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SYMPOSIUM

Amino Acid $\delta^{13}C$ Analysis Shows Flexibility in the Routing of Dietary Protein and Lipids to the Tissue of an Omnivore

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Synopsis Stable-isotope analysis (SIA) has revolutionized animal ecology by providing time-integrated estimates of the use of resources and/or habitats. SIA is based on the premise that the isotopic composition of a consumer's tissues originates from its food, but is offset by trophic-discrimination (enrichment) factors controlled by metabolic processes associated with the assimilation of nutrients and the biosynthesis of tissues. Laboratory preparation protocols dictate that tissues both of consumers and of their potential prey be lipid-extracted prior to analysis, because (1) lipids have carbon isotope ($\delta^{13}C$) values that are lower by approximately 3–8‰ than associated proteins and (2) amino acids in consumers' proteinaceous tissues are assumed to be completely routed from dietary protein. In contrast, models of stable-isotope mixing assume that dietary macromolecules are broken into their elemental constituents from which non-essential amino acids are resynthesized to build tissues. Here, we show that carbon from non-protein dietary macromolecules, namely lipids, was used to synthesize muscle tissue in an omnivorous rodent (*Mus musculus*). We traced the influence of dietary lipids on the synthesis of consumers' tissues by inversely varying the dietary proportion of C_4-based lipids and C_3-based protein while keeping carbohydrate content constant in four dietary treatments, and analyzing the $\delta^{13}C$ values of amino acids in mouse muscle after 4 months of feeding. The influence of dietary lipids on non-essential amino acids varied as function of biosynthetic pathway. The source of carbon in ketogenic amino acids synthesized through the Krebs cycle was highly sensitive to dietary lipid content, with significant increases of approximately 2–4‰ in Glutamate and Aspartate $\delta^{13}C$ values from the 5% to 15% dietary lipid treatment. Glucogenic amino acids (Glycine and Serine) were less sensitive to dietary lipid, but increased by approximately 3–4‰ from the 25% to 40% lipid diet. As expected, the $\delta^{13}C$ values of essential amino acids did not vary significantly among diets. Although lipids provide a calorie-rich resource that fuels energy requirements, our results show that they also can be an important elemental source of carbon that contributes to the non-essential amino acids used to build structural tissue like muscle. As such, the calculation of trophic-discrimination factors for animals that consume a lipid-rich diet should consider lipid carbon as a building block for proteinaceous tissues. Careful consideration of the macromolecular composition in the diet of the consumer of interest will help to further refine the use of SIA to study animal ecology and physiology.

Introduction

Stable-isotope analysis (SIA) is now a common ecological tool used to study resource-use and habitat-use by animals. Both physiological and statistical advances have contributed to SIA's recent expansion in ecology. First, a mechanistic understanding of the eco-physiological processes that result in variation in trophic-discrimination factors ($\delta X_{tissue\text{-}diet}$) and isotopic incorporation (Cerling et al. 2007; Martínez del Rio et al. 2009) has enabled ecologists to apply SIA to a wide variety of contexts, especially in situations when other dietary methods are limited by infrequent

observations or the inability to study temporal variation in diet at the individual level (e.g., scat or gut/stomach contents). Second, statistical advances in how stable-isotope data can be analyzed and interpreted have also fueled the explosive growth of this tool. The use of spatial metrics (Layman et al. 2007; Jackson et al. 2011) and mixing models that incorporate potential isotopic variation among inputs to the model (Phillips and Gregg 2003; Moore and Semmens 2008; Parnell et al. 2010; Newsome et al. 2012) has been used extensively to more accurately characterize the diet composition of wild animal populations.

Mixing models have been especially prolific, and these interpretive tools are now easy to use and when utilized properly can yield robust estimates of diet composition. Any mixing model, including simple linear or Bayesian-based models, make three general assumptions regarding how dietary macromolecules are assimilated and used to biosynthesize tissues, most of which are built of protein such as keratins, collagen, muscle, liver, and red blood cells. First, mixing models assume that trophic-discrimination factors are known for the species of interest and that they do not vary. The recent adoption of Bayesian-based models (Moore and Semmens 2008; Parnell et al. 2010) enables ecologists to constrain this potential source of error by including estimates of how much trophic-discrimination factors vary as *a priori* inputs into the model. Second, mixing models assume that the elemental concentration is constant among dietary sources, which is violated when examining omnivores that consume food with wildly different nitrogen concentrations (Phillips and Koch 2002). Again, the recently developed Bayesian-based models incorporate the carbon and nitrogen concentrations of potential dietary sources to provide more accurate estimates of diet composition.

