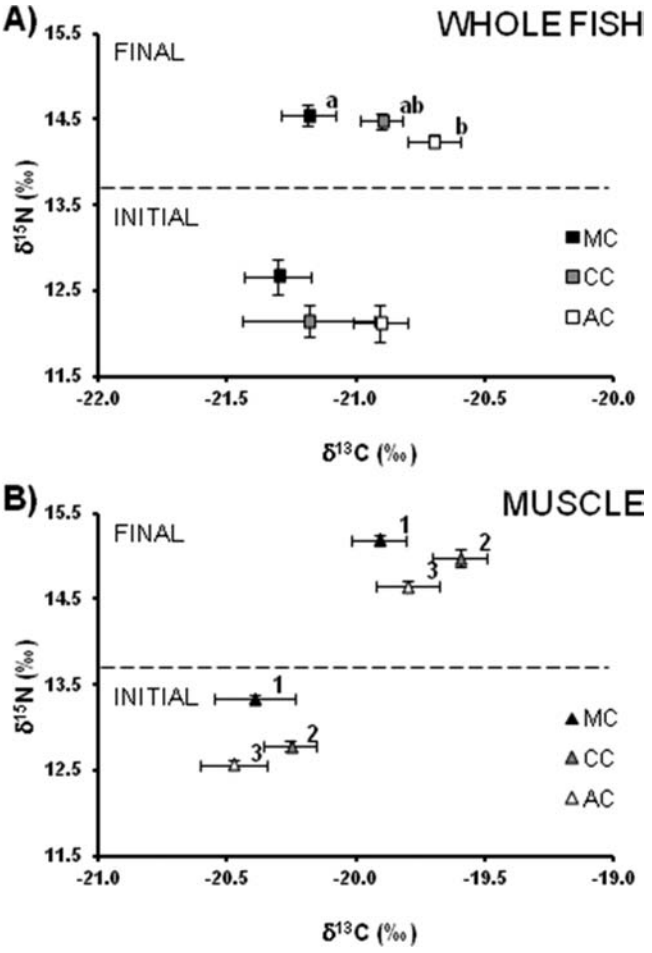

Figure 2. Evolution of the isotopic signature of bulk samples of whole fish (A) and muscle (B) of sea bream fingerlings produced in three hatcheries during the homogenization period. Symbols are reported as means and bars as SEM (initial $N = 3$ pools of five fish; final $N = 9$ fish per group), and a different letter indicates significant differences in δ^{13}C ($P < 0.05$), while different numbers indicate significant differences in δ^{15}N ($P < 0.05$).

Protein δ^{15}N also increased significantly and reflected the same differences observed in bulk δ^{15}N, showing the AC group with the lowest levels ($p < 0.05$) in whole fish and muscle samples throughout the homogenization period (Table 4). Similarly, protein δ^{13}C showed the same pattern as protein δ^{15}N but only in whole fish was the increase in protein δ^{13}C significant. In contrast, lipid δ^{13}C increased significantly in whole fish and muscle samples throughout the homogenization period, and initial differences disappeared. The changes in lipid content modified bulk δ^{13}C, showing an inverse relationship between these variables in whole fish ($r = 0.589$, $n = 27$, and $p < 0.001$) and in muscle ($r = 0.532$, $n = 27$, and $p < 0.005$) samples after homogenization (Figure 3).

The differences in δ^{15}N between the diets were greater than those observed in fish samples. Thus, the isotopic fractionation of nitrogen ($\Delta\delta^{15}$N: $\delta^{15}N_{tissue} - \delta^{15}N_{diet}$) in initial hatchery conditions was highest ($p < 0.05$) for the CC group (2.71 and 3.34‰) and lowest for the MC group (0.25 and 0.93‰) in whole fish and muscle, respectively (Table 4). After the homogenization period with the same diet and ration, $\Delta\delta^{15}$N converged to ∼2 and ∼2.5‰ for these tissues, respectively, decreasing in the

