

Hens Produce Artificially Enriched ^{13}C Egg Proteins for Metabolic Tracer Studies

Marshall D. McCue¹, Brian Arquisola¹, Erik Albach¹ & Erik D. Pollock²

¹ Department of Biological Sciences, St. Mary's University, One Camino Santa Maria, San Antonio, Texas, USA

² University of Arkansas Stable Isotope Laboratory, University of Arkansas, Fayetteville, AR, USA

Correspondence: Marshall D. McCue, Department of Biological Sciences, St. Mary's University, One Camino Santa Maria, San Antonio, Texas 78228, USA. Tel: 1-210-431-8005. E-mail: mmccue1@stmarytx.edu

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Abstract

Clinicians and researchers studying protein metabolism *in vivo*, typically use isotopically-labeled free amino acids as metabolic tracers rather than isotopically-labeled proteins because such proteins are commercially unavailable. However, the use of free amino acids in lieu of protein tracers violates the critical assumption that tracer molecules undergo the identical biochemical reactions as the tracee molecules of interest. To address this problem we synthesized ^{13}C -labeled proteins using egg laying hens and investigated the relationship between tracer dose and method of delivery on ^{13}C -protein production. We enriched hens with one of two isotope tracers (^{13}C -1-leucine or a uniformly labeled ^{13}C -amino acid mixture) mixed in their food or dissolved in their drinking water at different dosing levels (86-432 mg day⁻¹). The recovery of ^{13}C in egg white proteins of the hen fed ^{13}C -leucine ranged from 14% to 21%; recovery rates were highest at the lowest dosing level. At the highest dosing level egg whites were enriched more than 150‰ above background levels of ^{13}C . The time required for half maximal ^{13}C enrichment depended chiefly on the mode of tracer administration, and ranged from 2.5 days for ^{13}C -leucine dissolved in water to 4.9 days for ^{13}C -leucine mixed in food. Relative rates of ^{13}C recovery in the egg protein were lowest for hens fed the uniformly ^{13}C labeled amino acid mixture, presumably because of the high proportion of nonessential amino acids. The time required for the ^{13}C -enrichment in eggs to return to background levels at the end of the enrichment period was about twice that required to initially reach isotopic equilibrium with the diet, indicating significant biochemical discrimination of endogenous ^{13}C amino acids. We conclude that delivering small amounts of ^{13}C amino acid tracers in the drinking water of hens is the most effective way to produce ^{13}C -enriched proteins to for tracer studies that do not require $\delta^{13}\text{C}$ -enrichment above 200‰.

Keywords: diet switch, leucine, metabolic tracer, protein synthesis, stable isotope

1. Introduction

1.1 Isotopes and Breath Testing

$^{13}\text{CO}_2$ -breath testing is increasingly used in nutritional research and as a supplement to diagnose various medical conditions including bacterial infections, digestive disorders, and metabolic dysfunction (Amarri & Weaver, 1995; Anania et al., 2008; Braden, 2007, 2010; Koletzko et al., 1988; Parra & Martinez, 2006; Romagnuolo et al., 2002; Weaver, 1998). This technique relies on the assumption that the ^{13}C -labeled tracer molecules introduced into the body (usually orally) are eventually oxidized and exhaled as $^{13}\text{CO}_2$. Researchers collect breath samples and quantify the [$^{13}\text{CO}_2$] at specific time intervals after dosing. The pharmacokinetics (e.g., magnitude and time course) of the $^{13}\text{CO}_2$ excretion may then be used to identify various pathophysiological states. An increasing number of studies in comparative physiology have also begun using $^{13}\text{CO}_2$ -breath testing to explore the nutrient oxidation and bioenergetics in animals (Carleton et al., 2006; Hughes et al., 2008; McCue et al., 2010, 2011a; Sponheimer et al., 2006; Voigt et al., 2008a, 2010; Welch et al., 2008; Welch & Suarez, 2007). ^{13}C is a stable isotope thus $^{13}\text{CO}_2$ -breath testing offers a safe alternative to the use of radioactive, ^{14}C -labeled tracers, an important factor for pediatric or pregnant patients (Bodamer & Halliday, 2001; Braden et al., 2007; Christian et al., 2002; Evenpoel et al., 2000; Rating & Langhans, 1997) and studies involving wild animals (Ayliffe et al., 2004; Hatch et al., 2002; McCue, 2011; Sponheimer et al., 2006; Voigt et al., 2003, 2008a).

1.2 Amino Acid Tracers Are Not the Same as Protein Tracers

One of the inherent assumptions in any tracer methodology is that the *tracer* molecule follows the same biochemical reactions and metabolic fate as the *tracee* molecule (Boirie et al., 1995, 1996; Magkos & Mittendorfer, 2009; Patterson, 1997; Rosenblatt & Wolfe, 1988; Saris et al., 1993; Wolfe & Chinkes, 2005). However, most research exploring the oxidative fates of exogenous proteins *in vivo* does not employ isotopically labeled proteins, but rather purified ^{13}C -labeled amino acid tracers under the assumption that these free amino acids ‘behave’ exactly like those amino acids that are biochemically integrated into proteins (Braden, 2009; Fischer & Wetzel, 2002; Ghos & Beaufre, 1998; McCue, 2011). Recent studies have shown that this assumption may not be appropriate (Boirie et al., 1996; Conceicao et al., 2007; Daenzer et al., 2001; Dangin et al., 2001; Deglaire et al., 2009; Hughes et al., 2008; Koopman et al., 2009; Metges et al., 2000; Ronnestad et al., 2000; Zarate et al., 1999). Researchers and clinicians who routinely use free ^{13}C -amino acid tracers in lieu of proper ^{13}C -protein tracers (including the authors of the present paper) openly acknowledge that this practice violates the primary assumption of tracer methodology. However, these individuals justify their actions by citing the fact that large amounts (> 100 grams) of nutritionally complete, artificially enriched, ^{13}C -labeled proteins remain commercially unavailable (Berthold et al., 2011; Braden, 2010; Fromentin et al., 2011a; Jonderko et al., 2005).