A remaining challenge for the isotope-mixing models concerns the assimilation of dietary macromolecules used to build proteinaceous tissues, likely because they are more difficult to quantify. This concept is often referred to as ''routing'' in the literature (Schwarz 1991) and is best visualized as a spectrum of potential scenarios. On one end of the spectrum is direct and complete (100%) routing in which neither essential nor non-essential amino acids contained in dietary protein are altered during assimilation and are directly incorporated into synthesized tissues. This assumption suggests that only dietary protein should be used as source (prey) inputs in mixing models, whereas other dietary macromolecules (lipids or carbohydrates) do not substantially contribute to the biosynthesis of tissues. The other end of the spectrum assumes complete mixing of dietary macromolecules, which van der Merwe (1982) termed the ''scrambled egg hypothesis.'' This assumption stipulates that all dietary macromolecules are broken into their elemental constituents from which non-essential amino acids are resynthesized to build proteins used in tissue synthesis. Most mixing models are based on the scrambled egg hypothesis, which is potentially problematic for omnivores and even carnivores that consume diets containing a high proportion of non-protein macromolecules that vary in their isotopic composition. Note that the assumption of 100% mixing of dietary macromolecules inherent to mixing models runs contrary to widely adopted protocols for preparing samples; such protocols dictate that lipids should be removed from potential prey prior to the analysis of stable isotopes (Post et al. 2007).

Conceptually, some degree of protein routing is assumed to occur because essential amino acids that cannot be synthesized by eukaryotes must be sourced from dietary protein, or provided by prokaryotic microflora in the gut that can synthesize essential amino acids (Metges 2000). In contrast, non-essential amino acids can be synthesized *de novo* by the consumer and thus may contain carbon sourced from any dietary macromolecule. Previous work on a variety of animals generally shows that the $\delta^{13}C$ of non-essential amino acids in consumer tissues is higher than dietary non-essential amino acids, whereas little to no $\delta^{13}C$ discrimination occurs between essential amino acids in consumer tissues and those available from dietary protein. Since most proteinaceous tissues (muscle, liver, hair, and whole blood) analyzed by ecologists are constructed largely from non-essential amino acids (~60–72%; Wolf N, Newsome SD, Peters J and Fogel ML, submitted for publication), the degree to which protein is routed is likely context-dependent and is primarily influenced by dietary macromolecular content. And because the $\delta^{13}C$ value of dietary protein and lipids can vary by 3–8‰ (e.g., Cherry et al. 2011), the effects of routing are an important consideration when examining animals that can consume high proportions of dietary lipids, such as many marine carnivores and some terrestrial frugivores and omnivores.

Protein-routing has been examined through SIA of bulk tissues (e.g., Podlesak and McWilliams 2006; Voigt et al. 2008; Kelly and Martinez del Rio 2010) and individual compounds (e.g., O'Brien et al. 2002; Jim et al. 2006; Newsome et al. 2011). All of these studies conclude that protein routing occurs to some degree, but as expected is dependent on dietary macromolecular content. The controlled feeding study on rats by Jim et al. (2006) is the most comprehensive

examination to date of routing in an omnivore. Focusing on compound specific amino acid $\delta^{13}C$ analysis of collagen, this study found that at least 50% of amino acids in rat bone collagen were routed from dietary amino acids. Since rats' bone collagen is constructed of approximately 22% essential amino acids, and rats were fed adequate amounts of protein (20%), essential amino acids account for at least half of the total routing observed. Thus, the remaining portion (>30%) of the collagen was routed from dietary non-essential amino acids. This study also found that the degree of routing varied among individual non-essential amino acids; estimates of 42% and 28% were observed for Glycine and Aspartate, respectively. Overall, such studies suggest that routing does occur in omnivores fed on mixed diets of protein and carbohydrates, and models built on a 100% "scrambled egg" assumption of mixing typically underestimate the contributions of dietary protein to tissues.