Table 4. Initial (Hatchery) and Final (After Homogenization Period) Stable Isotope Ratios (δ^{13}C and δ^{15}N) of Reserves and Isotopic Fractionation ($\Delta\delta^{13}$C and $\Delta\delta^{15}$N) of Bulk Samples in Whole Fish and Muscle of Sea Bream Fingerlings from the Three Hatcheries[a]

		whole fish		muscle	
		initial	final	initial	final
		isotopic composition of reserves			
protein δ^{15}N	MC	14.12 ± 0.12 a	15.44 ± 0.06 a,*	14.19 ± 0.10 a	16.36 ± 0.04 a,*
	CC	12.86 ± 0.05 b	15.49 ± 0.08 a,*	13.75 ± 0.03 b	16.21 ± 0.06 a,*
	AC	13.29 ± 0.18 b	15.19 ± 0.06 b,*	13.55 ± 0.07 b	15.96 ± 0.08 b,*
protein δ^{13}C	MC	−19.52 ± 0.01 a	−19.33 ± 0.05 a,*	−19.69 ± 0.04 a	−19.85 ± 0.04 a
	CC	−19.82 ± 0.04 b	−19.35 ± 0.09 a,*	−19.96 ± 0.06 b	−19.88 ± 0.04 a
	AC	−20.13 ± 0.05 c	−19.71 ± 0.05 b,*	−20.29 ± 0.09 b	−20.23 ± 0.04 b
lipid δ^{13}C	MC	−24.45 ± 0.06	−24.16 ± 0.05*	−24.47 ± 0.07 a	−24.13 ± 0.02*
	CC	−24.53 ± 0.09	−24.11 ± 0.03*	−24.54 ± 0.03 ab	−24.14 ± 0.06*
	AC	−24.70 ± 0.04	−24.20 ± 0.03*	−24.76 ± 0.06 b	−24.21 ± 0.03*
		bulk isotopic fractionation			
$\Delta\delta^{15}$N	MC	0.25 ± 0.21 c	2.13 ± 0.12*	0.93 ± 0.04 c	2.78 ± 0.06 a,*
	CC	2.71 ± 0.19 a	2.07 ± 0.09*	3.34 ± 0.06 a	2.57 ± 0.05 b,*
	AC	1.34 ± 0.21 b	1.83 ± 0.07*	1.79 ± 0.05 b	2.24 ± 0.06 c,*
$\Delta\delta^{13}$C	MC	−0.41 ± 0.13 b	−0.29 ± 0.11 b	0.50 ± 0.16 b	0.98 ± 0.10*
	CC	−0.18 ± 0.25 b	0.01 ± 0.08 ab	0.75 ± 0.10 ab	1.29 ± 0.11*
	AC	0.88 ± 0.11 a	0.20 ± 0.10 a,*	1.31 ± 0.13 a	1.09 ± 0.12

[a] Values are reported as means ± SEMs (initial $N = 3$ pools of five fish; final $N = 9$ fish). Values with different letter in each row are significantly different (Tukey's posthoc test; $P < 0.05$), and an asterisk indicates significant differences (t test, $P < 0.05$) after (initial) and before (final) the homogenization period. Results without any letter or symbol indicate no significant differences.

CC group while increasing in the other two. In spite of this convergence, significant differences were observed in the $\Delta\delta^{15}$N of muscle samples. $\Delta\delta^{13}$C in initial conditions was highest for the AC group (1.34 and 1.79‰) and lowest for the MC group (−0.41 and 0.50‰) in whole fish and muscle samples, respectively ($p < 0.05$). After homogenization, $\Delta\delta^{13}$C also converged (∼0 and ∼1.1‰ for whole fish and muscle tissue, respectively), but only the AC group showed a decrease in $\Delta\delta^{13}$C in both kinds of tissue.

Changes in Metabolic Parameters of Muscle. The lowest RNA/protein and RNA/DNA ratios under initial conditions were also found in the AC group, indicating lower protein synthesis and transcriptional capacity (Figure 4). COX and CS activity did not differ significantly between groups in initial conditions. This observation indicates that energy production capacity of the three hatcheries was similar, although the CC group tended to show higher activity for these two enzymes.