1.3 Producing ^{13}C -Proteins

Although commercially unavailable, ^{13}C -labeled proteins can be synthesized in the laboratory. Several published studies report synthesizing artificially enriched, ^{13}C -labeled proteins in sufficient amounts to be used for clinical breath testing (Berthold et al., 1991; Boirie et al., 1995; Evenpoel et al., 1997; Fromentin et al., 2011b; Geboes et al., 2004; Irving et al., 1988). Yet, despite our progress in method development, we still know very little of how simple experimental factors can influence ^{13}C -protein production (Fromentin et al., 2011b). Such information is necessary if we intend to implement best practices in breath testing (Braden, 2007, 2010) and metabolic tracer studies (McCue, 2011).

Previous studies describe two general approaches for producing high quality, ^{13}C -labeled protein by supplementing diets with ^{13}C -amino acid tracers; one involves egg white proteins (primarily ovoalbumin) and the other involves milk proteins (*i.e.* whey protein and casein). Here we focus on producing ^{13}C -labeled egg proteins rather than milk proteins for several reasons. First, maintaining adult dairy cows or lactating women (Irving et al., 1988) for extended periods of time poses unique logistical obstacles for most laboratories such as housing and the ethical concerns with humans. Second, hens are capable of generating ^{13}C -labeled proteins with significantly lower daily dosages of ^{13}C -amino acids than required by cows. Additionally, egg proteins are better suited for making relatively small batches of labeled proteins as required. Third, egg whites are virtually lipid free and composed of over 91% protein (dry, ash-free mass; USDA, 2000) thus eliminating additional purification steps required for removing the lipid fraction of milk. Finally, egg proteins are considered the ‘gold standard’ to which all other dietary proteins are scored with regard to amino acid composition, digestibility, and nutritional value (Chibnall et al., 1943; Mitchell & Block, 1946; Rao et al., 1964; Satterlee et al., 1979; Young et al., 1975) – important characteristics for metabolic tracers.

The immediate goal of this project was to synthesize relatively large amounts of ^{13}C -protein (*e.g.*, several hundred grams) that can be used in future studies of breath testing and nutrient allocation. The ultimate goal was to increase our understanding of ^{13}C -protein synthesis in the hen model. The following experiments were designed to allow us to 1) characterize the incorporation and washout kinetics of ^{13}C -tracers, 2) develop a dose-response curve for a commonly used ^{13}C -tracer in breath testing (*i.e.*, ^{13}C -1- L-leucine), 3) compare the efficacy of different dosing techniques (*i.e.*, mixed with food or dissolved in drinking water), and 4) compare ^{13}C recovery rates between an essential amino acid and a mixture of essential and nonessential amino acids. We hope that by improving the existing protocols for synthesizing ^{13}C -proteins, these tracer materials can become more accessible to researchers and allow them to track the physiological fates of exogenous proteins *in vivo* more accurately.

1.4 Tracer Selection

We chose to use ^{13}C -1-L-leucine (hereafter: ^{13}C -leucine) as the primary tracer molecule in this study for several reasons. First, ^{13}C -leucine is one of the most commonly used tracers in amino acid breath testing. Second, leucine is an essential amino acid and is therefore less likely to be oxidized during ‘first pass’ splanchnic oxidation than nonessential amino acids (Berthold et al., 1991; Daenzer et al., 2001; Matthews et al., 1993; McCue et al., 2011b; Yu et al., 1992). Third, leucine is the most abundant essential amino acid and the third most abundant amino acid in egg proteins, comprising 8.4% and 8.8% of the amino acids in egg white and yolk

proteins, respectively (USDA, 2000). Finally, ^{13}C -leucine is widely available from commercial suppliers of stable isotope tracers and can be purchased in bulk for as low as $\$100 \text{ gram}^{-1}$.

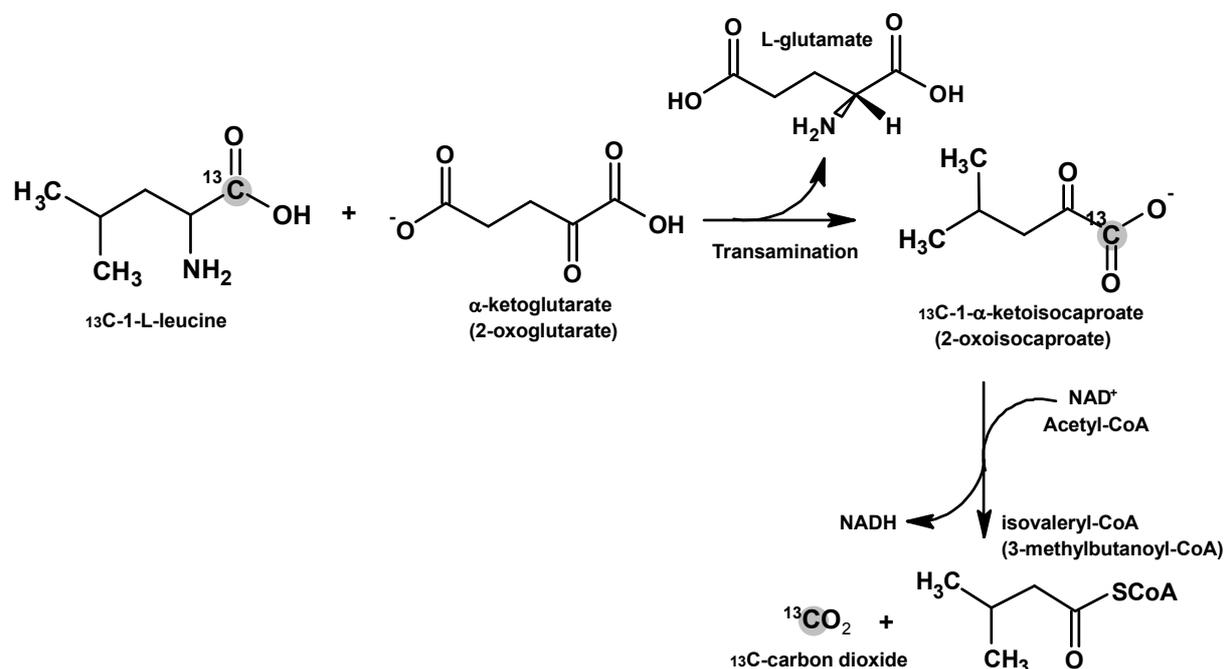


Figure 1. Series of reactions illustrating the fate of the number-one-carbon in leucine during oxidation

