Studies on carbon-13 turnover in eggs and blood of commercial layers[#]

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ABSTRACT

This paper aimed at evaluating the influence of diets containing different isotopic values of carbon-13 turnover on the half-life of egg (yolk + albumen), yolk and albumen individually, and blood of poultry using $\delta‰^{13}C$ isotopic variation. Commercial layers fed four experimental isocaloric and isonitrogenous diets (RC_4, RC_3, RMC_4 and RMC_3) containing different isotopic values, during an experimental period of 56 days. Turnover of the studied tissues was influenced by the experimental diets. Blood and albumen were more influenced by dietary treatments as compared to egg and yolk. The RMC_3 diet induced better performance (better feed intake and higher egg production) due faster rate of carbon substitution than the RC_4 diet, and lower half-life for egg (yolk + albumen), yolk, and albumen.

INTRODUCTION

Most of turnover studies using the natural variations of the delta per thousand of carbon-13 $(\delta‰^{13}C)^*$ of foods are related to ecological issues, focusing on the substitution velocity of tissue carbon from food with different isotopic ratios due migrations and changes in trophic levels (Mizutani *et al.*, 1991, Hobson 1995). However, stable isotopes technique can be useful in physiology and animal nutrition studies as the substitution rates of tissue carbon can be influenced by environmental, nutritional, and health factors (Carrijo *et al.*, 2000).

The period during which the isotopic concentration of a tissue reflects the isotopic signature of a diet is partially dependent on the isotopic turnover rate of that tissue (Hobson and Clark, 1992a). Isotopic turnover rate depends on the absorption, synthesis, and catabolism rates of each tissue component (Gannes *et al.*, 1998). Tissues with fast isotopic turnover rates reflect more recent diets, while those with slower turnover rates reflect diets fed during previous periods (Hobson and Clark, 1992a, b). Most metabolically active tissues (liver, pancreas, and adipose tissue) present faster turnover rates than the less metabolically active tissues, such as bone collagen (Hobson & Clark, 1992a).

Eggs, as other animal products, such as like milk and feces, are not considered tissues, and therefore it is not possible to calculate its tissue renewal rate (turnover). However, turnover studies in these products indicate that the renewal rate of nutrients for their synthesis reflect the metabolism of the tissues that generated these products.

Carrijo *et al.* (2000), studying carbon turnover in eggs and liver of laying hens, verified that carbon substitution time was similar in those tissues. This indicates that eggs could be used as an indirect measure of liver turnover rate, thereby avoiding the sacrifice of the birds. These

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[*] Content of carbon-13 expressed in parts per thousand (‰) relative to the Peedee Belemnite (PDB) standart.

authors recommend the separate isotopic rate analysis of egg fractions (albumen and yolk), because values found in eggs could result from isotopic dilution of fractions with different substitution velocity.

When working with diets based on grains of plants of the C_3 and C_4 photosynthetic cycle, a feed formulation problem arises in diets containing C_4 plants. There are few C_4 ingredients that can replace soybean meal (C_3), which is the main protein source of balanced diets for commercial layers. An alternative ingredient to replace soybean meal is corn gluten, which is a by-product of corn starch removal, and which contains 60% crude protein. However, corn gluten presents some restrictions, as it is deficient in lysine and tryptophan for chickens, and due to its bitter flavor.

Ducatti *et al.* (2002), based on literature results , proposed a theoretical model to express the results of relative carbon enrichment ($\delta‰^{13}C$) in terms of feeding time by supplying diets with different ^{13}C isotopic values, and comparing this with adult animals. We hypothesized the metabolism of certain animal tissues depends on the input rate of dietary carbon compounds and the substitution rate of the pre-existent compounds. This model is suitable for determining the total or partial (half-life) carbon substitution time in tissues in studies with poultry and small animals.

Kennedy & Krouse (1990) stated that stress related to external environmental factors, physical conditions, and diseases could cause variations in the isotopic fractionation.

The present study aimed at evaluating the influence of diets with different isotopic values on carbon-13 turnover of egg (yolk + albumen), yolk and albumen individually, and blood half-lives of commercial laying hens, using $\delta‰^{13}C$ isotopic variation.

