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Natural abundance of ^{15}N and ^{13}C in fish tissues and the use of stable isotopes as dietary protein tracers in rainbow trout and gilthead sea bream

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Abstract:

For developing efficient diets, two sets of experiments examined whether the use and allocation of dietary protein can be traced by labelling with stable isotopes (^{15}N and ^{13}C) in two culture fish (Oncorhynchus mykiss and Sparus aurata). In the first experiment, natural abundance and tissue distribution of these isotopes were determined, by measuring the $\delta^{13}C$ and $\delta^{15}N$ values by isotopic ratio mass spectrometry, in fingerlings (14–17 g) adapted to diets differing in the percentage of fish meal replacement by plant protein sources. For both species, $\delta^{15}N$ and $\delta^{13}C$ were greater in tissues with higher protein and lower lipid content. Delta ^{15}N of diets and tissues decreased as replacement increased, suggesting $\delta^{15}N$ can be used as a marker for dietary protein origin. The ^{15}N fractionation ($\delta^{15}N$ fish − $\delta^{15}N$ diet) differed between groups, and could thus be used to indicate protein catabolism. In the second experiment, fish (75–90 g) of each species ingested a diet enriched with ^{15}N-protein (10 g kg^{-1} diet) and ^{13}C-protein (30 g kg^{-1} diet). These proportions were suitable for determining that the delta values of tissue components were high enough above natural levels to allow protein allocation to be traced at 11 and 24 h after feeding, and revealed clear metabolic differences between species.

Keywords: $\delta^{13}C$ • $\delta^{15}N$ • diet • protein • rainbow trout • sea bream

Introduction

The replacement of fishmeal by plant ingredients is a naturally occurring tendency in aquaculture nutrition, even in fish species that are considered carnivorous. Improving protein utilisation requires an understanding of the metabolic routing of the protein and energy supply for metabolism and growth. The use and fate of individual molecules (amino acids) or nutrients can be studied with radioactivity. However, labelling feed

ingredients with radioactive markers is harmful to users, experimental animals and the environment. A safe alternative method for tracing dietary protein allocation in fish involves stable isotopes (^{13}C and ^{15}N). However, many aspects must be considered before using stable isotopes as tracers of intermediary metabolism. Stable isotopes have been extensively used in ecological studies. It is generally accepted that the $\delta^{13}C$ content of an organism reflects the $\delta^{13}C$ content of its diet with little (1‰) or no change (DeNiro & Epstein 1978; Fry & Sherr 1984). However, the ^{15}N content is more enriched (3-4‰) (Minagawa & Wada 1984). In the literature, many studies have been done using $\delta^{13}C$ and $\delta^{15}N$ as natural tracers with the assumption that these ratios are homogeneously distributed in all tissues of an organism. However, some potential sources of variation in the isotopic composition of animals have recently been found. In many species, $\delta^{13}C$ or $\delta^{15}N$ values of an animal vary with the type of food, trophy level or producer-consumer relationships (Vanderklift & Ponsard 2003). Values also vary among the organs and tissue components of an organism (Gannes et al. 1998). In fish, the natural abundance of stable isotopes depends on the proportion of principal tissue components (Pinnegar & Polunin 1999, in rainbow trout) and on the nutrient sources (Gaston & Suthers 2004, in rocky reef fish). All variations are due to the isotopic fractionation that occurs during the metabolic processes of deamination and decarboxylation. Thus, higher isotopic fractionation, or enrichment, of ^{15}N has been related to stress conditions, such as fasting (Hobson & Clark 1992; Hobson et al. 1993) or low-protein diets (Gaye-Siessegger et al. 2004a).

The first part of this study aims to establish the relationships between the natural abundance of ^{15}N and ^{13}C stable isotopes and the main sources of variation (diet, organs and the main composition of tissues) in two fish species: rainbow trout (*Oncorhynchus*

mykiss Walbaum 1792) and gilthead sea bream (*Sparus aurata* L. 1758). These species are common in freshwater and marine aquaculture respectively. We studied diet effects on each species by comparing six diets with different sources of protein. Diets ranged from a 100% plant diet to a diet with 100% of the protein provided by fish meal. Differences among organs and the effects of tissue composition were analyzed by comparing the levels of ^{15}N and ^{13}C in the whole body; in three tissues (liver, muscle and gut); and in the main tissue components (protein, lipids, and glycogen) with the delta values of the respective diets. Although most studies on protein metabolism have been carried out with ^{15}N, the use of ^{13}C can also provide information about protein transformation into glycogen and lipids. To evaluate the use of both stable isotopes as dietary protein tracers in these species, the second part of the present study analyses the distribution of both tracers 11 and 24 hours after feeding a meal artificially enriched with 1% ^{15}N- and 3% ^{13}C-protein. The study shows that these amounts could be used for metabolic studies of protein routing in fish.

Materials and Methods

Natural abundance of ^{15}N and ^{13}C in fish: experimental protocol and sampling

Juvenile rainbow trout (17.5 ± 0.05 g) were reared in an experimental freshwater fish farm (INRA, Donzaq, France) for three months (March-June), under a natural photoperiod in a flow-through system. Freshwater was supplied from natural springs at a constant temperature of 17 ± 1ºC. Juvenile sea bream (15.9 ± 0.05 g) were reared for three months (April-July) in tanks of 500 L in an indoor open marine water system at CSIC (Torre de la Sal, Spain). Day length and temperature changes followed natural variations, the latter increasing from 17ºC to 25ºC. In both cases, fish were fed twice a

day with the six experimental diets shown in Table 1 for Rainbow trout and in Table 2 for Gilthead sea bream. Diet 1 was based mainly on fishmeal protein. In diets 2 to 6, fishmeal was progressively replaced by a mixture of plant protein ingredients. Diet 6 did not contain any fishmeal at all. After three months, fish were anaesthetized and sacrificed by sectioning their spinal cord. Portions of the liver, white muscle, gut plus perivisceral fat (G+VF) and the rest of the fish (named the carcass) were excised and frozen in liquid nitrogen and stored at –80ºC until later analysis.