Leucine is a non-glucogenic amino acid (Murray et al., 2003) and losses of the number-1-carbon into carbohydrate and lipid pools should be low (compare with Gruhn & Hennig, 1984). Figure 1 illustrates how the number-1-carbon of a ^{13}C -1-leucine tracer molecule is converted into $^{13}\text{CO}_2$. The number-1-carbon of leucine is thought to only leave the body as $^{13}\text{CO}_2$ (Cheng et al., 1985; Matthews & Bier, 1983; Matthews et al., 1981; Parra & Martinez, 2006; Wetzel & Fischer, 2005), however during metabolic alkalosis, a small amount of HCO_3^- can be excreted by the renal system (De Benoist et al., 1984; Yu et al., 1990). Nevertheless, we test the assumption of negligible 'leakage' of the number-1-carbon of leucine in to the lipid pool by analyzing the ^{13}C of the adipose tissue in hens.

We also conducted ^{13}C -protein enrichment experiments using mixture of 16 uniformly ^{13}C -labeled amino acids isolated from algal proteins (CNLM-452, $\$1250 \text{ g}^{-1}$, Table 1; Cambridge Isotopes Inc. USA). This trial was conducted to compare the incorporation, kinetics and ^{13}C recovery rate between a highly enriched, mixed amino acid tracer (^{13}C atom percent = 100%) and a single amino acid tracer containing one ^{13}C label (*i.e.*, ^{13}C -leucine; ^{13}C atom percent = 16.7%).

Table 1. Amino acid profiles for the uniformly-labeled 16-amino acid mixture

Amino acid	Content (%)
Alanine	7
Arginine	7
Aspartic acid	10
Glutamic acid	10
Glycine	6
Histidine	2
Isoleucine	4
Leucine	10
Lysine	14
Methionine	1
Phenylalanine	4
Proline	7
Serine	4
Threonine	5
Tyrosine	4
Valine	5
Total	100

2. Methods

Three mature egg-laying hens (ISA Brown; ~2 years old) were obtained from a commercial farm (O5-Livestock, Hondo, Texas). Within one week of arriving in the laboratory, the hens were dewormed with three daily doses (60 mg) of Ivermectin (Panacur, Hoechst) dissolved in their drinking water (Carpenter et al., 2001). Before experiments food was offered *ad libitum* and the mass consumed each day was recorded. Hens were housed individually in stainless steel lagomorph cages, fed a complete, balanced diet (Naturewise Layer; Neutrena; $\delta^{13}\text{C} = -17.21\%$), and acclimated to constant conditions for six weeks (14L:10D; 27°C). Water consumption was measured each day.

2.1 Experiment 1

The feeding experiments consisted of supplementing the pelleted diet of Hen 1 with ^{13}C -1-L-leucine (Cambridge Isotope Laboratories, Inc.). A pyramid dosing regimen was implemented for Experiment 1 as a means to verify the dose responses of ^{13}C -1-L-leucine. Because daily food intake ranged from ~115 to 145 grams during the acclimation period, a daily ration of 150 grams of food was used for the experiments to ensure *ad libitum* availability. Each day, known amounts of ^{13}C -leucine (*i.e.*, 100, 200, or 300 mg) were mixed with the hen's daily ration. Six sequential dosing treatments were examined over 14-day periods (Table 2). Two of the feeding treatments (*i.e.*, 100 and 200 mg day⁻¹) were repeated several weeks apart to explore the effects of long-term tracer integration. In one treatment, fresh drinking water was replaced with a solution of 1 g L⁻¹ of ^{13}C -leucine and offered *ad libitum*. Once per week, vitamins and minerals (Avian Super Pack, Animal Science Products) were added to the drinking water according to the manufacturer's suggestion (80 mg L⁻¹). Each morning, between 0700-0930 hours, all uneaten food was collected and weighed.

Table 2. Summary of the ^{13}C -leucine dosing regimen for one hen (Hen 1)

Diet	Day	Food offered	Solid ^{13}C Leu	Aqueous ^{13}C Leu	^{13}C Leu ingested
A	1-14	150g	-	-	0 mg
B	15-28	150g	100 mg	-	86 mg
C	29-42	150g	200 mg	-	173 mg
D	43-63	150g	300 mg	-	259 mg
E	64-78	150g	200 mg	-	172 mg
F	79-92	150g	100 mg	348 mg	432 mg
G	93-105	150g	100 mg	-	86 mg

The experimental diets correlate with the $\delta^{13}\text{C}$ of egg white and yolk illustrated in Figure 2.

Eggs were collected twice each day and labeled with the time and date before being weighed and refrigerated. Approximately every two weeks the refrigerated eggs were hardboiled for 20 minutes to halt the protease (e.g. trypsin and chymotrypsin) inhibiting characteristics of raw eggs (Evenpoel et al., 1999; Evenpoel et al., 1998; Liu et al., 1971; Osuga & Feeney, 1968). Eggs were then peeled and the white and yolk fractions were separated, minced, and dried to a constant mass in a convection oven (70°C). The dried tissues were transferred into 50 ml centrifuge tubes and stored at 10°C until further processing. Subsamples (~10 mg) of each egg white and yolk were homogenized using a Wig-L-Bug amalgamator (Dentsply North America, Inc.), packaged into 2 ml centrifuge tubes, and analyzed at the University of Arkansas Stable Isotope Laboratory (Fayetteville, AR). The $\delta^{13}\text{C}$ values (*sensu* Craig, 1957) and carbon content of samples were determined using a Finnigan Delta Plus continuous-flow IRMS interfaced to a Carlo Erba NC2500 elemental analyzer (Thermo Scientific, Bremen Germany, Milan, Italy). Measured ^{13}C isotope ratios were corrected to VPDB (Coplen, 1994). Internal standards were run in parallel with the unknown samples and had an analytical error of < 0.1‰.