After the homogenization period, the RNA/protein ratio decreased in all groups as a consequence of growth. The transcriptional capacity of the AC group increased significantly, but the protein synthesis capacity of this group was still the lowest. The COX:CS ratio decreased in all groups ($p < 0.05$), without significant differences among them (Figure 4). Nevertheless, the enzyme activities of the AC group showed a different pattern to that of the other two groups. While CS and COX activities decreased significantly in the MC and CC groups, the CS activity of AC fingerlings increased significantly, and the COX activity remained unchanged. These observations suggest that the AC group presented higher energy waste.

Principal Components Analysis (PCA). PCA was used to determine the capacity of the variables (chemical, metabolic, and isotopic measurements in muscle) to discriminate between fingerlings from the three hatcheries after the homogenization period. The first three factors accounted for over 70% of the variability within the data (Table 5), and bulk δ^{15}N was the variable with the highest load on the first factor (Table 6). Plots of factor 1 versus factors 2, 3, and 4 all provided good discrimination among fingerlings from the three hatcheries (Figure 5); in particular, AC fingerlings were clearly separated from the other two groups, with factor 1 providing the greatest discrimination.

DISCUSSION

Isotopic Characterization of Fingerlings in Hatchery Conditions. The initial ^{15}N and ^{13}C composition of juveniles from the three fish farms differed. Given the differences in diets and rearing conditions, especially food ration and water temperature, these isotopic differences at origin must be supported by other metabolic markers. The three diets in the initial condition were isoproteic and isolipidic, but the significant differences in isotopic composition should reflect differences in the proportion and type of the raw materials used. When the plant component in a diet increases, total δ^{15}N falls, and the δ^{13}C values of the lipid fraction also decrease.[9,13] Plant meal based on C3 plants (i.e., wheat) gives lower δ^{13}C values than C4 plants (i.e., corn).[21] Therefore, the diet given to the CC group, which had the lowest δ^{15}N, had the highest proportion of plant ingredients, whereas that given to the AC group, which had the lowest δ^{13}C values, had a higher amount of C3 plant-based ingredients. Despite the wide range of variation in δ^{15}N among these fish-meal based diets, the differences in whole fish and muscle δ^{15}N between the three groups were small. Consequently, variations in isotopic fractionation of

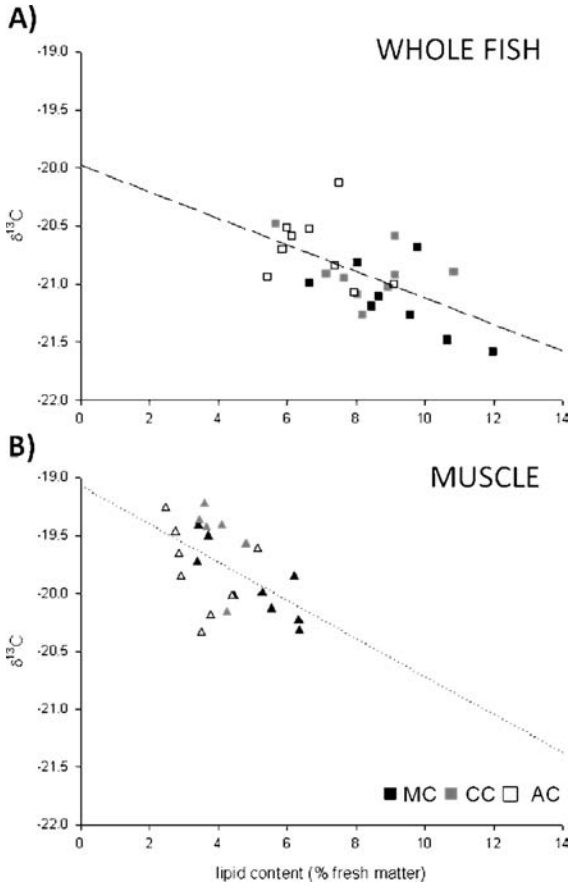

Figure 3. Relationship between lipid concentration and $\delta^{13}C$ in whole fish (A, dashed line: $r = 0.589$, $P < 0.001$) and muscle (B, dotted line: $r = 0.532$, $P < 0.005$) of sea bream fingerlings after the homogenization period.