MATERIAL AND METHODS

The study was carried out at Lageado Experimental Farm, in the School of Veterinary Medicine and Animal Science (FMVZ) of São Paulo State University (UNESP), Botucatu campus.

Ninety-six *Hy Line W36* 120-week-old layers were housed in metallic cages placed in four rows. Cages were1.00m long x 0.45m deep x 0.40m high. Cages had two internal compartments, housing four birds each. Birds were designated to four treatments with three replicates of eight birds each. Food and water were available *ad libitum* in feeders and nipple drinkers.

Layers were adapted to the new facilities during a 28 day-period, and were fed 101.5 g/bird/day of a commercial feed (based on corn, soybean meal, and wheat). This diet had a $\delta‰^{13}C$ isotopic signal = -16.06 ± 0.23 (n = 2). Average egg production was 52%. As these birds were at the end of their productive cycle, their egg production was low.

This trial lasted 84 days, including a 28 days of pre-experimental period. According to Carrijo *et al.* (2000), this pre-experimental period is sufficient for isotopic marking of eggs to happen. The objective of this period objective was to replace carbon derived from the commercial feed in the studied tissues by carbon derived from the new diets. Birds were divided in two groups of 48 each, with one group receiving a diet based on grains of C_3 (RC_3) photosynthetic cycle plants, whereas the other was fed with a diet consisting of grains of C_4 (RC_4) photosynthetic cycle plants. At the end of the adaptation period, birds started to receive the experimental diets.

The group fed the RC_3 feed during the pre-experimental period was divided in two treatments (n=24), which were fed RC_4 and RMC_4 diets afterwards. The other group, which was being fed RC_4, was also divided in two treatments, and were fed RC_3 and RMC_3 diets (see treatment description below).

Treatments consisted of four isocaloric and isonitrogenous diets with different isotopic values, which were fed during the entire experimental period (56 days).

Treatments:
Treatment 1 (RC_4) - Feed consisting of grains of C_4 photosynthetic cycle plants.
Treatment 2 (RC_3) - Feed consisting of grains of C_3 photosynthetic cycle plants.
Treatment 3 (RMC_4) - Mixed feed consisting of 67% C_4 grains and 33% C_3 grains.
Treatment 4 (RMC_3) - Mixed feed consisting of 67% C_3 grains and 33% C_4 grains.

The mixed diets were used to obtain intermediate $\delta‰^{13}C$ values as those of the pure diets with the objective of evaluating the influence of the mixed diets and the possible improvement of the quality of the C_4 diet.

Nutritional composition of ingredients used in feed formulation, as well as nutritional requirements followed recommendations of Rostagno *et al.* (2000). The percentage composition, calculated nutritional levels, and $\delta‰^{13}C$ values of the experimental diets are shown in Table 1.

On the 0th, 1st, 2nd, 4th, 7th, 11th, 16th, 21st, 28th, 35th, 42nd, 49th, and 56th day of the experimental period,

eight eggs were randomly collected per treatment group on each collection day, with four eggs used for yolk and albumen sampling, and the other four eggs were homogenized in blender (yolk + albumen). Additionally, on each collection day, blood samples were taken from the wing vein of four birds per treatment. The samples were labeled, frozen, and stored at -18°C. The collections were concentrated in the first days due the higher velocity of carbon incorporation. Day zero was assumed when samples were obtained immediately before feed change, and its purpose was to express the isotopic composition of feed offered at the pre-experimental period

Table 1 – Composition of the experimental diets (calculated levels and values of δ‰^{13}C).