2.2 Calculation of Tracer Kinetics

Rates of ^{13}C -leucine incorporation into the egg white and yolk fractions were modeled (SigmaPlot 11.0, Systat Software Inc.) using the following 2-parameter, exponential equation (Patterson, 1997):

$$\delta^{13}\text{C}(x) = (\delta^{13}\text{C}_{\infty} - \delta^{13}\text{C}_{\text{Bkg}}) * (1 - e^{-k * t}) \quad (1)$$

where $\delta^{13}\text{C}(x)$ is the ^{13}C enrichment at time x , $\delta^{13}\text{C}_{\infty} - \delta^{13}\text{C}_{\text{Bkg}}$ refers to the maximal ^{13}C enrichment at equilibrium above the initial, background value (Bkg), k is the rate constant for tracer incorporation, and t is time in days. The time required for one-half of the isotopic enrichment to occur (t_{50}) was calculated according to the following equation:

$$t_{50} = \ln(2) / k \quad (2)$$

We evaluated the possibility that prolonged exposure to artificially enriched ^{13}C -leucine in the diet could cause gradual, long-term enrichment in egg products using paired t-tests to compare the $\delta^{13}\text{C}$ of egg whites and egg yolks at the same dosing level (i.e. 200 mg day⁻¹) at different points in the experiment.

Trophic fractionation $\Delta^{13}\text{C}$ for egg white and yolk was calculated only for the background diet according to the following equation:

$$\Delta^{13}\text{C} = \delta_{\text{tissue}} - \delta_{\text{diet}} \quad (3)$$

The chemical composition of dried egg whites (NDB No. 01173) and yolks (NDB No. 01137) are well characterized and available through the USDA (National Nutrient Database; <http://www.nal.usda.gov/>). Values including proximate analysis and amino acid composition were used for subsequent calculations (see Appendix for equations). For example, the amino acid content of egg whites allowed us to calculate the ^{13}C tracer recovery and the specific ^{13}C enrichment of individual leucine residues in the whole protein mixture.

Hen 1 was euthanized just prior to the end of the scheduled experiment after it broke its leg on day 104. We subsequently harvested and measured the $\delta^{13}\text{C}$ of several tissues (i.e., pectoral and neck muscle, large and small intestine, liver, lung, cecum, blood, brain, skin, long bone marrow, and abdominal fat depot) from this animal. These tissues were dried to constant mass at 70°C and coarsely ground. One-gram subsamples were further homogenized using an amalgamator as described above. Lipids were separated from all tissues (except whole blood) by vortexing for 30 minutes in an excess of 2:1 chloroform:methanol (Oppel et al., 2010). After

centrifugation, the supernatant was discarded, with the exception of adipose tissue and recovered by evaporation under a dry stream of N₂. Pelleted lean tissue samples were also dried under a stream of N₂. The δ¹³C of all tissue samples were analyzed as described above for the egg tissues.

2.3 Experiment 2

A second experiment was conducted to compare the ¹³C integration kinetics at the start of dosing with the ¹³C washout kinetics following terminus of ¹³C tracer dosing. Hen 2 was fed *ad libitum* and given 100 mg of ¹³C-leucine dissolved in 750 ml of drinking water each day for 21 days. Because we had previously determined that the *ad libitum* water consumption for Hen 2 was > 800ml day⁻¹, the use of 750 ml to guarantee optimum utilization of tracer was justified. On day 22, ¹³C-leucine dosing was halted and Hen 2 was instead given 750 ml of fresh water each day. The eggs were collected daily and processed as described above. Hen 2 was euthanized 48 days following her first ¹³C isotope treatment. We did not harvest Hen 2 tissues because it is likely that many of these tissues would not have had time to reach isotopic equilibrium (Phillips & Eldridge, 2006).

2.4 Experiment 3

Hen 3 was fed *ad libitum* and given 50 mg day⁻¹ of uniformly ¹³C-labeled amino acid mixture dissolved in 750 ml of water for 20 days. On day 21 ¹³C-labeled amino acid dosing was halted and Hen 3 was returned to the background diet. The eggs were collected daily and processed as described above. Hen 3 was euthanized 48 days following the first isotope treatment, and like Hen 2 her tissues were not harvested.

Rates of ¹³C incorporation into the egg white (not egg yolk) of Hen 2 and 3 were modeled as described above and the rates of ¹³C washout from egg whites were modeled (SigmaPlot 11.0, Systat Software Inc.) using the following 2-parameter, exponential equation:

$$\delta^{13}\text{C} (x) = (\delta^{13}\text{C}_{\infty} - \delta^{13}\text{C}_{\text{Bkg}}) * (1 - e^{-k * t}) \quad (4)$$

where δ¹³C_∞ - δ¹³C_{Bkg} was treated as a shared parameter between the incorporation and washout models. The t₅₀ for both of these models were calculated using Equation (2). Mean values are reported followed by standard deviation, unless indicated otherwise.

3. Results

3.1 Experiment 1: Trophic Fractionation, Incorporation Kinetics, and Dosing Response

Hen 1 (1137 g initial; 1186 g final) consumed an average of 125.0 g day⁻¹. As such the actual ingested tracer doses were 86.3, 172.7, and 259.0 mg ¹³C-leucine day⁻¹ for the three dosing levels, respectively. For the simultaneous dose of tracer mixed with food and dissolved in water, a total of 432 mg of ¹³C-leucine day⁻¹ was ingested. A total of 72 intact eggs (mass: 65.2 ± 1.2 g) were collected over the enrichment period yielding a mean net production rate of one egg every 1.25 days. Eight eggs were broken or cracked during laying; the egg white and yolk were salvaged when possible.

