Tracing Metabolic Routes of Feed Ingredients in Tissues of Broiler Chickens Using Stable Isotopes

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ABSTRACT The present study aimed to quantify the proportion of ^{13}C from energy and protein feed ingredients that follow the metabolic routing of the liver and muscle in broiler chickens. A stable isotope of carbon technique was used that is based on the isotopic discrimination that occurs in the plants during the photosynthesis process. One-day-old male chicks were subjected to treatments based on free choice of energy and protein sources. Rice bran (R) and soybean meal (S), C_3 plants, have higher isotopic ratios than corn (C), a C_4 plant, and corn gluten meal (G). Choices were R+S, C+G, R+G, C+S, or R+C+G+S. A complete feed (CF) was a sixth treatment. Feed intake and BW were measured at 30 d of age, when liver and breast muscle were collected for isotopic analysis. Treatments affected the amount of feed intake and the choices of energy or protein sources. Complete feed had the largest intake, differing from the other treatments that had free-choice feeding. Final BW was a direct reflection of consumption by these birds in all treatments. The isotopic results indicated that the $^{13}C/^{12}C$ ratio was generally higher in breast muscle than in liver, probably because of higher protein content. Moreover, in the liver, the proportion of ^{13}C retained from the energy ingredient was greater than the proportion from the protein ingredient. That is in contrast to muscle, where the proportion of ^{13}C retained from the protein ingredient was greater than from the energy ingredient that was self-selected.

(*Key words*: broiler, carbon-3 (C_3), carbon-4 (C_4), nutrient partition, stable isotope)

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INTRODUCTION

In dietary studies of birds, the applicability of some results is linked directly to the availability of data for nutritional requirements and diet composition for birds. In that context, the stable isotope technique is indicated for situations where 2 isotopically distinct dietary sources are available to subjects. This technique is being used increasingly as a tool to delineate dietary patterns in terrestrial and marine ecosystems because stable isotopic compositions of subject tissues often can be predictably related to stable isotopic compositions of diet (DeNiro and Epstein, 1978).

Different plants have different major pathways for carbon fixation during photosynthesis. In C_3 plants, a compound with 3 carbons is the immediate product of carbon fixation. Carbon dioxide is added to ribulose 1,5-bisphosphate to produce 2 molecules of 3-phosphoglycerate, which eventually goes to glucose and then starch. In C_4 plants, a compound with 4 carbons is the immediate product of carbon fixation. Carbon dioxide is added to phosphoenolpyruvate to yield oxaloacetate, which is reduced to malate or transaminated to aspartate. These compounds can be converted to glucose, then to starch, or be incorporated into protein. Carbon-3 plants have a higher $^{13}C/^{12}C$ ratio than C_4 plants such as corn (Minson et al., 1975). These isotopic demarcations are preserved during the feed passage by the digestive tract of the animal. Thus, it is possible to evaluate the C_3 and C_4 proportion directed for each tissue through its isotopic analysis.

Isotopic values of specific tissue components may not always follow bulk diet values. The reason is that the carbon skeletons of different dietary constituents (protein, lipids, and carbohydrates) can be shunted to different tissue constituents. This effect has been termed isotopic routing (Schwarcz, 1991).

A number of studies emphasized the problems that metabolic routing poses in the interpretation of isotopic data in diet reconstruction (Sillen et al., 1989; Parkington, 1991; Schwarcz and Schoeninger, 1991). However, differential routing of carbon skeletons in animals fed

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Abbreviation Key: C = corn; CF = complete feed; G = corn gluten meal; R = rice bran; S = soybean meal.

TABLE 1. Calculated nutrient content (DM basis) and determined ^{13}C-content[1] of ingredients used for self-selection treatments

	High ^{13}C content		Low ^{13}C content	
Nutrients[2]	Rice bran	Soybean meal	Corn	Corn gluten meal
ME, kcal/kg	2,453	2,540	3,371	3,775
CP, %	13.21	48.00	8.57	59.85
Crude fiber, %	10.32	4.50	1.95	1.03
Calcium, %	0.11	0.33	0.03	0.046
Available phosphorus, %	0.32	0.22	0.08	0.16
Methionine, %	0.23	0.67	0.17	1.44
Methionine + cysteine, %	0.45	1.40	0.37	2.50
Lysine, %	0.58	2.92	0.25	1.00
Tryptophan, %	0.14	0.66	0.06	0.28
Threonine, %	0.48	1.88	0.33	2.11
$\delta^{13}C$ (‰), (n = 3)	−29.50 ± 0.13	−25.87 ± 0.26	−11.66 ± 0.40	−13.77 ± 0.10