A similar issue that has received comparably little attention is the possibility that animals may use carbon in ingested lipid to synthesize non-essential amino acids—an important consideration in the study of carnivores that consume (and often prefer) calorie-rich prey with high lipid contents. Ecologists routinely extract lipids from prey samples prior to isotopic analysis, which in essence assumes that amino acids in tissues are completely routed from dietary amino acids. Recent studies on wild carnivores that consume diets with high lipid contents (≥10%) suggest that animals may use dietary lipid carbon to build non-essential amino acids that form the foundation of most tissues. Newsome et al. (2010) showed that $\delta^{13}C$ trophic-discrimination factors, or $\Delta^{13}C_{tissue\text{-}diet}$, in a wild population of California sea otters (*Enhydra lutris nereis*) were significantly related to dietary lipid content, suggesting that otters are using ^{13}C-depleted lipid carbon to build tissues made of pure protein (vibrissae). Similar patterns have been observed in captive wolves (*Canis lupus*) (Fox-Dobbs et al. 2007), polar bears (*Ursus maritimus*) (Polischuk et al. 2001; Cherry et al. 2011), and California sea lion pups (*Zalophus californianus*) (Newsome et al. 2006) that consumed lipid-rich diets. Cherry et al. (2011) used a sophisticated mixing model to account for the contribution of dietary protein and lipids, which systematically differed by approximately 6–8‰ irrespective of prey type, to tissue (blood) synthesis in polar bears and concluded that using only prey protein in mixing-models results in erroneous estimates of diet composition. Such examples suggest that for animals that consume diets high in lipid content (≥10%), one should use bulk samples of prey that have not been lipid-extracted to estimate appropriate $\Delta^{13}C_{tissue\text{-}diet}$ values.

In this study, we used compound-specific $\delta^{13}C$ analysis to investigate the effects of non-protein dietary macromolecules on the isotopic composition of non-essential and essential amino acids. Our study is a continuation of a controlled-feeding experiment on mice by Wolf N, Newsome SD, Peters J and Fogel ML (submitted for publication) that examined (1) the effects of dietary macromolecular content on $\delta^{13}C$ trophic-discrimination factors and (2) the influence of amino-acid composition on $\Delta^{13}C_{tissue\text{-}diet}$ among tissues irrespective of diet, a phenomenon known as tissue-specific discrimination (Koch 2007; Caut et al. 2009). We created diets that contained a C_3-derived protein and a C_4-derived lipid and we varied the protein:lipid content among four diets to trace dietary lipid carbon into the non-essential and essential amino acids used to build consumers' (mice) muscle tissue; the content of dietary carbohydrates remained constant at approximately 35% among treatments. In general, our goal was to further highlight the need for ecologists to carefully consider dietary macromolecular content, including isotopic variation among individual amino acids, lipids, and carbohydrates when using SIA to reconstruct the composition of animals' diets through mixing-models. In some cases, this may result in a reassessment of $\delta^{13}C$ trophic-discrimination factors for consumers that routinely ingest prey rich in lipids or carbohydrates.

Methods

Experimental design

All work was conducted with the approval of the University of Wyoming Animal Care and Use Committee (*#A-3216-01*). In addition to being commonly used to study mammalian physiology, mice were chosen for our experiment because they thrive in captivity and are capable of eating a wide variety of synthetic diets. Because the mice were rapidly growing juveniles, we assumed they would incorporate dietary components into their tissues at a much faster rate than would adults (Martínez del Rio et al. 2009). Thirty-eight recently weaned mice (*Mus musculus*) approximately 3 weeks old (Charles River Laboratories International, Wilmington, MA, USA) were housed at the University of Wyoming Animal Care Facility. Mean (±SD) starting mass of the all mice was 13.9 ± 1.7 g. Mice were randomly divided into four treatment groups ($n = 10$ for treatment groups 1 and 4 and $n = 9$ for treatment groups 2 and 3) and housed in one of four plastic cages (11 inches × 16.5 inches × 8.5 inches) at 25°C with a 12L:12D