Use of ^{15}N and ^{13}C as dietary protein tracers: experimental protocol and sampling

Animals fed diet 2 (RT2, GSB2) were selected for this part of the study. Diet 2 was comprised of 33% plant protein, which reflects currently available commercial diets. Diets were labelled with 1% ^{15}N-*Spirulina* protein and 3% ^{13}C-*Spirulina* protein (*Martek Biosciences, Columbia, MD, USA*). Doses of 1% ^{15}N-protein and 3% ^{13}C-protein were chosen to maintain the natural ratio of these stable isotopes (0.36% for ^{15}N and 1.1% for ^{13}C). Eighteen trout and 18 sea bream were fasted for 24 hours, lightly anaesthetised (2,3 – diphenoxyethanol 1:2500) and then force-fed with a gastric cannula an amount equivalent to 1% of their whole body weight. After force-feeding, fish were allowed to recover in separate tanks. Light sedation and force-feeding took less than 4 minutes for each fish. Another four animals from each species received the same dietary ration containing 4% non-labelled *Spirulina*. These fish were used to determine the natural background of samples (blank), which was subtracted from the corresponding treated samples. Fish were sampled 11 and 24 hours after oral administration. They were sacrificed as described above. Samples of liver, white muscle and G+VF were taken and immediately frozen in liquid nitrogen with the carcass. All samples were

stored at –80ºC until analysis. All fish handling procedures were in accordance with current Catalan legislation on animal use for experimentation (DOGC nº 2450, 214/1997).

Principal components of tissue samples

Tissue samples were homogenised in liquid nitrogen using a pestle and mortar to obtain a fine powder. The rest of the fish was homogenised at -20ºC using a food processor *(Pacojet AG, Switzerland).* Tissue-water content was determined gravimetrically after drying the samples at 105ºC for 24 h. Lipids were extracted according to the method described by Folch et al. (1957). The washed lipid extracts were dried under N_2 and the lipid content was determined gravimetrically. Glycogen was purified by alcoholic precipitation after tissue hydrolysis with KOH. It was then quantified according to Fraga (1956). Protein purification was carried out using defatted samples via precipitation with trifluoroacetic acid (10%). Bound protein was separated from free amino acids after centrifugation at 1060 *g* for 30 minutes. The protein content was calculated from the nitrogen obtained by elemental analysis (Elemental Analyser *Flash 1112*) as N x 6.25. Aliquots of the purified lipid, glycogen and protein, as well as aliquots from diets and whole tissues, were lyophilized and used for isotopic analysis.

Analysis of ^{15}N and ^{13}C by EA-IRMS

Dried samples were ground into a homogenous powder. Samples ranging from 0.3 - 0.6 mg were accurately weighed in small tin capsules. Analyses of the different samples were performed to determine the carbon and nitrogen isotope composition using an Elemental Analyzer *Flash 1112* coupled to a *Finnigan MAT Delta C* Isotope Ratio Mass

Spectrometer via a *Conflo III* interface (*ThermoFinnigan, Germany*). International standards of known isotopic composition were included to correct for analytical and instrumental variations. The same reference material analysed over the analysis period was measured with ±0.2‰ precision for natural materials and ±0.4‰ precision for enriched materials. Results are presented in delta notation (δ): i.e. the values relative to PDB for carbon and to N air for nitrogen, in parts per thousand. Delta values for the whole body are estimates of the true values because they are calculated as the added values obtained from the different samples into which the fish were divided. Fractionation values were calculated as δ fish – δ diet.

Statistics

All statistical analyses were performed using commercial software (SPSS 12.0). Data were tested separately for each species. For the study of ^{15}N and ^{13}C natural abundance, the influence of the diet and the tissue on $\delta^{15}N$ and $\delta^{13}C$ values for the whole body, the tissues, and the tissue components were tested by two-way ANOVA. Post-hoc multiple comparison Tukey or T3-Dunnett tests were conducted when needed. The relationships between two variables were tested using linear regressions. To study the use of ^{15}N and ^{13}C as dietary protein tracers, a T-test for non-related samples was conducted to compare the increase in labelling of ^{15}N and ^{13}C tissue protein. Delta values of ^{15}N and ^{13}C at 0, 11, and 24 hours in tissue protein, lipid, and glycogen were tested by one-way ANOVA and the post-hoc Tukey test when needed.

Results

Natural abundance of ^{15}N and ^{13}C in fish

The increase in plant protein levels led to a decrease in $\delta^{15}N$ diet values. In addition, $\delta^{15}N$ fish values were higher than and directly related to $\delta^{15}N$ diet values (Fig. 1). Trout fed RT3 had higher nitrogen fractionation (3.16 ‰) than the other trout groups (1.75 ± 0.08 ‰ on average). Gilthead sea bream fed GSB1, GSB4, GSB5 and GSB6 had higher nitrogen fractionation (4.5 ± 0.23 ‰) than sea bream fed GSB2 and GSB3 (1.8 ± 0.14 ‰). As in observations for the whole body, $\delta^{15}N$ tissue values decreased in proportion to the replacement of fishmeal by plant protein sources. A positive relationship was found between $\delta^{15}N$ tissue values and protein content (Fig. 2). Table 3 shows $\delta^{15}N$ tissue values and $\delta^{15}N$ protein values in both species adapted to the six experimental diets. In both species, $\delta^{15}N$ tissue values depended on the diet consumed ($P<0.05$) and on the tissue ($P<0.05$). The gut + perivisceral fat (G+PV) had the lowest $\delta^{15}N$ value ($P<0.05$).

In contrast to ^{15}N, $\delta^{13}C$ diet values were not related to the percentage of protein substitution (Fig. 3). A significant relationship between $\delta^{13}C$ diet values and $\delta^{13}C$ fish values was only seen in rainbow trout. In addition, the fractionation for carbon (-0.60 to 1.04 ‰) was lower than that for nitrogen. Table 4 shows the $\delta^{13}C$ tissue values and $\delta^{13}C$ protein values in both species adapted to the six experimental diets. The type of diet affected the ^{13}C content in trout tissues, but not in sea bream. There were differences in ^{13}C content in the tissues of both species. The gut (plus perivisceral fat) values were lower than those of other tissues. The gut values were also lower than the $\delta^{13}C$ of the corresponding diets (-1.9±0.2 ‰ for trout, -2.2±0.2 ‰ for sea bream). In both species, $\delta^{13}C$ tissue values (Fig. 4A) and $\delta^{13}C$ protein values (Fig. 5A) were directly related to

protein content. This relation was observed with the nitrogen isotope, while $\delta^{13}C$ tissue values were negatively related with lipid content (Fig. 4B). Among the main components of tissues, only $\delta^{13}C$ glycogen values (Fig. 5B) showed high variability. Lipids were heavily depleted of ^{13}C (-27 to -25 ‰) compared with $\delta^{13}C$ protein values (-23 to -18‰) and $\delta^{13}C$ glycogen values (-23 to -17‰).