[1]Content of ^{13}C expressed in parts per thousand (‰) relative to the Peedee Belemnite (PDB) standard.
[2]Calculated nutrient composition described by Rostagno et al. (2000).

low vs. high protein diets is exciting from a physiological standpoint because it may provide clues about how animals allocate nutrients to their tissues. Animals on high protein diets can rely almost entirely on dietary amino acids to provide carbon skeletons for protein, but animals on low-protein diets synthesize the carbon skeletons of amino acids from a combination of protein and carbohydrate carbon sources (Gannes et al., 1998).

The fate of dietary components can be traced by feeding animals diets with components of contrasting isotopic values. The isotopic composition of different tissues and body components reflects how dietary nutrients are allocated to different tissues. Little is known about the mechanisms that lead to routing and how the magnitude of fluxes of carbon skeletons among dietary pools and the pools in different tissues vary (Gannes et al., 1998). Consequently, the objective of this study was to determine the routing of stable isotopes in energy and protein feed ingredients to the liver and breast muscle of broilers.

MATERIALS AND METHODS

Birds and Treatments

One-day-old male broiler chicks (Ross, n = 216) were housed in a metallic battery containing 3 floors with 6 cages/floor, with an initial density of 12 broilers/cage. The dimensions of the cages were 0.30 m high, 0.95 m wide, and 0.50 m deep. The experimental design was randomized in complete blocks with 3 replications/treatment (replications = blocks = floors).

The broilers were divided into 6 experimental treatments based on the self-selection of energy and protein sources, in addition to a supplement. Rice bran (R) and soybean meal (S) are rich sources of ^{13}C, because they are from C_3 plants, whereas corn (C) and corn gluten meal (G) are poor sources of ^{13}C. Five combinations of these ingredients were used as treatments: R+S, C+G, R+G, C+S, and R+C+G+S. The calculated nutrient content and the determined ^{13}C content are presented in Table 1. Chicks were allowed to self-select the proportions of these ingredients by using a feeder with either 3 or 5 separate compartments. The final compartment in the feeder contained a vitamin and mineral supplement (Table 2). The sixth treatment was a complete feed (CF) that was similar to a commercial broiler feed. Its calculated nutrient content and analyzed ^{13}C content are presented in Table 3.

Water was supplied ad libitum by a nipple and the feed supply was through feeders equipped with 3 compartments to separate the energy sources from those of protein and supplement. The lighting program was 24L:0D, using incandescent lamps of 100 W. Until 10 d of age, heat was provided by 100-W bulbs.

Isotopic Procedures

At 30 d of age, 1 chicken per cage (3 chickens/treatment) was randomly selected and killed by cervical dislocation to collect liver and breast muscle samples, which were identified and frozen at −20°C.

To prepare the samples for isotopic analysis, the liver and breast muscles (n = 3) were thawed, rinsed in distilled water and dried in a forced air oven at 56°C for 48 h. All samples were individually ground to a powder

TABLE 2. Composition of the vitamin and mineral supplement for self-selection treatments

Ingredients, %	
Mineral mix[1]	0.10
Vitamin mix[2]	0.50
Salt	0.35
Limestone	1.00
Dicalcium phosphate	2.00

[1]Mineral mix would provide the following per kilogram of diet if selected in the same proportions as used in a complete feed: Cu, 70 mg; Fe, 50 mg; I, 1.25 mg; Mn, 60 mg; Se, 0.2 mg; Zn, 50 mg; vehicle, 1 g.
[2]Vitamin mix would provide the following per kilogram of diet if selected in the same proportions as used in a complete feed: vitamin A, 7,500 IU; vitamin D_3, 2,500 IU; vitamin E, 15 mg; vitamin K_3, 1.2 mg; vitamin B_{12}, 12.5 mcg; thiamine, 1.5 mg; riboflavin, 5.5 mg; pyridoxine, 2 mg; niacin, 35 mg; calcium pantothenate, 10 mg; folic acid, 0.6 mg; biotin, 0.06 mg; choline chloride, 350 mg; methionine, 1,550 mg; coccidiostat, 100 mg; growth promoter, 40 mg; antioxidant, 20 mg; vehicle, 4 g.