Table 1 Composition (weight percent) and $\delta^{13}C$ values (‰) of dietary ingredients and bulk diets of the four experimental diet-treatments of varying protein (P) and lipid (L) contents

Ingredient	40P:5L diet	30P:15L diet	20P:25L diet	5P:40L diet	$\delta^{13}C \pm SD$
Brewer's yeast	2.4	2.4	2.4	2.4	−23.5 ± 0.8
USP fortification salt	4.2	4.2	4.2	4.2	–
Vitamin fortification mixture	1.2	1.2	1.2	1.2	−12.0 ± 0.1
Corn oil	1.2	1.2	1.2	1.2	−11.0 ± 0.1
Cellulose	9.6	9.6	9.6	9.6	−11.0 ± 0.1
Casein	40.4	30.1	20.4	5.4	−27.0 ± 0.1
Lipid	4.8	15.1	25.1	39.8	−14.5 ± 0.1
Sucrose	36.1	36.1	35.9	36.1	−11.6 ± 0.1
Bulk diet $\delta^{13}C$ (‰)	−24.8	−23.3	−22.1	−19.3	–

photoperiod. Each of the treatment groups received one of four diets that varied in protein:lipid content and in isotopic composition (Table 1). The total dietary content of C_4-based lipids ($\delta^{13}C = -14.5$‰) and C_3-based proteins ($\delta^{13}C = -27.0$‰) varied inversely between 5% and 40%. The dietary content of C_4-based carbohydrates ($\delta^{13}C = -11.6$‰) remained constant (35%) among diet-treatments. For each treatment, dietary components were thoroughly mixed with a little water and presented to mice as a slurry to avoid individuals from selectively consuming particular dietary components. The mice were fed *ad libitum* and food and water were replaced daily. The mice were also weighed weekly to monitor growth and health.

Amino acid $\delta^{13}C$ analysis

We measured the $\delta^{13}C$ value of individual amino acids in mouse muscle and dietary protein (casein) using gas chromatograph combustion isotope ratio mass spectrometry. Approximately 1.5 mg of dried mouse muscle or dietary casein was hydrolyzed to amino acids in 1 ml of 6N hydrochloric acid at 110°C for approximately 20 h. Hydrolyzed amino acids were then derivatized to N-trifluoroacetic acid isopropyl esters (Fantle et al. 1999; O'Brien et al. 2002) in several batches and run in triplicate. Derivatized samples were injected into a Thermo Scientific Trace GC Ultra gas chromatograph for separation, converted to CO_2 gas through a combustion interface, and analyzed with a Thermo Scientific Delta V isotope-ratio mass spectrometer at the University of Wyoming Stable Isotope Facility (Laramie, WY, USA). Isotopic results are expressed as δ values, $\delta^{13}C = 1000 \times [(R_{sample} - R_{standard})/R_{standard}]$, where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratios of the sample and standard, respectively. The internationally accepted standard is Vienna-Pee Dee Belemnite limestone (V-PDB) for carbon and the units are expressed as per mil (‰).

Using this derivatization method, measurements of $\delta^{13}C$ value for a given amino acid include carbon both from the amino acids and the derivatization reagents (e.g., isopropanol), which can be heavily fractionated depending on the synthesis pathway and on the number of exchangeable versus non-exchangeable carbon atoms in each amino acid. Amino-acid standards of known $\delta^{13}C$ composition were derivatized and analyzed along with each batch of samples. The results of the standards reflect the effect of carbon added by the derivatization reagents and provided the information required to calculate the $\delta^{13}C$ value of amino-acid carbon in the samples. Individual amino acid $\delta^{13}C$ values ($\delta^{13}CAA_{sample}$) were calculated using the equation:

$$\delta^{13}CAA_{sample} = \frac{\delta^{13}CAA_{dsa} - \delta^{13}CAA_{dst} + \delta^{13}CAA_{std} \times p_{std}}{p_{std}}$$