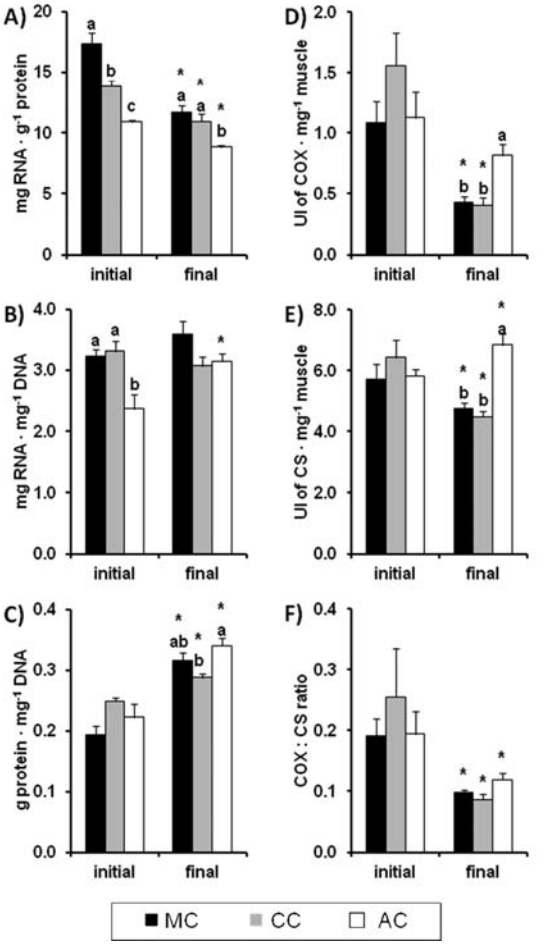

Figure 4. Initial (hatchery) and final (after the homogenization period) muscle growth (RNA:protein ratio, A; RNA:DNA ratio, B; and protein:DNA ratio, C) and metabolic (COX activity, D; CS activity, E; and COX:CS ratio, F) indices of muscle samples of sea bream fingerlings produced in the three farms. Columns assigned a different letter are significantly different between groups ($P < 0.05$), and an asterisk indicates significant differences after (initial) and before (final) the homogenization period ($P < 0.05$).

nitrogen ($\Delta\delta^{15}N_{tissue\text{-}diet}$), within the range reported in sea bream[9] or in other species,[25,26] would indicate differences in fish metabolism,[15,16] these possibly related to rearing conditions. The isotopic composition of animals has been reported to depend mainly on food composition;[7,8] however, temperature and ration also influence $\delta^{15}N$ and $\delta^{13}C$ of sea bass tissues.[14] The protein intake is proportional to ^{15}N enrichment in fish muscle,[27] and tilapias fed diets with a high plant meal content show increased $\delta^{15}N$,[28] thereby suggesting greater transformation of plant-derived dietary amino acids into animal protein. This observation could explain the finding that the CC group with the highest $\Delta\delta^{15}N$ (2.71 and 3.34‰ for whole fish and muscle, respectively) was fed the highest ration (6% bw day^{-1}) of the diet most depleted in $\delta^{15}N$, that is, with the highest plant protein content. In fact, this group also presented greater energy demands, an observation consistent with the direct relationship between ration and COX and CS activities, as previously found in saithe.[18] The MC group showed the lowest $\Delta\delta^{15}N$, presenting values of 0.25‰ in whole fish and 0.93‰ in muscle, much lower values than the general 3‰ assumed for one trophic shift in the food web.[8] This finding would suggest that this group had the lowest transformation of dietary protein, which is consistent with the diet having the highest fish meal content.