Ingredients,	T1 (RC$_3$)^3	T2 (RC$_4$)^3 %	T3 (RMC$_3$)^3	T4 (RMC$_4$)^3
Rice, broken	62.86	-	42.11	20.74
Soybean meal	23.50	-	15.75	7.76
Corn	-	62.15	20.51	41.63
Corn gluten meal	-	17.00	5.61	11.39
Kaolin	-	8.50	2.81	5.70
Limestone	9.25	9.50	9.33	9.42
Dicalcium Phosphate	1.70	1.65	1.68	1.67
DL - methionine	0.14	0.05	0.11	0.08
L - lysine	-	0.60	0.20	0.40
Salt	0.35	0.35	0.35	0.35
Soybean oil	2.00	-	1.34	0.66
Mineral premix[1]	0.10	0.10	0.10	0.10
Vitamin premix[2]	0.10	0.10	0.10	0.10
TOTAL	100	100	100	100
Calculated Nutritional Levels				
ME, MJ/kg	11.56	11.56	11.56	11.56
CP, %	16.00	16.00	16.00	16.00
Met, %	0.44	0.40	0.43	0.41
Met+Cys, %	0.68	0.69	0.68	0.68
Lys, %	0.81	0.80	0.80	0.80
Trp, %	0.22	0.08	0.18	0.13
Ca, %	4.05	4.04	4.05	4.04
Available P, %	0.37	0.37	0.37	0.37
Na, %	0.20	0.20	0.20	0.20
Isotopic Values				
δ‰^{13}C (n=4)	-26.70±0.04	-12.36±0.10	-22.40±0.02	-17.22±0.11

1 - Composition by kg of the mineral supplement: Copper: 8,000mg; Iron: 50,000mg; Manganese: 70,000mg; Zinc: 50,000mg; Iodine: 1,200mg; Selenium: 200mg; QSP Excipient: 1,000g. 2 - Composition by kg of the vitamin supplement: Vitamin A: 7,000,000 UI; Vitamin D$_3$: 2,000,000 UI; Vitamin E: 5,000 mg; Vitamin K$_3$: 1,600mg; Vitamin B$_2$: 3,000mg; Vitamin B$_{12}$: 8,000mcg; Niacin: 20,000mg; Panthotenic Acid: 5,.000mg; Antioxidant: 15,000mg; QSP Excipient: 1,000g. 3 - T1 (RC$_4$) - Feed consisting of grains of C$_4$ photosynthetic cycle plants; T2 (RC$_4$) - Feed consisting of grains of C$_3$ photosynthetic cycle plants.;T3 (RMC$_4$) - Mixed Feed consisting of 67% C$_4$ grains and 33% of C$_3$ grains; T4 (RMC$_3$) - Mixed Feed consisting 67% C$_3$ grains and 33% C$_4$ grains.

In order to perform isotopic analyses, egg and yolk samples were thawed, dried in forced ventilation oven (Marconi - MA 035 model) at 56°C for 48 hours, and defatted in ethyl ether using the Soxhlet apparatus for 4 hours. It is necessary to remove lipids from the sample because the presence of fat makes the isotopic signature lighter in ^{13}C in relation to the diet. For albumen and blood, the drying period was 24 hours, and these were not defatted because they have low fat content. All samples were ground in cryogenic mill (Spex- freezer/mill 6750 model) for three minutes at -190ºC, in order to obtain homogeneous material with microscopic aspect (Ducatti, 2004).

Isotopic composition of samples was determined using a DELTA-S (Finnigan Mat) mass spectrometer coupled to an EA 1108 CHN Elemental Analyzer, in the Stable Isotopes Center facilities of the Biosciences Institute of UNESP, Botucatu campus. The results were expressed as δ‰^{13}C relative to the *Peedee Belemnite* (PDB) standard, with analysis error of 0.2‰, according to equation 1:

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

Where:
δ‰^{13}C = enrichment of the ^{13}C/^{12}C ratio of the sample in relation to the PDB standard.
R = isotopic ratio (^{13}C/^{12}C) of the sample and the standard.

In order to quantitatively measure the carbon substitution speed of the diets after certain time, the exponential function expressed by equation 2 (Ducatti *et al.*, 2002) was used:

$$\delta‰^{13}C(t) = \delta‰^{13}C(f) + [\delta‰^{13}C(i) - \delta‰^{13}C(f)]e^{-kt} \quad (2)$$

Where:
δ‰^{13}C(t) = isotopic enrichment of the tissue at any time (t).
δ‰^{13}C(f) = isotopic enrichment of the tissue at the balanced base, or final status.
δ‰^{13}C(i) = isotopic enrichment of the tissue, at the initial condition.
k = change constant (turnover) in time units^{-1}
t = time (in days) since feed was replaced.