TABLE 3. Composition and calculated analysis of the complete feed (CF)

Ingredient	%
Corn	63.00
Soybean meal	33.00
L-Lysine	0.10
Limestone	1.10
Dicalcium phosphate	1.83
DL-Methionine	0.12
Salt	0.35
Mineral mix[1]	0.10
Vitamin mix[2]	0.40
Calculated analysis	
ME, kcal/kg	2,972
CP, %	21.41
Crude fiber, %	2.71
Calcium, %	0.98
Available phosphorus, %	0.45
Methionine, %	0.45
Methionine + cysteine, %	0.81
Lysine, %	1.20
$\delta^{13}C$ (‰),[3] (n = 3)	−14.87 ± 0.07

[1]Mineral mix provided per kilogram of complete diet: Cu, 70 mg; Fe, 50 mg; I, 1.25 mg; Mn, 60 mg; Se, 0.2 mg; Zn, 50 mg; vehicle, 1 g.

[2]Vitamin mix provided per kilogram of complete diet: vitamin A, 7,500 IU; vitamin D_3, 2,500 IU; vitamin E, 15 mg; vitamin K_3, 1.2 mg; vitamin B_{12}, 12.5 mcg; thiamine, 1.5 mg; riboflavin, 5.5 mg; pyridoxine, 2 mg; niacin, 35 mg; calcium pantothenate, 10 mg; folic acid, 0.6 mg; biotin, 0.06 mg; choline chloride, 350 mg; methionine, 1,550 mg; coccidiostat, 100 mg; growth promoter, 40 mg; antioxidant, 20 mg; vehicle, 4 g.

[3]Content of ^{13}C expressed in parts per thousand (‰) relative to the Peedee Belemnite (PDB) standard.

in a cryogenic mill[2] of liquid nitrogen, at –190°C. In this mill, each sample was placed in a different tube and each material was powdered for 3 min at maximum frequency to obtain a homogeneous material of fine granular powder (Ducatti, 2000). After milling, the liver and muscle samples (1.0 g powdered) had the lipids removed using a Soxhlet apparatus with ethyl ether for 4 h (AOAC, 1990). After preparation, the samples were stored in a freezer at –18°C.

For the determination of the isotopic ratio (δ ‰ $^{13}C/^{12}C$), a DELTA-S mass spectrometer[3] was used, according to the method described by Ducatti et al. (1979). The values were expressed in parts per thousand (‰) relative to the Peedee Belemnite (PDB)[4] standard, with error of analysis on the order of 0.1 ‰ and calculated as follows:

$$\delta \text{ ‰ } {}^{13}C_{(\text{sample, standard})} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3,$$

where δ ‰ ^{13}C = relative ratio of sample in relation to standard PDB, and R = isotopic ratio ($^{13}13C/^{12}12C$) of sample and standard.

[2]Model 6700, SPEX Certiprep, Metuchen, NJ.

[3]Finnigan MAT GmbH, Bremen, Germany.

[4]PDB is a Cretaceous belemnite, *Belemnitella americana*, from the Peedee formation of South Carolina.

Feed ingredients and broiler tissues were analyzed using this procedure.

To measure the isotopic contribution and relative amount of each source (isotope dilution principle), the mass-balance and isotopic equation was used, expressed in the form δa (A) + δb (B) = δP. Here, δa and δb represent the value of the δ ‰ ^{13}C of the different food sources, A and B indicate the percentage contribution of each food source in the formation of tissue, and δP corresponds to the δ‰ ^{13}C of the analyzed tissue. As only 2 primary sources contributed to the formation of the product (A + B = 1), the contribution of any one of them in relation to the product P can be expressed. The percentage of ingredient A that contributed to the formation of body tissue was determined by the ^{13}C content of the tissue. Then the percentage contribution of ingredient B to the tissue was calculated as 100 − A.

When the treatment was C+G+R+S, it was necessary to adapt the previous formula considering the tissue δ of 2 associated treatments (R+S and C+G, which together possess the 4 ingredients relating to treatment C+G+R+S). Here δa and δb represent the δ ‰ ^{13}C value of tissues in the treatment R+S and C+G, respectively, A and B indicate the percentage contribution of each treatment in the formation of tissue, and δP corresponds to the δ‰ ^{13}C of the analyzed tissue. Letting R+S = % A, the proportion of R and the proportion of S can be calculated using the rule of three. Letting C+G = % B permits solving for proportions of C and G.