The terms $\delta^{13}C_{dsa}$ and $\delta^{13}C_{dst}$ refer to the derivatized sample and standard, respectively, and std refers to the standard. p_{std} is equal to the proportion of carbon in the derivative that was sourced from the amino acid (Fantle et al. 1999; O'Brien et al. 2002). We resolved 10 amino acids using these methods, 6 of which are typically considered non-essential for most eukaryotes—Glycine, Serine, Alanine, Aspartate, Glutamate, and Proline—and 4 of which are considered essential, including Valine, Leucine, Isoleucine, and Phenylalanine. Glutamine and Asparagine are converted to glutamic acid (Glutamate) and aspartic acid (Aspartate), respectively, during derivatization. A set of amino-acid isotopic standards were analyzed for every three

unknown samples. The standard deviation of $\delta^{13}C$ values among standards ranged from 0.3‰ for Isoleucine and Phenylalanine to 1.8‰ for Alanine ($n = 25$). The mean $\delta^{13}C$ standard deviation for all amino acids measured was 0.7‰.

Bulk mouse muscle tissue was lipid-extracted with repeated rinses of a 2:1 chloroform:methanol solvent solution, rinsed repeatedly in deionized water, and lypholized. Approximately 0.5–0.6 mg of dried muscle was weighed into 3×5 mm tin capsules and $\delta^{13}C$ and $\delta^{15}N$ values measured using a Costech 4010 or CarloErba NC2500 elemental analyzer interfaced with a Finnegan Delta Plus XL mass spectrometer at the University of Wyoming Stable Isotope Facility (Laramie, WY, USA).

Finally, we used a linear mixing model to quantify the percentage of mouse muscle carbon derived from protein and non-protein dietary sources. Model inputs include two dietary end-members, casein protein ($\delta^{13}C_{Protein} = -27‰$) and non-protein ($\delta^{13}C_{Non\text{-}Protein} = -13‰$) sources, the latter of which was estimated by averaging the $\delta^{13}C$ value of dietary carbohydrates (sucrose, −11.5‰) and lipids (−14.5‰). The model takes the form of the equation, $\delta^{13}C_{Mouse\ Muscle} = ((f_{Protein})(\delta^{13}C_{Protein}) + (1-f_{Protein})(\delta^{13}C_{Non\text{-}Protein})) + \Delta^{13}C_{Muscle\text{-}Diet}$, where $f_{Protein}$ and $1-f_{Protein}$ are the proportions of protein and non-protein (lipids and carbohydrates) dietary carbon, respectively. $\Delta^{13}C_{Muscle\text{-}Diet}$ represents the $\delta^{13}C$ trophic-discrimination factor for mouse muscle, which we assumed was 1‰ (Caut et al. 2009).

Amino-acid concentrations in mouse muscle

To measure amino-acid concentrations in mouse muscle, we randomly chose samples from two mice in each diet-treatment (total $n = 8$) from which approximately 1.0 mg of lipid-extracted muscle tissue was hydrolyzed in 6N hydrochloric (HCl) acid at 110°C for 20 h. 100 µl of amino-acid hydrolysate was dried in a centrifugal evaporator (Labconco micro centrivap IR) and taken back up in 100 µl 20 mM HCl. Samples were sonicated for 5 min and any insoluble material was pelleted by a 5-min spin at full speed in a microcentrifuge (Eppendorf 5415D). Ten microliters of the redissolved samples were combined with Borate buffer (100 mM sodium tetraborate, pH 8.8) to a total volume of 80 µl. Twenty microliters of Waters AccQ-Tag reagent (~10 mM 6-Aminoquinolyl-*n*-hydroxysuccinimidyl carbamate in acetonitrile) was added. The reactions were mixed, incubated at room temperature 1 min, then at 55°C for 10 min. Amino-acid standards were derivatized with the AccQ-Tag reagent in a similar manner and diluted to generate a standard curve of concentrations from 6.25 pmole/µl to 100 pmole/µl in the final reaction volume. Derivatized amino acids were analyzed on a Waters Acquity UPLC system. One microliter aliquots were injected on a Waters AccQ-Tag Ultra C18 column (2.1×100 mm) run at 0.7 ml/min at 55°C. AccQ-Tag Eluents A and B are proprietary Waters reagents. From the MSDS, Eluent A, when properly diluted, appears to be an ammonium formate buffer in 0.5% acetonitrile. Eluent B appears to be the same buffer in 60% acetonitrile. Derivatives of amino acids were detected at 260 nm in an Acquity system photodiode array detector with a 500-µl flow cell. Peaks were identified, integrated, and quantified with the Waters Empower software package.