Carbon-13 half-life for egg, yolk, albumen, and blood, in the condition of 50% of each diet in t = T was calculated by equation 3:

$$T = \ln 2/k \quad (3)$$

where,
T represents half-life in days

ln is the Neperian logarithm, and constant k presents time unit^{-1}, providing an idea of "speed" in the exchange process of stable isotopes in the tissues (Tieszen *et al.*, 1983; Hobson & Clark, 1992a, b; Ducatti *et al.*, 2002; Ducatti, 2004).

Isotopic results were analyzed using regression equation method of Origin® 6.0 Professional (Microcal Software, 1999) software, whereas performance results (feed intake, egg production, and egg mass) were statistically analyzed using analysis of variance (ANOVA), with the aid of the GLM procedure of the SAS software (1999). The test of Tukey at the probability level of 5% was used to compare treatment means.

RESULTS AND DISCUSSION

Table 2 shows the regression equations of the treatments in the different studied tissues. δ‰^{13}C value changes in tissues along time occurred gradually until a new balance was reached. This change can be seen in Figure 1, which illustrates turnover behavior in blood, egg, yolk, and albumen.

Blood carbon half-life was 13.3 days in RC_4 treatment, and 5.3 days in RC_3 treatment. Turnover period was 2.5 times slower in the blood of RC_4 treatment layers as compared to layers fed the RC_3 diet. These results are consistent with those of Carrijo *et al.* (2000), who evaluated liver and egg turnover rates of layers, and found higher half-life values when a C_3 feed was replaced by a C_4 diet as compared to the inverse replacement. This slower turnover can be partly explained by the drastic feed intake reduction of layers fed RC_4 (Table 3). Corn gluten may have reduced palatability, and nutritionally unbalanced the feed mainly due to the level of tryptophan–, which was
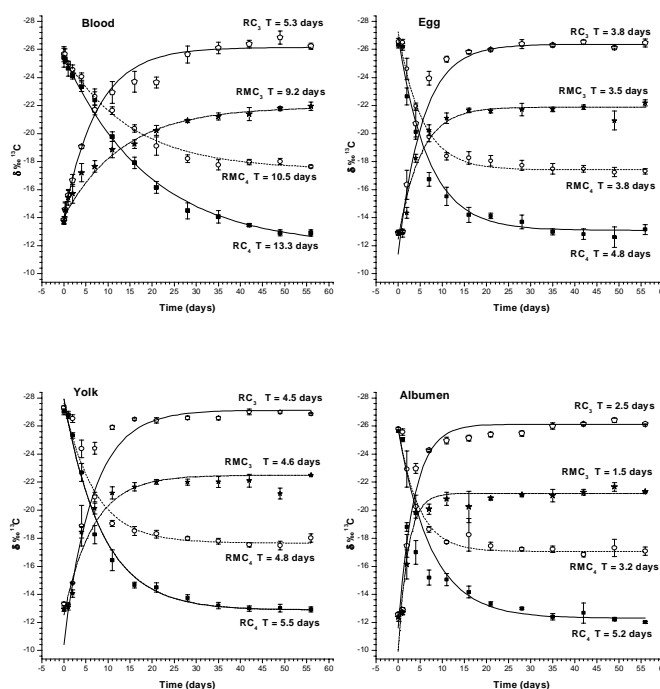

Figure 1 – Experimental model of the turnover of carbon stable isotopes* (mean + standard-deviation, n = 4) of blood, egg, yolk, and albumen of commercial laying hens with respective half-life values, in days, of treatments RC_3, RMC_3, RMC_4, and RC_4.

* Microcal Software Origin® 6.0 Professional. Origin Data Analysis and Technical Graphics. USA, Microcal Software Inc. 1999.

not considered when the experimental feeds were formulated – was probably the reason of low feed intake.