Use of ^{15}N and ^{13}C as dietary protein tracers

Actual ^{15}N enrichment for the labelled diets was 0.96 % for RT2 and 0.84 % for GSB2. Enrichment of ^{13}C was 2.90 % for RT2 and 2.90 % for GSB2. This is in agreement with the proposed labelling (1 N:3 C). In both species, $\delta^{15}N$ and $\delta^{13}C$ tissue protein values (Fig. 6) were much higher at 11 and 24 hours ($P<0.05$) than the natural abundance levels (at 0 h). Liver proteins were enriched in ^{13}C and in ^{15}N in an equivalent amount within a species. However, there was a between-species difference (trout: $\Delta_{0\text{-}11\ h}$: $\delta^{15}N$ = 61, $\delta^{13}C$ = 65, $\Delta_{0\text{-}24\ h}$: $\delta^{15}N$ = 60, $\delta^{13}C$ = 62; sea bream: $\Delta_{0\text{-}11\ h}$: $\delta^{15}N$ = 142, $\delta^{13}C$ = 163, $\Delta_{0\text{-}24\ h}$: $\delta^{15}N$ = 128, $\delta^{13}C$ = 125). This was also true of muscle proteins (trout: $\Delta_{0\text{-}11\ h}$: $\delta^{15}N$ = 5.4, $\delta^{13}C$ = 7.8, $\Delta_{0\text{-}24\ h}$ $\delta^{15}N$ = 11.2, $\delta^{13}C$ = 11.7; sea bream: $\Delta_{0\text{-}11\ h}$ $\delta^{15}N$ = 10.3, $\delta^{13}C$ = 14), except for the muscle proteins of sea bream from 0 to 24 hours (sea bream: $\Delta_{0\text{-}24\ h:}$ $\delta^{15}N$ = 12.8, $\delta^{13}C$ = 18, $P<0.05$). For both tracers, the incorporation of labelling into the protein was higher in sea bream than in trout. In addition, the incorporation of ^{13}C as hepatic glycogen was higher in sea bream (Fig. 7). This reflects the differences between both species in the fate of dietary protein. Moreover, in force-fed trout, $\delta^{13}C$ glycogen values of liver (-5.3 to 7 ‰) and muscle (-16 to -9‰) were not markedly higher than natural abundance levels in blank samples (-22 to -19‰). For both tracers,

the labelling rose quickly in liver whereas in muscle the incorporation was slower and continued to increase from 11 to 24 hours.

Discussion

Natural abundance of ^{15}N and ^{13}C in fish

The time needed to reflect diet isotopic composition depends basically on the turnover of tissues and growth rates (Sakano et al. 2005). In fish, Herzka et al. (2000) observed that whole red drum larvae respond to dietary shift in 10 days, while the time lag required for muscle of adult salmon was almost 40 days (age 2+) (Sakano et al. 2005). Juvenile fish in the present study were in a fast growth phase. They adapted to the different diets in a three-month period. This was long enough to reflect the diet isotopic composition. Diets, whole body fish and fish tissues had more ^{15}N as the fishmeal content in the diet increased. Therefore, ^{15}N content in fish can be an indicator of protein source (fishery or plant by-products). In contrast, carbon isotopic composition in diets and fish did not relate to the percentage of plant content in diets. Instead, $\delta^{13}C$ tissue values were probably the product of the metabolic routing of carbon from several macronutrients. Differences among $\delta^{13}C$ values in rainbow trout diets can be attributed to the type of plant by-products: C_3 plants (wheat, peas, soybean, and rapeseed) or C_4 plants (corn). Plants with the C_3 pathway presented lower ^{13}C values than C_4 plants (Oleary 1988). This may not be the only factor conditioning the delta values of diets, as minor changes in the percentage of each ingredient of the diet and the final proportion of macronutrients (proteins, sugars, lipids) could in some cases reinforce the differences among diets (as observed in the case of trout diets) or reduce them (as in sea bream diets).

The range of dietary nitrogen fractionation in both fish species (1 - 5.7‰) was in accordance with previously reported values in other animals (Gannes et al. 1998). Discrimination factors are commonly used in ecology to construct trophic relationships (Post 2002). Nevertheless, several studies have shown that nitrogen fractionation is related to both nutritional and metabolic aspects. Thus, higher fractionation of dietary nitrogen, lower growth and higher protein mobilization occurs in: crows (*Corvus brachyrhynchos)* under fasting conditions (Hobson & Clark 1992); Ross' geese (*Chen rosii)* under nutritional stress (Hobson & Clark 1993); and red-backed voles (*Clethrionomys gapperi*) fed low-protein diets (Sare et al. 2005). In fish, low-protein diets cause higher nitrogen fractionation in tilapia and carp (Gaye-Siessegger et al. 2004a, b). In addition, Trueman et al. (2005) showed that nitrogen fractionation in growing salmon is not constant, but changes inversely with growth rate. Similarly, the higher nitrogen fractionation found in the present work in trout fed RT3 could be related to a lower specific growth rate in this condition (Martin et al. 2003) than in other conditions (Vilhelmsson et al. 2004; Gomez-Requeni et al. 2005). Therefore, the highest nitrogen fractionation and the lowest growth rate found in trout fed the RT3 diet may reflect higher protein turnover and catabolism than in the other groups. In sea bream, the specific growth rates found by Gomez-Requeni et al. (2003; 2004) can be related to nitrogen fractionation in all groups except for GSB6. The protein diet of the GSB6 group was 100% vegetable-based. In this condition, fish reduced their food intake (Gomez-Requeni et al. 2004). Low food consumption might explain the results for the GSB6 group, as it has been observed in carp that isotopic ratios in animals are not only affected by the composition of the diet, but also by the amount of food consumed (Gaye-Siessegger et al. 2004b). In the case of carbon, fractionation values were lower

for both species than nitrogen values. This is consistent with results observed in other animals (DeNiro & Epstein 1978; Fry & Sherr 1984) and is due to the isotopic routing of the carbon skeletons of dietary constituents.