Statistical Analysis

Feed intake and BW data were analyzed by a one-way ANOVA (SAS Institute, 1996). Significant differences among treatment means were further analyzed using the Tukey test with a 5% level of significance.

Isotopic data were analyzed using the Origin 6.0 (Microcal Software, 1999) nonlinear regression fitting procedure. The substitution curve of the ^{13}C for each tissue was fitted (Hobson and Clark, 1992; Ducatti et al., 2002) to the regression equation ($Y = A + B\ e^{-kt}$), where Y represents the $\delta^{13}C$ value of the tissue in question, A represents the initial condition, B is the asymptotic value of $\delta^{13}C$ for that tissue, k is the turnover rate of carbon in the liver or pectoralis muscle associated with the variable growth, and t is time (d) since the first day of experiment (1 d old). Data are presented as means ± SDM.

RESULTS AND DISCUSSION

The results in Table 4 showed that there was difference among total feed ingredient intake (g/chicks) that were consumed by the chicks in each treatment. Chicks fed CF had the largest intake, differing from the other treatments. Treatments R+S, C+S, and R+C+G+S did not differ statistically in feed intake. Diets with soybean meal as the protein source presented a better balance of

TABLE 4. Feed consumed, percentage ingredient consumed, and BW (mean ± SEM, n = 3) of broilers at 30 d

Treatments[1]	Ingredients	Ingredient intake (g/chick)	Ingredient intake (%/chick)	BW (g/chick)
R + S	R	577 ± 84	39.2	865 ± 58[b]
	S	895 ± 112	60.8	
	Total	1,472[b]	100.00	
C + G	C	221 ± 9	92.4	89 ± 4[d]
	G	18 ± 4	7.6	
	Total	239[d]	100.00	
R + G	R	527 ± 41	90.5	243 ± 17[c]
	G	56 ± 29	9.5	
	Total	583[c]	100.00	
C + S	C	1,023 ± 136	65.7	811 ± 133[b]
	S	535 ± 84	34.3	
	Total	1,558[b]	100.00	
R + C + G + S	R	50 ± 23	3.2	901 ± 137[b]
	C	838 ± 122	54.0	
	G	176 ± 50	11.3	
	S	489 ± 107	31.5	
	Total	1,553[b]	100.00	
CF	CF	1,972 ± 45[a]	100.00	1,204 ± 26[a]

[a–d]Means ± SEM within columns with no common superscript differ significantly ($P < 0.05$).

[1]R = rice bran; S = soybean meal; C = corn; G = corn gluten meal; CF = complete feed.

essential amino acids. Diets C+G and R+G, although different, provided the smallest intakes, probably because corn gluten meal, the protein source of these diets, presented an imbalance of essential amino acids.

The birds that received diet C+S selected proportions of C and S that were similar to those in CF (63.00% corn and 33.00% soybean meal), demonstrating the capacity of these birds to self-select protein and energy sources to meet nutritional requirements. The intake of different ingredients observed in the present experiment is in agreement with the results obtained by Sakomura et al. (1997); however, our results differ from those of Mastika (1992) in a similar experiment, who found no difference in intake due to selection of feed ingredients. Birds fed diet R+S consumed more S than R, possibly because of a better balance of amino acids (Table 2).

For the birds that received diet C+G and ones that received R+G, the energy ingredient intake was greater than the G intake. This was due to its low palatability or because G did not meet the birds' nutrient requirements. At the end of 30 d, the birds fed with R+G were heavier than those fed with C+G, which may be due to the superior biological value of protein in the rice bran relative to that of corn and its CP content compared with corn (Rostagno et al., 2000).

The birds subjected to the treatment R+C+G+S showed throughout the experiment a preference for corn and soybean meal, which in the beginning is larger for the intake of the protein food (soybean meal + corn gluten meal) and in the end, larger for the intake of the energy food (corn). With the option of a second protein source (corn gluten meal), the birds of this treatment showed the clear capacity to self-select protein and energy sources to meet nutritional requirements. Final BW directly reflected the consumption by these birds in all treatments (Table 4).