Gonzales (2002) states that very sweet (20% of sucrose) or bitter water is usually rejected by poultry. This is also true for solid feed; poultry reject very sweet or bitter foods (Balog & Millar, 1989).

In addition, amino acids balance in the RC_3 diet was different from that in the RC_4 diet (Table 1), where

Table 2 - Regression and half-life equations of blood, whole egg, and egg fractions (yolk and albumen) of commercial laying hens.

Tissues	Treatments	Equations (δ^{13}C)	R^2	CarbonHalf-life (days)
Blood(n=4)	RC_4	$-11.95‰ - 13.63‰e^{-0.052t}$	0.99	13.3
	RC_3	$-26.15‰ + 12.14‰e^{-0.132t}$	0.98	5.3
	RMC_4	$-17.43‰ - 8.17‰e^{-0.066t}$	0.99	10.5
	RMC_3	$-21.90‰ + 7.87‰e^{-0.076t}$	0.98	9.2
Egg(n=4)	RC_4	$-13.10‰ - 13.82‰e^{-0.146t}$	0.99	4.8
	RC_3	$-26.36‰ + 14.95‰e^{-0.181t}$	0.99	3.8
	RMC_4	$-17.41‰ - 9.83‰e^{-0.185t}$	0.99	3.8
	RMC_3	$-21.89‰ + 9.42‰e^{-0.200t}$	0.98	3.5
Yolk(n=4)	RC_4	$-13.01‰ - 14.99‰e^{-0.127t}$	0.99	5.5
	RC_3	$-27.13‰ + 16.70‰e^{-0.153t}$	0.99	4.5
	RMC_4	$-17.67‰ - 9.97‰e^{-0.143t}$	0.99	4.8
	RMC_3	$-22.50‰ + 9.96‰e^{-0.151t}$	0.99	4.6
Albumen(n=4)	RC_4	$-12.30‰ - 13.48‰e^{-0.133t}$	0.95	5.2
	RC_3	$-26.14‰ + 14.54‰e^{-0.277t}$	0.98	2.5
	RMC_4	$-17.06‰ - 8.86‰e^{-0.220t}$	0.99	3.2
	RMC_3	$-21.20‰ + 11.26‰e^{-0.467t}$	0.94	1.5

only methionine and lysine requirement levels were corrected. However, RC_4 diet tryptophan levels were below (calculated tryptophan level was 0.08%) the nutritional requirement, which is 0.18% for layer in this stage (Rostagno *et al.*, 2000).

Table 3 – Feed intake, in g/bird/day, at the end of the pre-experimental period (22-28 days), beginning (29-35 days) and end of the experimental period (78-84 days).

Days	Treatments			
	RC_4	RC_3	RMC_4	RMC_3
22-28	97.26abA	93.81bC	100.83a	84.01cC
29-35	84.23cB	141.67aA	105.61b	142.69aA
78-84	81.63bB	112.24aB	112.24a	109.78aB

Different small letters in the same row are different by the test of Tukey (p < 0.05). Different capital letters in the same column are different by the test of Tukey (p < 0.05).

The high feed intake of birds in RC_3 and RMC_3 treatments was probably due to thefeed offered in the pre-experimental period , when these birds were fed a diet formulated exclusively with C_4 ingredients. After feed change (29-35 days), feed intake was dramatically increased to compensate the low feed intake of the last phase.

Studies demonstrated that poultry can control their feed intake based on feed protein amount and quality. Feed intake regulation theory by dietary protein considers the principle that the detection of plasmatic levels of amino acids, particularly of the nutritionally essential amino acids, takes place by activating control systems in the central nervous system (Gonzales, 2002).

Feed intake regulation mechanism is essentially homeostatic, with variations in feed intake depending on protein quality and amount in the feed. Feeds with serious amino acids unbalance induce a marked decrease in intake (Boorman, 1979). These factors may have contributed for a reduction in the layer metabolism rate, and consequently blood carbon turnover.