To our knowledge, this study presents the first values for the isotopic composition of gilthead sea bream. Regardless of the diet composition and ration, nitrogen fractionation values were higher in gilthead sea bream than in trout, at least when comparing fish fed to satiety with diets exhibiting similar delta values. This suggests that isotope fractionation is species-specific. This is in agreement with Gaston & Suthers (2004). There were also some similarities between the profile of changes in both species. For instance, tissues with high protein and low lipid content (muscle) presented higher $\delta^{13}C$ and $\delta^{15}N$ values than tissues enriched in lipids (gut). In addition, there was a direct relationship between protein content, $\delta^{15}N$-protein and $\delta^{13}C$-protein values in both species. Low $\delta^{13}C$ values have previously been reported in tissues with high lipid content, both in mammals (Tieszen et al. 1983) and in shellfish (Lorrain et al. 2002). The high ^{15}N and ^{13}C enrichment in muscle is in accordance with published results (Pinnegar & Polunin 1999; Lorrain et al. 2002; Gaston & Suthers 2004). It could be associated with the relative abundance of different amino acids in tissues. Essential amino acids are known to exhibit little change from their isotopic composition in the diet. However, other amino acids that are wholly synthesized or at least partially modified within the body may undergo changes in isotopic composition of varying magnitude (Gaebbler et al. 1966).

Use of stable isotopes as dietary protein tracers

The addition of 1% ^{15}N-protein and 3% ^{13}C-protein to diets allowed us to compare the isotope distribution during the post-feeding period. ^{15}N-labelling in tissue proteins was always higher than ^{13}C-labelling, due to the isotopic routing of ^{13}C. However, both isotopes presented similar profiles of changes between 0 and 11 hours and between 11 and 24 hours. As observed in gerbils by Tieszen et al. (1983), the turnover of metabolites in liver is higher than in muscle, explaining the higher enrichment of biochemical components in the former tissue.

^{13}C was also used to study dietary protein allocation into lipid and glycogen components. Thus, a marked difference between rainbow trout and gilthead sea bream was observed in dietary protein incorporation into the glycogen of liver and muscle. Gilthead sea bream exhibited a higher gluconeogenic capacity than trout. In addition, the turnover of ^{13}C-labelled glycogen in the liver of gilthead sea bream was higher. This result is consistent with the different capacity of sea bream (Meton et al. 1999) and rainbow trout (Panserat & Kaushik 2002) to produce and utilize glucose.

In conclusion, the proposed experimental protocol (adding 1% ^{15}N- and 3% ^{13}C-protein to the diet) is suitable for tracing protein allocation in all fish tissue components. Moreover, blank samples are not needed to measure protein allocation in main components (protein, lipid and glycogen), as natural isotopic compositions in these fractions are negligible compared to the values obtained when stable isotopes are artificially administered. However, the low capacity of rainbow trout to synthesize glycogen from dietary protein and the high variability in the natural abundance of ^{13}C in glycogen leads to inaccurate measurements of gluconeogenic and glycogenic fluxes.

Therefore, in this species, the natural ^{13}C levels in this component should be taken into consideration.

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FIGURE LEGENDS:

Figure 1. Relationship between $\delta^{15}N$ fish whole body values (Δ for rainbow trout and ○ for gilthead sea bream) and $\delta^{15}N$ diet values. The diet name is indicated besides the symbol. Trout (--) y = 0.82x + 3.10, r=0.97; sea bream (—) y = 0.93x + 4.07, r=0.84; P<0.05.

Figure 2. Relationship between $\delta^{15}N$ tissue values and the percentage of tissue protein content in fish fed the six experimental diets. Values are for trout (open symbols) and for sea bream (black symbols) liver (●○), gut plus perivisceral fat (◊♦), muscle (▼▽) and carcass (□■). Trout (--) y = 0.17x + 5.87, r = 0.42, sea bream (—) y = 0.3x + 6.26 r = 0.48; P<0.05.

Figure 3. Relationship between $\delta^{13}C$ fish whole body values (Δ for rainbow trout and ○ for gilthead sea bream) and $\delta^{13}C$ diet values. The diet name is indicated besides the symbol. Trout (--) y = 0.63x - 8.09, r=0.78; P<0.01

Figure 4. Relationship between $\delta^{13}C$ tissue values and the percentage of protein and lipid in tissues. Fish fed with all diets are considered. Open symbols correspond to trout tissues and black symbols to sea bream tissues: liver (○●), G+VF (◊♦), muscle (▼▽) and carcass (□■). Linear relationship between $\delta^{13}C$ tissue values and protein percentage: trout (--) y = 0.34x – 27.3, r = 0.89; sea bream (—) y = 0.28x – 26.57, r = 0.78; P<0.05.

Linear relationship between $\delta^{13}C$ tissue values and lipid percentage: trout (--) y = -0.1x – 20.73, r = -0.76; sea bream (—) y = -0.1x – 20.64, r = -0.83; P<0.01.

Figure 5. Relationship between $\delta^{13}C$ tissue components values (protein, glycogen, and lipid) and the percentage of tissue components in fish fed the six experimental diets. Open symbols correspond to trout tissues and black symbols to sea bream tissues: liver (○●), G+VF (◊♦), muscle (▼▽) and carcass (□■). Linear relationship between $\delta^{13}C$ protein and protein percentage: trout (--) y = 0.07x – 21.51, r = 0.36; sea bream (—) y = 0.04x – 21.28, r = 0.33; P<0.05. Linear relationship between $\delta^{13}C$ lipid and lipid percentage: sea bream (—) y = 9.8x – 25.93, r = 0.39; P<0.01.

Figure 6. $\delta^{13}C$ (□) and $\delta^{15}N$ (■) in the protein fraction of liver (A) and muscle (B) in rainbow trout (RT) and gilthead sea bream (GSB) after consuming a diet enriched in ^{13}C and ^{15}N. Values represent the mean ± s.e.m of n= 2. Letters indicate the significant differences (P<0.05) over time.