In contrast to ^{15}N, the $\delta^{13}C$ values of consumer tissues depend not only on protein but also on carbohydrates, and especially on lipids, which are depleted in ^{13}C.[29] Furthermore, the carbon skeletons of several dietary constituents (proteins, lipids, and carbohydrates) can be routed to diverse tissue constituents, that is, "isotopic routing".[30] Indeed, the differences in lipid $\delta^{13}C$ between the three groups reflected those of dietary protein more closely than dietary lipid. This finding is consistent with results

Table 5. Table of Eigenvalues for PCA of Chemical, Metabolic, and Isotopic Data from Muscle of Sea Bream Fingerlings after the Homogenization Period

	eigenvalue	variability (%)	cumulative eigenvalue	cumulative %
F1	6.88	43.01	6.88	43.01
F2	2.75	17.18	9.63	60.18
F3	1.74	10.88	11.37	71.06
F4	1.26	7.90	12.63	78.96
F5	0.93	5.80	13.56	84.75
F6	0.82	5.11	14.38	89.87
F7	0.51	3.22	14.89	93.09

reported by Kelly and Martinez del Rio,[27] who observed that more than 50% of the carbon in lipids extracted from muscle was derived from dietary protein. All of these findings explain the lack of differences in initial bulk $\delta^{13}C$ in muscle and whole fish between the three groups, despite variations in dietary isotopic composition and lipid content of tissues. Concerning ^{13}C fractionation, our results were in agreement with reviewed literature.[25,26] Gaye-Siesseger et al. [13] proposed changes in the anabolism−catabolism ratio, which could be referred to the inverse relationship between $\Delta\delta^{13}C$ and ration. Hence, the observation that the AC group with the lowest ration in hatchery conditions (3% bw day^{-1}) showed the highest $\Delta\delta^{13}C$ in the two tissues sampled can be explained by the observation that it showed the lowest protein synthesis capacity of the three groups. The AC group was subjected to the lowest rearing temperature, which would explain this lower capacity, as also observed in Atlantic wolffish.[31]

Table 6. Factor Loadings for Muscle of Seabream Fingerlings[a]

variable	F1	F2
bulk $\delta^{15}N$	**0.868**	−0.007
$\Delta\delta^{15}N$	**0.866**	−0.008
protein $\delta^{13}C$	**0.863**	0.025
CS	**−0.850**	−0.097
protein $\delta^{15}N$	**0.845**	−0.144
% water	**−0.826**	0.240
% lipid	**0.817**	−0.456
COX	**−0.816**	−0.121
COX·CS^{-1}	−0.680	−0.276
RNA·protein^{-1}	0.581	0.190
bulk $\delta^{13}C$	0.020	**0.962**
$\Delta\delta^{13}C$	0.000	**0.958**
% protein	0.350	0.570
protein·DNA^{-1}	−0.352	−0.175
RNA·DNA^{-1}	0.219	−0.357
lipid $\delta^{13}C$	0.295	0.011

[a] Bold type indicates loadings >0.7 and <−0.7.

Evolution of Isotopic Composition after the Homogenization Period. After submitting the fingerlings to the same diet and identical rearing conditions for 35 days, the isotopic signature changed as a result of growth, but the initial differences observed in isotopic signature remained. These differences were maintained, especially in muscle $\delta^{15}N$, which was the variable with the highest load on the first principal component of the PCA analysis. The time lag required to reflect diet isotopic composition in fish depends on the speed at which new material is added to tissue (growth rate) and the replacement of materials exported from tissues as a result of catabolism (metabolic turnover).[32]