Carbon half-life values in the blood of layers in the intermediate treatments (RMC_4 and RMC_3) were 10.5 days and 9.2 days, respectively. These values were not different from RC_4 treatment. These results are consistent with those obtained by Hobson & Clark (1992a), who, working with substitution of predominant C_3 diet for C_4 in quails, obtained blood carbon half-life of 11.4 days. These half-life values were expected to be similar to those found in the RC_3 treatment. Feed intake results of these treatments presented similar values. Tryptophan balance was probably less harmful to these birds as compared to the diet including

exclusively C_4 due the interference of the diet on metabolism. This fact is probably more related to the supply of nutritional requirements than to photosynthetic cycle type of the plants included in the diets.

The half-life value for the egg (yolk + albumen) of layers fed the RC_3 diet was 3.8 days, and 4.8 days birds in RC_4 treatment. These results agree with those of Carrijo *et al.* (2000), who evaluated liver and egg turnover rates of layers, and obtained half-life values of 3.7 days when a C_4 diet was replaced for a C_3 diet. These authors also found a value of 4.0 days when the C_3 diet was replaced for the C_4 diet. In the present study, carbon half-life values of eggs from RMC_4 and RMC_3 treatments were 3.8 days and 3.5 days, respectively.

The yolk carbon half-life of the RC_3 treatment was 4.5 days, whereas 5.5 days were calculated for the RC_4 treatment (Table 2). Intermediary and values similar to the RC_3 treatment were found for yolks from layers in treatments RMC_4 and RMC_3, which were 4.8 days and 4.6 days, respectively.

The albumen of layers in the RC_4 treatment had carbon half-life of 5.2 days, a while for the birds in treatments RC_3, RMC_4, and RMC_3, values were 2.5, 3.2, and 1.5 days, respectively. The possible reason for the slower turnover of albumen in RC_4 treatment is that, as amino acids for albumen formation are directly taken from blood plasma and blood, and these birds may still have had carbon from the previous diets in the body (slow turnover), this carbon was used for albumen formation in these birds.

Hobson (1995), studying turnover of quail egg components, stated that total carbon replacement from egg yolk took eight days, and from albumen, from three to five days. These data are different from the findings of this study, which may be due to the fact that Hobson (1995) studied different species and diets. In addition, quails were producing eggs daily, whereas in the present study, layer hens produced one egg every other day.

Egg carbon turnover values are intermediate to yolk and albumen values, showing the influence of each component (yolk and albumen) in their formation process, where the yolk takes longer to reflect the new diet.

During the physiological process of egg formation, the ovary programs a sequence of ova in different development stages (approximately eight days of production). Therefore, in the eggs collected in the beginning of the trial, yolk had a large influence of

carbon-13 isotopic ratio from the previous feed. Albumen deposition around the yolk takes about three hours in the magnum, where proteins are synthesized from amino acids directly derived from blood plasma (Hobson, 1995). This explains the lower half-life of the albumen.

Blood and albumen showed to be strongly influenced by diet composition as compared to egg and yolk. This is why RC_4 treatment turnover was slower than the other treatments. The possible explanation is that, since blood transports nutrients, there was intense mobilization of reserves in layers in this treatment to try to maintain egg production. As albumen synthesis is directly dependent from plasma, this reflects the blood turnover.

Moreover, blood turnover is slower than the turnover of the egg and its fractions. A possible explanation is the blood consists of a cellular fraction and plasma, and the cellular fraction takes longer to exchange existing carbon as compared to plasma. This reflects nutrient transit in the body (Hobson and Clark, 1993), which is similar to what occurs during the formation of the egg and its fractions.

CONCLUSION

The RMC_3 diet was better because it promoted better productive performance (high feed intake and high egg production), faster carbon substitution rate than the unbalanced diet (exclusively C_4), and shorter half-life of egg (yolk + albumen), yolk, and albumen.

Turnover is influenced by the quality of the diet fed to the birds. In addition, diet composition has less influence on egg and yolk turnover, as compared to blood and albumen. Carbon substitution rate is faster for egg and its components (yolk and albumen) than for blood.

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