Figure 7. $\delta^{13}C$ in lipids and glycogen of liver (A) and muscle (B) after consuming a diet enriched in ^{13}C: ○ lipids rainbow trout, ▽ glycogen rainbow trout, ● lipid gilthead sea bream, ▼ glycogen sea bream. Values represent the mean ± s.e.m of n = 2. Letters indicate significant differences (P<0.05) over time.

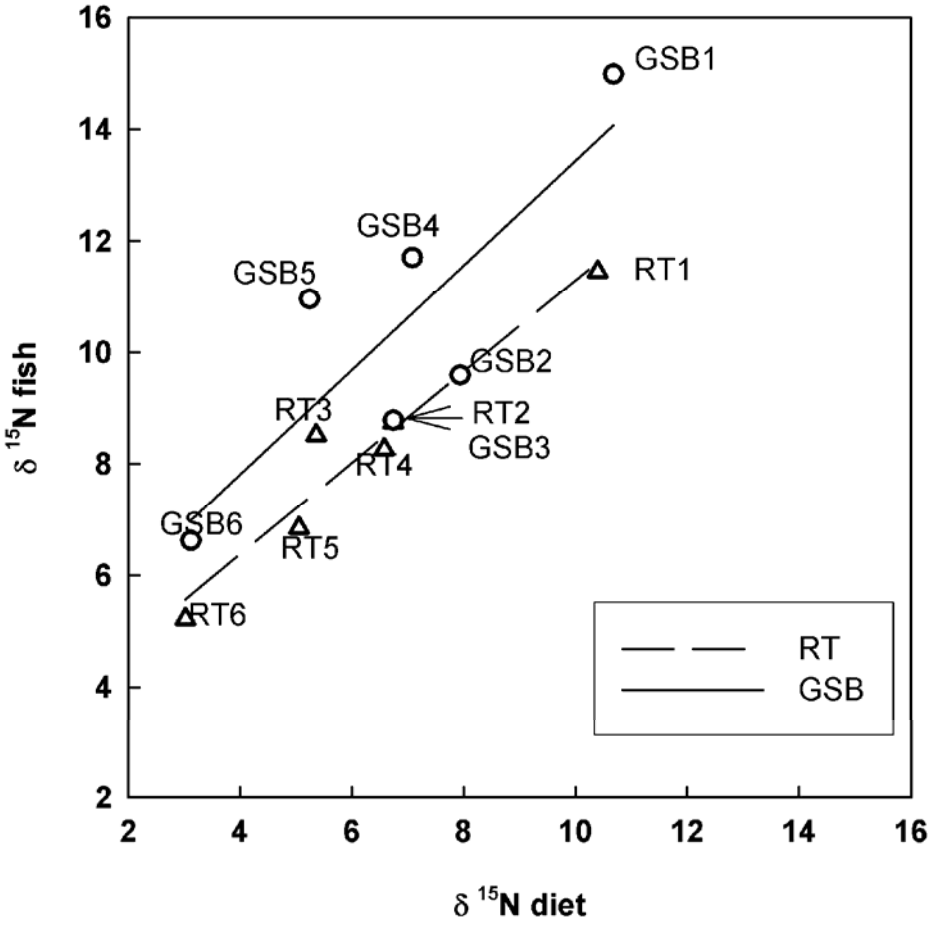

Figure 1. Relationship between δ 15N fish whole body values (△ for rainbow trout and ○ for gilthead sea bream) and δ 15N diet values. The diet name is indicated besides the symbol. Trout (--) y = 0.82x + 3.10, r=0.97; sea bream (□) y = 0.93x + 4.07, r=0.84; P<0.05.

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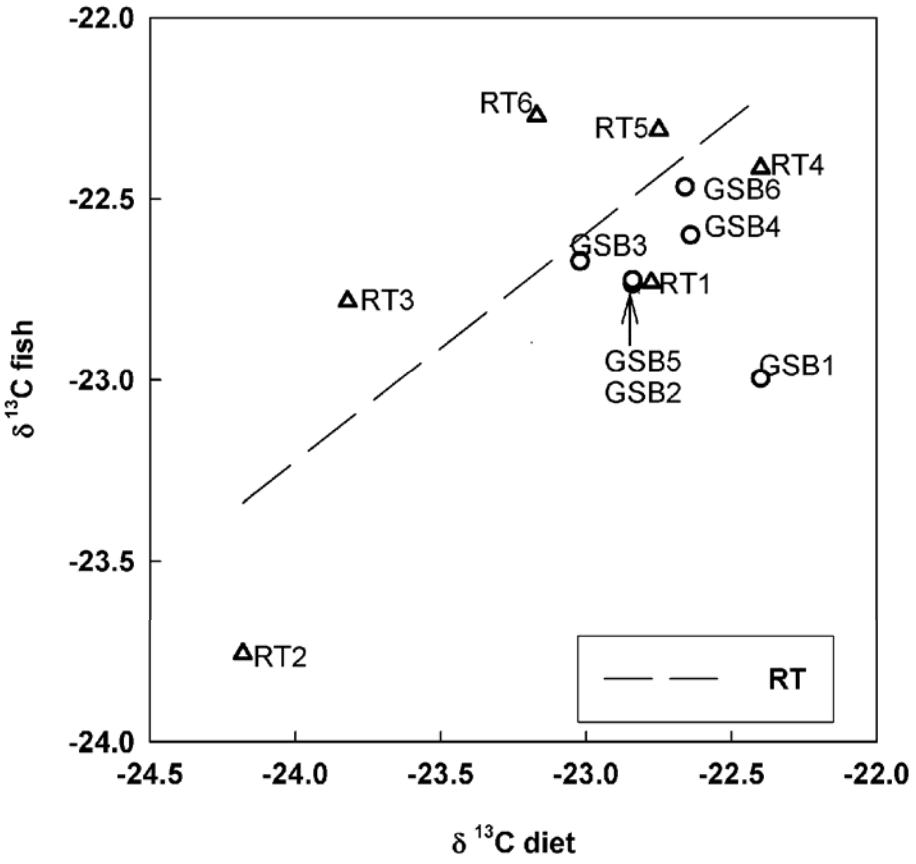

Figure 2. Relationship between δ 15N tissue values and the percentage of tissue protein content in fish fed the six experimental diets. Values are for trout (open symbols) and for sea bream (black symbols) liver (○ ●), gut plus perivisceral fat (), muscle (▽) and carcass (□ ■). Trout (--) y = 0.17x + 5.87, r = 0.42, sea bream (□) y = 0.3x + 6.26 r = 0.48; P<0.05.