Dried egg whites contained 45.9 ± 1.0% (n = 80) carbon by mass and dried egg yolks contained 64.3 ± 1.1% (n = 77) carbon by mass. The mean dry mass of intact egg whites was 6.70 ± 0.16 g. The δ¹³C of the background diet was -17.2 ± 1.4‰, the Δ¹³C_{white} was -1.9 ± 0.8‰, and the Δ¹³C_{yolk} was -2.6‰ ± 0.17‰. The δ¹³C of enriched egg whites and yolks was strongly influenced by the tracer dose (Figure 2) and reached maximal values of 133.7‰ and 26.5‰, respectively.

On day 56, our concern about diminished egg production in Hen 1 prompted us to offer additional dietary supplements: 100 mg of diatomaceous earth, 200 mg of crushed oyster shells and 15-30 live darkling beetle (*Zophobus morio*) larvae for one week (*i.e.* days 57-63; Figure 2). Although we collected and measured the ¹³C values in eggs produced during that week, we did not use those eggs (or the eggs laid during the following week) for modeling purposes or statistical analyses.

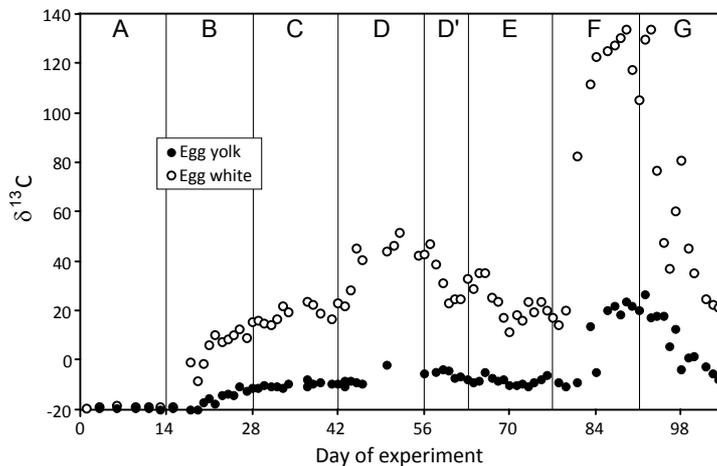


Figure 2. The $\delta^{13}\text{C}$ of egg whites and yolks from Hen 1 consuming ^{13}C -leucine tracers

The time divisions refer to different dosing regimens (see Table 1). Note that the period D' refers to a period in the experiments when reduced egg production prompted us to supplement the hen's diet with minerals and live insects. The data points within this period are not used for statistical analyses or modeling purposes.

A significant increase in the $\delta^{13}\text{C}$ of egg white was detected after just one day of dietary ^{13}C -leucine enrichment (one-tailed t-test; $df = 6$, $p < 0.0001$); However a significant increase in the $\delta^{13}\text{C}$ of egg yolk required an additional three days (one-tailed t-test; $df = 7$, $p < 0.0001$). The subsequent analyses and modeling of tracer integration into egg tissues accounted for the differences in lag periods between the timing of ovulation (e.g. when yolk is synthesized) and oviposition (e.g. when the egg white protein is synthesized). Exogenous ^{13}C -incorporation closely followed 2-parameter exponential models ($R^2 = 0.90\text{-}0.98$). The integration models for egg white and yolk had rate constants (k) of 0.141 and 0.179, yielding t_{50} of 4.92 and 3.87 days, respectively (Figure 3); neither of these values had a statistically significant difference.

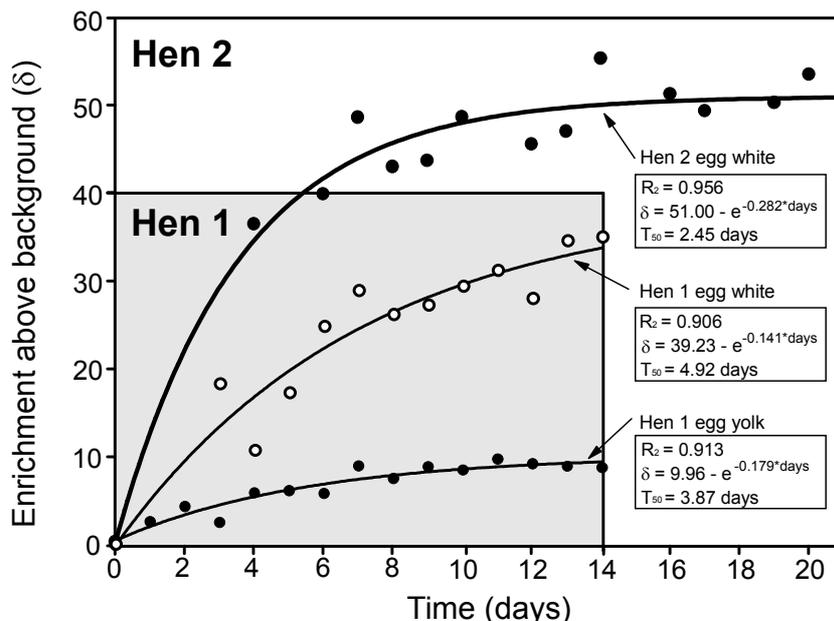


Figure 3. Incorporation curves for ^{13}C atoms into egg white and egg yolks of two hens receiving ^{13}C -leucine tracers

Hen 1 (shown in the gray box) ingested 86.3 mg day^{-1} of tracer mixed with food. Hen 2 ingested 100 mg day^{-1} of tracer dissolved in its daily water supply (750 ml). Curves are modeled using a first-order, 2 parameter kinetic model: $\delta^{13}\text{C}(x) = (\delta^{13}\text{C}_{\infty} - \delta^{13}\text{C}_{\text{Bkg}}) * (e^{-k \cdot t})$. See Methods for details.

Egg white exhibited larger increases in $\delta^{13}\text{C}$ than egg yolk, but this difference can be attributed to the high lipid content of the yolk (*i.e.* 55.8% dry mass). According to the ^{13}C -incorporation models the maximal enrichment at the lowest experimental dose (*i.e.* 86.3 mg day⁻¹) was 3.9 times higher for the egg white (*i.e.* 39.2‰ above background) than the egg yolk (*i.e.* 10.0‰ above background).