Table 5 shows the values of the isotopic ratio, expressed in delta per thousand, of $^{13}C/^{12}C$ of broiler liver and breast muscle samples subjected to different treatments. Muscle from all treatments except R+S had a higher $^{13}C/^{12}C$ ratio than the liver. Because such values as well as the isotopic mean value of the experimental diets are known (Tables 1 and 3), it was possible to establish the percentage contribution of the carbon from each food source in the metabolic route of the tissues in question.

In general, the energy foods resulted in a larger percentage of carbon partitioned to the liver (Table 6), and the protein foods resulted in a larger percentage of carbon partitioned to the muscle formation, confirming the biochemical theory of tissue formation (Lehninger et al., 1993).

In the broilers that only received C_3 feed ingredients (R+S), of the total carbon that followed the metabolic route of the liver, a larger carbon contribution was observed from the protein food (soybean meal) than from the energy food. Possibly, this occurred because they

TABLE 5. Content of ^{13}C in liver and breast muscle (mean ± SEM, n = 3) of broilers fed for 30 d[1]

Treatments[2]	Liver (‰)	Muscle (‰)
R + S	–26.3 ± 0.4	–25.7 ± 0.3
C + G	–11.1 ± 0.1	–12.3 ± 0.3
R + G	–26.3 ± 0.5	–27.4 ± 0.1
C + S	–17.2 ± 0.1	–18.8 ± 0.1
R + C + G + S	–15.8 ± 0.4	–17.6 ± 0.1
CF	–16.4 ± 0.2	–18.5 ± 0.3

[1]Content of ^{13}C expressed in parts per thousand (‰) relative to the Peedee Belemnite (PDB) standard.

[2]R = rice bran; S = soybean meal; C = corn; G = corn gluten meal; CF = complete feed.

TABLE 6. Percentage contribution of each feed source in the metabolic partitioning of carbon for liver and breast muscle of broilers (n = 3) at 30 d of age subjected to different treatments

	Liver		Muscle	
Treatments[1]	Energy (%)	Protein (%)	Energy (%)	Protein (%)
R + S	R 38.6	S 61.4	R 22.9	S 77.1
C + G	C 78.7	G 21.3	C 24.2	G 75.8
R + G	R 85.7	G 14.3	R 92.7	G 7.3
C + S	C 54.2	S 45.8	C 42.9	S 57.1
R + C + G + S	R 14.5 C 49.2	G 13.3 S 23.0	R 10.7 C 12.9	G 40.2 S 36.2
CF	C 59.3	S 40.7	C 45.2	S 54.8

[1]R = rice bran; S = soybean meal; C = corn; G = corn gluten meal; CF = complete feed.

are foods that differ little in energy terms, but in composition of amino acids, soybean meal is of superior biological value to rice bran, causing a larger percentage in the liver than in the muscle. Similarly, in the broilers that received a diet based on R and G, of the total carbon that followed the metabolic route of muscle formation, the contribution of the carbon of the energy food (rice bran) was larger than that of the carbon of the protein foods. This may have occurred because of the larger consumption of this food by the birds (Table 4), or because the protein from the rice was of superior biological value to the protein of the corn gluten meal.

It was suggested by Gannes et al. (1998) that the isotopic composition of muscle reflects dietary protein. The amino acids released from degraded proteins can be deaminated and oxidized directly by a tissue and used for energy or gluconeogenesis in the liver or used in the synthesis of other proteins (Goldberg and Chang, 1978). In contrast with the liver, which exports most of the carbon compounds received for the accomplishment of metabolic processes in other tissues, the muscle retains more of the amino acids it receives as protein.

The values of allocation found in this study for δ‰ $^{13}C_{,PDB}$ show that, independent of biological value, the proportion of ^{13}C retained from the energy ingredient in the liver was greater than the proportion of protein ingredient. This is in contrast to muscle where the proportion of ^{13}C retained from the protein ingredient was greater than the proportion of energy ingredient that was self-selected. Moreover, it was concluded that the $^{13}C/^{12}C$ ratio was generally higher in muscle than in liver, probably because of higher protein content.

This research reported one example of the use of stable carbon isotopic routing for studying metabolism. The sources of ^{13}C were plants that selectively incorporated higher or lower amounts of stable isotope in their products. When chicks eat these products, the ratio of $^{13}C/^{12}C$ in the tissues is altered by the ratio in the plant products eaten. Then, matching the appropriate stable isotope with the characteristics of the tissue to be studied will provide the possibility of studying isotopic routing in broilers and others animals.

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