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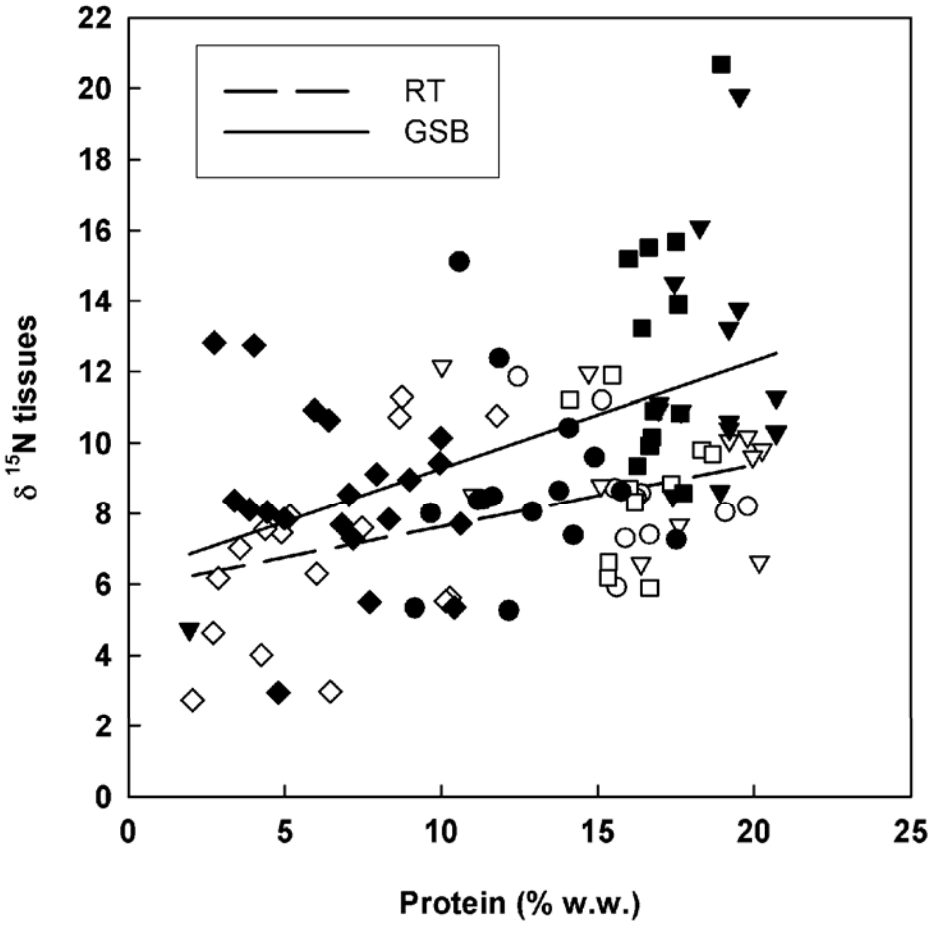

Figure 3. Relationship between $\delta^{13}C$ fish whole body values (△ for rainbow trout and ○ for gilthead sea bream) and $\delta^{13}C$ diet values. The diet name is indicated besides the symbol. Trout (--) y = 0.63x - 8.09, r=0.78; P<0.01

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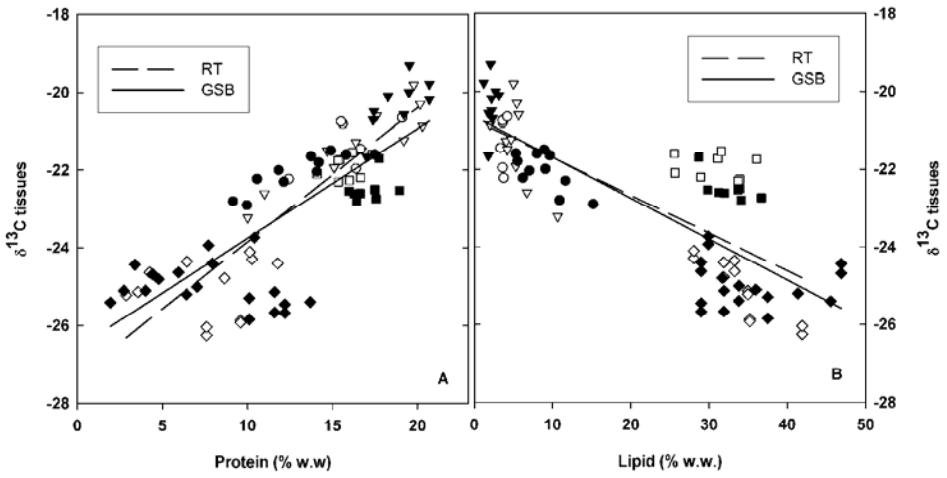

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707x383mm (150 x 150 DPI)