The mass of ^{13}C -leucine ingested and the ^{13}C -enrichment in the egg tissues exhibited a positive, but non-linear response. The relative ^{13}C -enrichment gradually decreased as the tracer dose in the food tripled from 86.3 mg day⁻¹ to 259 mg day⁻¹ (Figure 4).

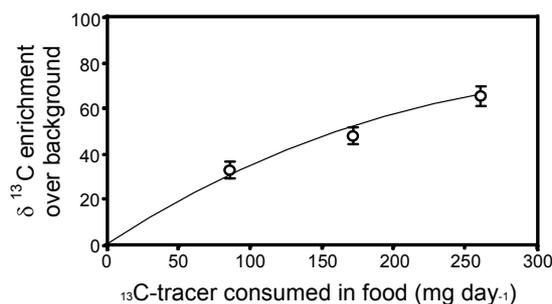


Figure 4. Dose sensitivity of leucine residues in egg whites for two hens under different dosing regimens. Circles and error bars refer to mean values \pm standard deviation

3.2 Experiment 2: Tracer Delivery Method

Hen 2 (1505 g initial; 1423 g final) consumed food *ad libitum* and was given access to 100 mg day⁻¹ ^{13}C -leucine dissolved in 750 ml of water for 21 days during which she laid 13 eggs (53.9 \pm 9.1 g). Unlike Hen 1, Hen 2 was repeatedly observed spilling water while drinking and we did not attempt to quantify total tracer recovery in Hen 2. Nevertheless, the rate constant (k) for ^{13}C incorporation was 0.282 resulting in a t_{50} of 2.45 days. This value is approximately half of that observed in Hen 1 while consuming a similar dose (86.3 mg day⁻¹) of ^{13}C -leucine mixed into her food (Figure 3). Moreover, the asymptotic enrichment calculated by the tracer integration model (51.0‰) was 30% higher than that calculated for Hen 1 (39.2‰). Assuming a linear dose response over this narrow range of doses (*i.e.*, 86.3 versus 100 mg day⁻¹; but see Fig. 4 across a wide range of doses) the egg white of the larger Hen 2 was still approximately 10% more enriched in ^{13}C than that of the smaller Hen 1. This difference could be attributed to differences in mean egg mass (17% smaller) or the increased bioavailability of dissolved ^{13}C -leucine. During the subsequent 27-day washout period Hen 2 only laid 2 eggs, thus precluding accurate pharmacokinetic modeling (but see Experiment 3 below).

3.3 Experiment 3: ^{13}C -Labeled Amino Acid Mixture

Hen 3 (2072 g initial, 2010 g final) consumed food *ad libitum* and was given access to 50 mg of uniformly ^{13}C -labeled amino acids dissolved in 750 ml of water for 20 days during which she laid 18 eggs. Hen 3 also produced 18 eggs during the 28-day washout period; overall generating over 200 g (dry mass) of ^{13}C -enriched egg white protein. Eggs had a mean mass (53.4 \pm 2.1g) that was 18% lower than those of Hen 1, but not significantly different from those of Hen 2 (unpaired t-test, $p = 0.951$, $df = 30$). According to the integration model, the asymptotic ^{13}C enrichment of the egg whites was 78.5‰ above background. The k and the t_{50} were 0.3965 and 1.75 days, respectively. The rate constant for loss of ^{13}C in egg whites after the tracer was removed was 0.227 with a t_{50} of 3.05 days. Excessive water spillage was not observed in Hen 3. The mean $\Delta^{13}\text{C}_{\text{white}}$ for Hen 2 and Hen 3 was $-1.50 \pm 1.0\text{‰}$ ($n = 6$), a value that differed by only 0.3‰ from that observed in Hen 1.

4. Discussion

4.1 ^{13}C -1-Leucine Tracers

In the first experiment, Hen 1 produced over 400 g (dry mass) of ^{13}C -leucine enriched egg white protein over a 90-day period. Because leucine composes 8.4% of whole egg white protein mass, the enrichment of the individual leucine residues in the protein was much greater than that of the whole protein. We calculated that the $\delta^{13}\text{C}$ of leucine residues increased from background levels (-19.1‰ ; 1.08 atom percent) to 1403.6‰ (2.62 atom percent) at the highest tracer dose. In other words, before artificial enrichment, 5.4% of all leucine molecules in the egg white contained at least one ^{13}C -atom, but after consuming the highest tracer dose (432 mg day⁻¹) for 14 days, 25.3% of all of leucine molecules contained one or more ^{13}C -atoms (see equations in Appendix).

Hen 2 produced over 100 g (dry mass) of ^{13}C -leucine enriched egg white protein over a 40-day period. The ^{13}C -atoms from the leucine tracer were incorporated more rapidly into egg tissues when delivered in the drinking water (Figure 3). The asymptotic $\delta^{13}\text{C}$ enrichment in egg proteins was greater than in Hen 1, but because the eggs of Hen 1 were larger it is difficult to make a formal comparison. It is important to note that $\delta^{13}\text{C}$ values of egg protein alone can be misleading since they do not account for the size of the egg which can vary by 66% between 'small' and 'jumbo sizes' (USDA, 2000). Moreover, since hen egg yolk mass exhibits less variation than egg white, the total protein content of eggs can vary by as much as 100%.

We calculated that at equilibrium the ^{13}C -tracer recovery in the egg white of Hen 1 ranged from 14.3% to 21.5% depending on the dose. As expected the fractional recovery rate (*i.e.*, δ enrichment per mg tracer) was the highest at the lowest dose of the ^{13}C -leucine tracer (Figure 4). The pattern of relatively diminishing enrichment with increasing dose is likely related to increased oxidation of the exogenous leucine tracer since it was present in increasing excess in the diet. Although the egg white proteins from Hen 2 had higher ^{13}C enrichment than those of Hen 1 when fed a similar dose of ^{13}C -leucine (Figure 3), the total fractional rates of ^{13}C recovery (20.0%) were similar.