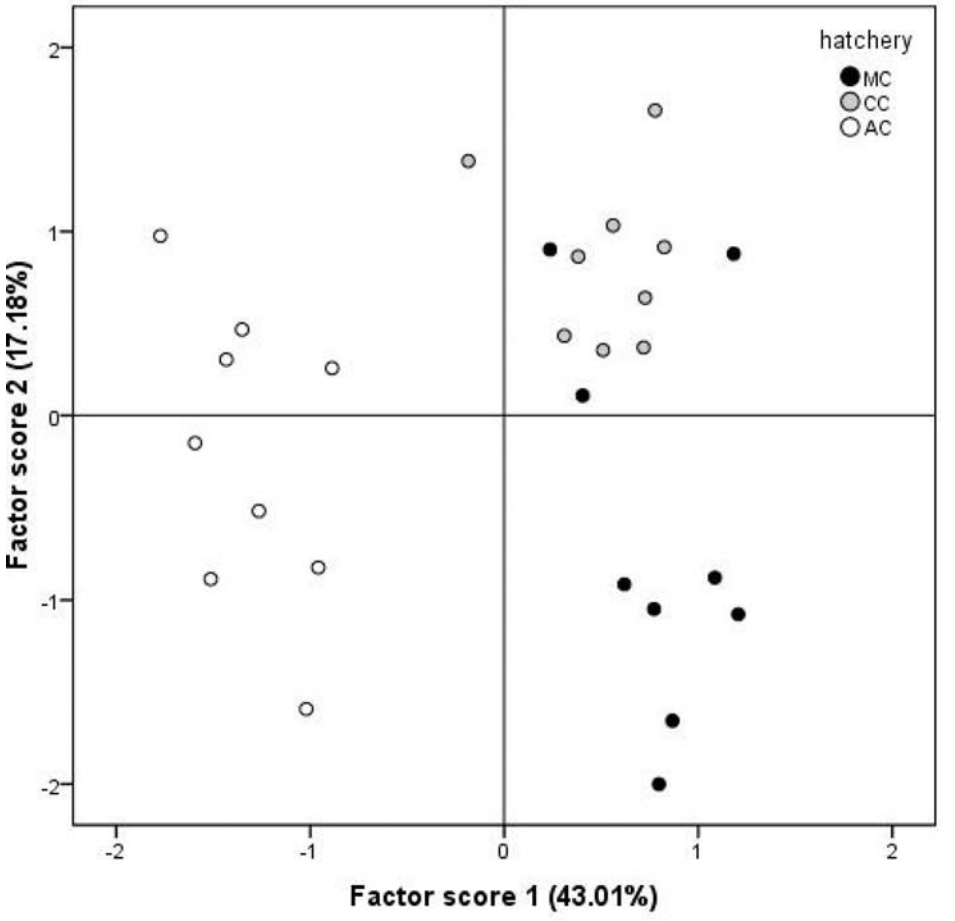

Figure 5. PCA plot of combined chemical, metabolic, and isotopic data of muscle from sea bream fingerlings from the three hatcheries after the homogenization period. Factors 1 and 2 represent the first and second principal components, with the percentage of explained variance indicated in parentheses.

Half-lives for ^{13}C and ^{15}N in juvenile fish are under 1 month, although the half-life of the former is shorter than that of the latter.[33−36] Thus, for the fingerlings in this study, which were in a fast growth phase, a trial lasting 35 days was sufficient to reflect the new conditions of isotopic composition in tissues. At this point, the isotopic composition of these fish, which follows an asymptotic evolution after a diet switch,[33−36] should be in the plateau phase (i.e., in isotopic equilibrium with diet). Moreover, all fish were fed the same ration to prevent an influence of this variable on isotopic composition.[13,16] Therefore, we propose that the final isotopic differences between the three fish groups are the result of the "isotopic imprint" that remains from the initial hatchery conditions (which is consequence of the previous physiological or metabolic conditions). In this regard, systematic differences in isotope composition have been found in individual animals over long periods of time as compared to other individuals with identical rearing (food and water) conditions.[37] These observations may reflect differences in physiology. However, the use of ^{15}N and ^{13}C isotopes as a discrimination tool, while useful at early growth stages, may be complicated by maturity and growth rate. Long-term studies in laboratory controlled conditions have to be carried out to clarify how long this isotopic imprint lasts.