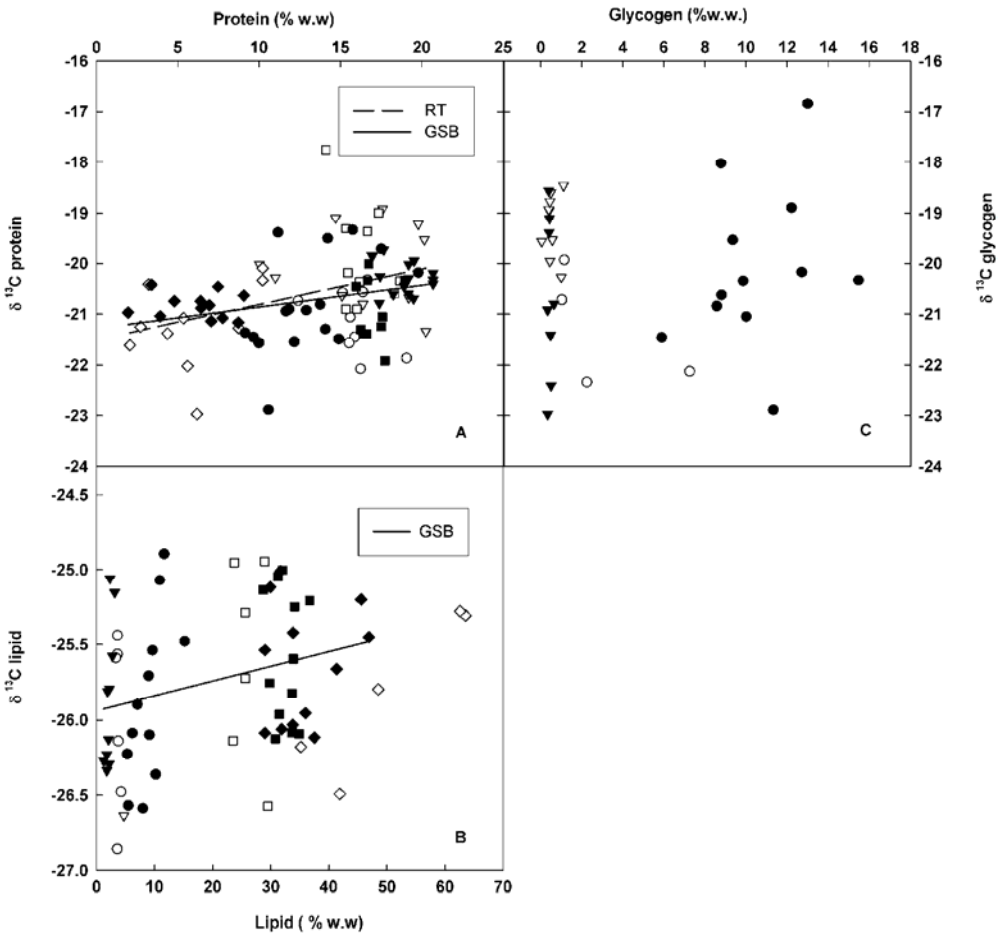

Figure 5. Relationship between δ^{13}C tissue components values (protein, glycogen, and lipid) and the percentage of tissue components in fish fed the six experimental diets. Open symbols correspond to trout tissues and black symbols to sea bream tissues: liver (○ ●), G+VF (), muscle (▽) and carcass (□ ■). Linear relationship between δ^{13}C protein and protein percentage: trout (--) y = 0.07x □ 21.51, r = 0.36; sea bream (□) y = 0.04x □ 21.28, r = 0.33; P<0.05. Linear relationship between δ^{13}C lipid and lipid percentage: sea bream (□) y = 9.8x □ 25.93, r = 0.39; P<0.01.

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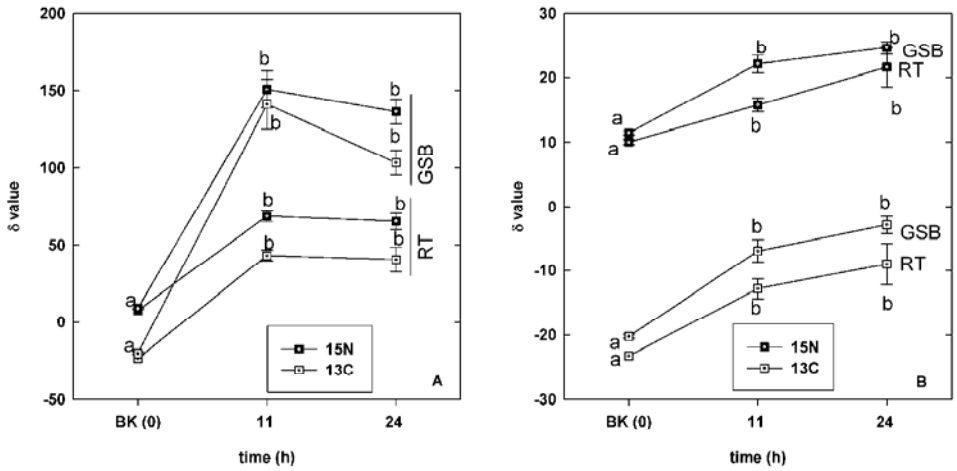

Figure 6. $\delta^{13}C$ (□) and $\delta^{15}N$ (■) in the protein fraction of liver (A) and muscle (B) in rainbow trout (RT) and gilthead sea bream (GSB) after consuming a diet enriched in ^{13}C and ^{15}N. Values represent the mean ± s.e.m of n= 2. Letters indicate the significant differences ($P<0.05$) over time.

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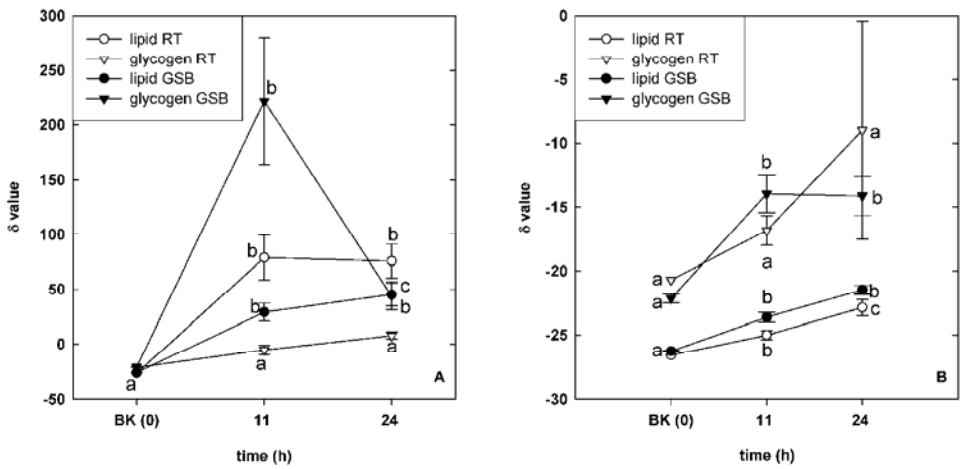

Figure 7. $\delta^{13}C$ **in lipids and glycogen of liver (A) and muscle (B) after consuming a diet enriched in ^{13}C: ○lipids rainbow trout, ▽glycogen rainbow trout, ●lipid gilthead sea bream, glycogen sea bream. Values represent the mean ± s.e.m of n = 2. Letters indicate significant differences (P<0.05) over time.**