In their experiment, Evenepoel et al. (1997) reported total tracer recovery rates as high as 40.2% by using a base diet that was deficient in leucine, which presumably minimized oxidative losses of the tracer (McCue, 2011; McCue et al., 2010). It is noteworthy that the previous study estimated, and did not quantify, the tracer recovered in the egg yolk. The egg yolks in this experiment had dry masses of 7.8 ± 0.4 g; $n = 71$. If we consider the egg yolks then the ^{13}C total recovery in this study would be as high as 33.7%, but without requiring a customized, leucine-deficient diet.

In this study, 16.7% of the food labeled with ^{13}C -leucine tracer was uneaten and was not reused. If this material could be recycled within the food chain, then the total yield of ^{13}C could be substantially increased. Furthermore, recycling some of the egg yolk back into the food chain (*i.e.*, by combining it with fresh food) could also increase the production of ^{13}C -proteins by up to 5%, however no studies have examined this possibility.

The aqueous ^{13}C -leucine was more rapidly incorporated into egg white proteins. This outcome is clear when comparing the t_{50} of Hen 1 (4.92 days; crystalline tracer) and Hen 2 (2.45 days; aqueous tracer; Figure 3) at 100 mg doses of ^{13}C -leucine. This pattern was also evident when Hen 1 switched dosing regimens. Between days 79 and 92, Hen 1 achieved a t_{50} of 2.26 when ^{13}C -leucine was administered via drinking water.

4.2 Uniformly ^{13}C -Labeled Amino Acids

The rate at which Hen 3 (fed a mixture of uniformly ^{13}C -labeled amino acids) incorporated ^{13}C into egg white proteins ($t_{50} = 1.75$ days) was similar, if not slightly higher than that documented in Hen 2 (Figure 5). Given that the masses of eggs produced by Hen 2 and 3 did not statistically differ, the differences in incorporation kinetics between the leucine and mixed amino acid tracers could stem from 1) differential incorporation kinetics of the individual amino acids and/or 2) *de novo* synthesis of nonessential amino acids from recycled carbon skeletons containing ^{13}C .

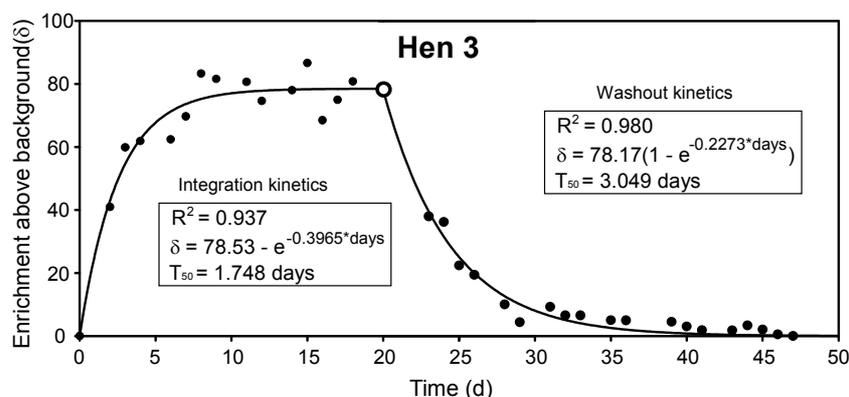


Figure 5. Illustration of the ^{13}C integration and washout kinetics for Hen 3 consuming 50 mg day^{-1} of a uniformly ^{13}C -labeled mixed amino acid mixture

The integration curve was modeled the same as in Figure 3 and the washout curve was modeled using a first-order, 2-parameter, exponential equation: $\delta^{13}\text{C}(x) = (\delta^{13}\text{C}_{\infty} - \delta^{13}\text{C}_{\text{Bkg}}) * (1 - e^{-k * t})$ where $(\delta^{13}\text{C}_{\infty} - \delta^{13}\text{C}_{\text{Bkg}})$ was treated as a shared parameter between the incorporation and washout models. See Methods for details.

Hen 3 consumed a daily dose of artificially enriched ^{13}C -tracers (50 mg d^{-1}) that was half that consumed by Hen 2, but because the concentration of ^{13}C -atoms in the tracer molecules was higher, the maximal ^{13}C enrichment of egg white proteins was higher in Hen 3 than Hen 2 (*i.e.*, 78.5‰ versus 51.0‰, respectively). Nonessential, exogenous amino acids are more likely to be oxidized in the postabsorptive phase (Brown-Mason et al., 1981; McCue et al., 2010; Voigt et al., 2008b), and thus the maximal ^{13}C enrichment of egg white proteins resulting from the uniformly labeled mixed amino acid tracer was lower than expected if solely based on the ^{13}C content of the two tracers [*i.e.*, uniformly ^{13}C -labeled mixed amino acid tracer (^{13}C atom percent = 100%) versus the ^{13}C -1-leucine tracer (^{13}C atom percent = 16.7%)]. Consequently, the net cost of ^{13}C enrichment in the total egg white protein was lower using ^{13}C -1-enriched leucine than the uniformly ^{13}C -labeled amino acid mixture. However, if the goal of a future experiment is to produce highly enriched (> 500‰) egg proteins, any dosing level of ^{13}C -1-labeled leucine (or even uniformly ^{13}C -labeled leucine) would be insufficient.

The ^{13}C washout curve for Hen 3 consuming the uniformly labeled ^{13}C amino acid mixture closely followed a first-order decay model (Figure 5). A near complete isotopic recovery of egg white proteins (*i.e.*, return to background values \sim -17‰) was identified 28 days after stopping tracer dosing. The t_{50} for the washout period was 3.05 days, nearly twice the value for tracer incorporation (*i.e.*, 1.75 days). Differences in integration and washout kinetics of egg protein were previously documented in hens fed ^{15}N tracers (Gruhn et al., 1979) and quail fed tritium (Brown-Mason et al., 1981), and most likely result from isotopic discrimination during amino acid transamination and deamination and/or the recycling of ^{13}C -carbons into de novo synthesis of nonessential amino acids (*e.g.*, Cherel et al., 2005a; Martinez del Rio & Wolf, 2005; Martinez del Rio et al., 2009; McCue & Pollock, 2008; Pearson et al., 2003; Sick et al., 1997).