Direct relationships between $\delta^{15}N$ and body weight, irrespective of dietary switches, have been interpreted as metabolic or physiological changes associated with age in walleye[38] and sea bass.[39] However, growth enhances de novo biosynthesis of dispensable amino acids, which become ^{13}C-enriched as a result of isotopic discrimination steps, and could significantly alter consumer tissue $\delta^{13}C$, as reported in mummichog.[40] Accordingly, the observation that all of the fish in our study almost doubled their weight after the homogenization period, thereby leading to an increase in bulk $\delta^{15}N$ and $\delta^{13}C$ of whole body and muscle samples, mirrored the δ values of deposited protein. These isotopic enrichments are linked to the decrease in the RNA:protein ratio observed in all groups at the end of the homogenization period. As body weight increases, the RNA content decreases, and a larger proportion of the RNA pool is involved in protein turnover.[17] Furthermore, deamination and transamination, together with decarboxylation, lead to isotopic enrichment of newly synthesized protein.[41,42] Therefore, the increase in protein turnover that occurs with growth could explain the increase in $\delta^{15}N$ and $\delta^{13}C$ in the three groups. Nonetheless, the group previously eating the diet used in the homogenization period showed smaller changes in $\delta^{15}N$ and $\delta^{13}C$ in whole fish and muscle samples; thus, increases in its isotopic fractionation of ^{15}N and ^{13}C can be explained solely by growth. In this regard, the dietary switch to an isotopically heavier diet in the other two groups caused greater increases in tissue isotopic values. However, of the groups that switched diet, the changes were slightly lower in the one with the lowest $\Delta\delta^{15}N$ values. As a result, this group maintained the lowest ^{15}N and ^{13}C protein values. In contrast, the isotopic values of the other group (CC) approached those of the one previously eating the diet used in the homogenization period. It is well accepted that growth and protein turnover are determinant factors of the rate of isotopic incorporation after a dietary switch in fish.[43] Therefore, the group with lowest protein turnover, indicated by its lower $\Delta\delta^{15}N$, together with a lower growth rate, could explain this discordance. Furthermore, the dietary switch of these two groups affected the evolution of their isotopic fractionation differentially. Martinez del Rio et al.[43] hypothesized that $\Delta\delta^{15}N$ decreases with increasing protein quality in diet and/or with the efficiency of nitrogen deposition. Thus, the observed decline of $\Delta\delta^{15}N$ in fingerlings previously fed the diet most depleted in ^{15}N can be related to the improved quality of the new diet during the homogenization period, as indicated by the higher dietary $\delta^{15}N$.[9] However, the increase in $\Delta\delta^{15}N$ in the group could be consequence of high metabolic waste, reflected in the highest COX and CS activities, which were maintained after homogenization. In contrast, these activities decreased in the other two groups (MC and CC) in an inverse relationship with growth, as observed in saithe.[18] Therefore, the group with highest metabolic cost for growth would show lower protein deposition efficiency, also evidenced by a trend to lower feed conversion efficiency and the lowest content of muscle protein. In agreement with this, this group showed the lowest RNA:protein ratio, which reflected lower protein synthesis capacity. Moreover, this group would show a higher proportion of growth by processes of hypertrophy instead of hyperplasia, as a result of its higher increase in protein:DNA and RNA:DNA ratios. In contrast, lipid-$\delta^{13}C$ increased in whole fish and muscle samples of the three groups, although unlike protein, differences in lipid $\delta^{13}C$ in muscle were not maintained after the homogenization period. This change would be produced by the "isotopic routing" of carbon skeletons described above.

Here, we demonstrate that after a 35 day homogenization period, fingerlings can be discriminated between hatcheries mainly on the basis of isotopic measures (especially $\delta^{15}N$) combined with metabolic parameters (COX and CS activities) and proximal composition (fat content) in muscle, as shown in PCA analysis. The group with the lowest isotopic values in hatchery conditions reached a distinct isotopic equilibrium to that of the other two groups. Lower protein synthesis capacity, lower hyperplasic processes, and higher energy metabolic costs of this group could explain these differences. The muscle isotopic signature is a more suitable indicator than whole fish for the discrimination between fingerlings produced in distinct hatcheries, although additional physiological information is required to determine fish seeds with the highest growth potential. Therefore, particular rearing conditions and/or genetic divergence between hatcheries, affecting the growth capacity of fingerlings, are defined mainly by the isotopic imprint. In summary, we conclude that the set of parameters analyzed here could be useful for potential buyers of aquaculture products.

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