707x373mm (150 x 150 DPI)

Table 1: Ingredients and chemical composition of the six experimental diets tested in rainbow trout

Ingredient ($g \cdot Kg^{-1}$)	RT1	RT2	RT3	RT4	RT5	RT6
Fish meal (CP 70%)	638.0	389.9	316.4	319.0	159.5	
Corn gluten meal				116.0	177.1	232.4
Wheat gluten [a]		71.4		100.0	150.0	200.0
Extruded peas [b]		215.1	56.8	80.0	120.0	163.3
Rapeseed meal [c]				46.9	75.0	100.0
Soybean meal (CP 42%)		25.3	331.3			
Extruded whole wheat	203.4	135.7	71.8	110.2	42.5	
Fish oil	128.7	101.6	109.5	143.6	151.1	158.7
Mineral mix [d]	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin mix [d]	10.0	10.0	10.0	10.0	10.0	10.0
CaHPO4.2H2O (18%)		10.9	16.1	16.1	37.8	40
L- amino acid mixture		20.1	68.2	37.5	56.9	75.7
Proximate composition						
Dry matter (%)	94.4	92.2	90.5	92.2	91.5	91.6
Proteins (% DM)	51.5	45.1	46.3	50.3	49.1	48.6
Lipid (% DM)	19.7	15.6	16.4	19.6	19.6	19.2
Gross energy ($KJ \cdot g^{-1}$ DM)	22.7	22.4	22.1	23.5	23.9	23.6

CP: crude protein. DM: dry matter. a: Amylum, Holland. b: Aquatex, France c: Primor00, France. d: according to the National Research Council (NRC, 1993).

Table 2: Ingredients and chemical composition of the six experimental diets tested in gilthead sea bream

Ingredient ($g \cdot Kg^{-1}$)	GSB1	GSB2	GSB3	GSB4	GSB5	GSB6
Fish meal (CP 70%)	703.7	500.0	478.1	352.0	176.0	
Corn gluten meal				120.0	180.0	250.0
Wheat gluten [a]				120.0	180.0	250.0
Extruded peas [b]		199.2		60.0	90.0	120.0
Rapeseed meal [c]				30.9	67.2	27.3
Soybean meal (CP 42%)		121.9	300.0			
CPSP-G [e]		50.0				
Lupin, sweet white						6.9
Extruded whole wheat	142.0		56.1	94.9	30.6	15.5
Mineral mix [d]	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin mix [d]	10.0	10.0	10.0	10.0	10.0	10.0
Fish oil	124.3	83.6	94.5	141.3	14.8	158
CaHPO4.2H2O (18%)				6.5	30.7	51.1
L- amino acid mixture		15.4	41.3	44.3	65.7	91.4
Proximate composition						
Dry matter (%)	93.7	92.1	93.7	94.3	91.9	90.1
Proteins (% DM)	46.1	52.7	53	47.5	44.8	42.8
Lipid (%DM)	16.5	15.7	16.4	16.6	17.2	16.8
Gross energy ($KJ \cdot g^{-1}$ DM)	20.4	22.5	22.5	21.3	21.4	21.7

CP: crude protein. DM: dry matter. a: Amylum, Holland. b: Aquatex, France c: Primor00, France. d: according to the NRC (1993). e: Fish-soluble protein concentrate with fat from Sopropêche (Boulogne sur Mer, France).

Table 3. $\delta^{15}N$ tissue levels and $\delta^{15}N$ protein levels in rainbow trout and gilthead sea bream adapted to the six experimental diets

TROUT	RT1	RT2	RT3	RT4	RT5	RT6	
				$\delta^{15}N$ of diet			
	10.4	6.7	5.4	6.6	5.1	3.0	
							Factor 1
				$\delta^{15}N$ of tissue			*(tissue)*
liver	11.5	8.7	8.1	8.5	7.4	6.0	*1,2*
muscle	12.1	10.1	9.7	9.5	8.1	6.6	*1*
carcass	11.6	9.8	9.7	8.8	7.5	6.0	*1,2*
gut pv. fat	10.9	7.5	7.1	6.9	5.3	3.2	*2*
Factor 2							
(diet)	*a*	*b*	*b*	*b*	*bc*	*c*	
				$\delta^{15}N$ of protein			
liver	11.8	8.0	5.4	8.7	7.7	6.5	
muscle	13.2	10.0	10.1	9.2	10.4	7.6	
carcass	10.8	7.8	7.1	7.0	10.7	8.8	
gut pv. fat	11.8	8.8	7.5	8.1	9.8	7.4	
Factor 2							
(diet)	*a*	*ab*	*ab*	*ab*	*ab*	*b*	
SEA BREAM	GSB1	GSB2	GSB3	GSB4	GSB5	GSB6	
				$\delta^{15}N$ of diet			
	10.68	7.94	6.74	7.08	5.24	3.12	
							Factor 1
				$\delta^{15}N$ of tissue			*(tissue)*
liver	13.8	8.5	7.9	10.0	9.4	5.3	*1*
muscle	19.8	11.1	10.4	14.2	14.7	8.6	*2*
carcass	18.2	10.9	10.1	14.2	14.7	9.0	*2*
gut pv. fat	12.1	8.6	7.6	9.5	7.0	4.6	*1*
Factor 2							
(diet)	*a*	*b*	*bc*	*ab*	*abc*	*c*	
						Interaction	*0.009*
							Factor 1
				$\delta^{15}N$ of protein			*(tissue)*
liver	13,6	9.0	8.2	10.3	10.0	6.1	*1*
muscle	20.2	11.5	10.8	15.5	15.3	9.9	*2*
carcass	18.5		9.9	14.5	14.9	9.4	*2,3*
gut pv. fat	14.2	7.8	7.7	10.6	10.4	6.4	*1,3*
Factor 2							
(diet)	*a*	*bc*	*bc*	*ab*	*ab*	*c*	
						Interaction	*0.001*

Values represent the mean of $\delta^{15}N$ values, with n=6 for diets and n=2 for each tissue and diet. Numbers on the right indicate differences ($P<0.05$) for Factor 1 (tissues). Letters at the bottom indicate differences ($P<0.05$) for Factor 2 (diets).