4.3 ^{13}C Enrichment in Somatic Tissues

The tissues of Hen 1 were harvested to compare their ^{13}C values. The $\delta^{13}\text{C}$ of the lipid fraction of the adipose tissue was -21.18‰, a value that matches the non-enriched values of the egg white and yolk and confirms that none of the ^{13}C -tracer 'leaked' into the carbon pool of the lipids. Long-term experiments raising mice on ^{13}C -leucine yielded the same outcome (unpublished observations). An intact egg was removed from the hen's oviduct and the $\delta^{13}\text{C}$ of the egg white was 13.45‰. Because this hen was undergoing a diet switch from high enrichment (432 mg day^{-1} ^{13}C -leucine) to moderate enrichment (86.3 mg day^{-1} ^{13}C -leucine), we would expect that the tissues with the most rapid protein turnover would most quickly reflect the changes of ^{13}C in the diet.

Hens are capital breeders (De Vink et al., 2011; Meijer & Drent, 1999) and synthesize egg products using recently ingested nutrients, but these nutrients mix with endogenous nutrients in circulation. Consequently, the proteins deposited in the egg white can serve as starting point to compare the $\delta^{13}\text{C}$ of the circulating amino acid pool with that of other tissues. When comparing the ratio of the $\delta^{13}\text{C}$ of the lean somatic tissues to the $\delta^{13}\text{C}$ of the egg white, 13 of the 15 tissues examined had values greater than 1.0 (Figure 6). The tissues with the highest apparent rates of protein turnover were smooth muscle tissues (*e.g.*, small intestine, large intestine, and oviduct) whereas the tissues with the slowest rates of protein turnover were the brain, heart, lung, and blood cells. The relative rates of tissue-specific protein turnover suggested by these data closely parallel those reported for non-galliform birds (reviewed in Bauchinger & McWilliams, 2012). Only the lean fraction of the skin and the adipocytes had ratios less than 1.0. Rather than concluding that the skin and adipocytes are synthesized more rapidly than egg whites, we presume these they contained little ^{13}C because the two-week duration of the previous 432 mg day^{-1} ^{13}C dosing was insufficient for these tissues to reach isotopic equilibrium (or near equilibrium) with the diet.

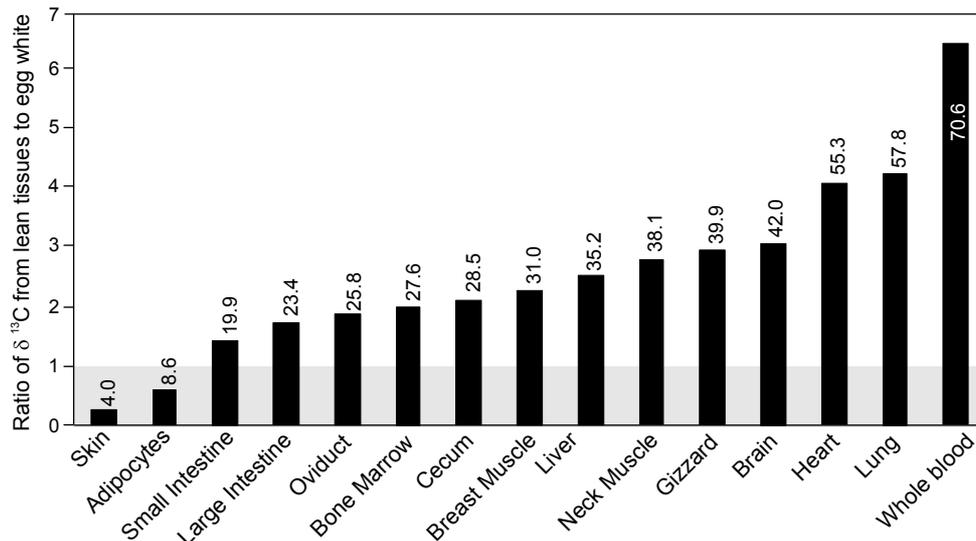


Figure 6. Ratio of the $\delta^{13}\text{C}$ values of the lean fractions of different somatic tissues from Hen 1 to that of egg white on day 105. Two weeks prior this hen was switched from $432 \text{ mg } ^{13}\text{C leucine day}^{-1}$ to $86 \text{ mg } ^{13}\text{C leucine day}^{-1}$ and the hen was not at an isotopic equilibrium with its diet. Numbers above the bars represent actual $\delta^{13}\text{C}$ values

5. Conclusion

This is the first study to compare a) more than two dosing levels of a particular tracer, b) multiple dosing techniques, c) different amino acid tracers, and d) incorporation and washout kinetics during the synthesis of ^{13}C -enriched egg proteins. The results of these experiments show that increasing the dose of a ^{13}C -tracer does not cause a concomitant linear increase in the $\delta^{13}\text{C}$ of egg proteins. This study also revealed that delivering tracers dissolved in the drinking water did not necessarily increase the final $\delta^{13}\text{C}$ of proteins, but decreased the time required for the eggs to reach isotopic equilibrium with the diet – a factor that could influence budgets involved with the large scale synthesis of these proteins. The use of highly enriched, uniformly ^{13}C -labeled mixtures of amino acids may be suitable if desired protein enrichments are above $\sim 500\%$ (> 1.5 atom percent), but because of the excessive oxidative losses of ^{13}C from nonessential amino acids (Fromentin et al., 2011b) and the lack of the positional specificity of ^{13}C -atoms (Berthold et al., 1991; Fromentin et al., 2011b; Kelly & Martinez del Rio, 2010), a ^{13}C -1-labeled essential tracer molecule is desirable for applications where desired ^{13}C enrichments are modest. While both the integration and washout kinetics of ^{13}C -atoms in egg proteins closely follow first-order models, the half-life for ^{13}C -atoms leaving the body is much lower than its entry into the body. Additional studies will be necessary to investigate the physiological basis of this isotopic discrimination *in vivo*. Overall, these experiments provide several new insights that should help make the production of ^{13}C -labeled proteins more efficient and increase the availability of these molecules for $^{13}\text{CO}_2$ breath testing in humans and animals.

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