Statistical analysis

Given the low sample sizes in each treatment ($n = 6$–7) and differences in $\delta^{13}C$ variance of specific amino acids among treatments, we used a non-parametric Wilcoxon test to assess statistical significance in amino acid $\delta^{13}C$ values among the four diet-treatments. Significance was assigned at a P-value of <0.05.

Results

Below we summarize amino acid $\delta^{13}C$ patterns among diet-treatments and the relative concentration of amino acids in mouse muscle. We also briefly describe synthesis pathways for the amino acids we analyzed. Mean (±SD) $\delta^{13}C$ values of amino acids in mouse muscle and dietary protein (casein), mean (±SD) bulk muscle $\delta^{13}C$ values, and trophic-discrimination factors between muscle and bulk diet ($\Delta^{13}C_{Muscle\text{–}Bulk\ Diet}$) and muscle and dietary protein ($\Delta^{13}C_{Muscle\text{–}Diet\ Protein}$) are presented in Table 2.

The $\delta^{13}C$ values of Glutamate and Aspartate, two non-essential ketogenic amino acids synthesized from intermediaries in the Krebs cycle, significantly increased from diets of 5% lipid to those of 15% lipid (Fig. 1). Glutamate and Aspartate are also major components of mouse muscle, contributing approximately 13% and 9% (weight percent), respectively (Fig. 2).

Proline is also considered a ketogenic amino acid because it is synthesized through four steps from Glutamate involving multiple reductase enzymes. Unlike Glutamate, Proline $\delta^{13}C$ values did not significantly increase until the 25% lipid diet-treatment (Table 2 and Fig. 1). Even though Proline can be

Table 2 Mean $\delta^{13}C$ values of amino acids in the dietary protein source (casein) and mouse muscle from each diet-treatment that varied in lipid content from 5% to 40%

			Dietary lipid content			
Amino acid	**Pathway**	**Casein**	**5% (6)**	**15% (7)**	**25% (7)**	**40% (6)**
Alanine (Ala)	Glucogenic (glycolysis)	−22.1 (0.1)	−21.0 (0.3)^{A}	−20.2 (0.7)^{A,B}	−18.9 (0.7)^{B}	−17.3 (1.3)^{B}
Glycine (Gly)	Glucogenic (glycolysis)	−13.2 (0.8)	−15.3 (0.4)^{A}	−15.5 (0.5)^{A}	−15.1 (0.3)^{A}	−12.1 (0.8)^{B}
Serine (Ser)	Glucogenic (glycolysis)	−17.0 (0.1)	−19.2 (0.7)^{A}	−19.6 (0.8)^{A}	−18.4 (0.7)^{A}	−14.0 (1.1)^{B}
Aspartate (Asp)	Ketogenic (Krebs)	−24.2 (0.1)	−22.7 (0.5)^{A}	−21.0 (0.3)^{B}	−18.6 (0.4)^{C}	−16.5 (0.9)^{C}
Glutamate (Glu)	Ketogenic (Krebs)	−19.8 (0.1)	−18.8 (1.0)^{A}	−14.1 (0.2)^{B}	−12.2 (0.7)^{C}	−11.6 (1.6)^{B,C}
Proline (Pro)	Ketogenic (Krebs)	−21.3 (0.1)	−18.5 (0.4)^{A}	−18.1 (0.3)^{A}	−16.2 (0.4)^{B}	−13.9 (1.1)^{B}
Leucine (Leu)	Essential	−30.6 (0.1)	−30.1 (0.5)^{A}	−29.1 (0.1)^{A}	−29.3 (0.2)^{A}	−28.9 (0.5)^{A}
Isoleucine (Ileu)	Essential	−27.3 (0.4)	−23.8 (0.6)^{A}	−24.0 (0.4)^{A}	−23.5 (0.4)^{A}	−22.9 (0.6)^{A}
Valine (Val)	Essential	−29.5 (0.2)	−23.9 (0.6)^{A}	−24.5 (0.5)^{A}	−24.6 (0.6)^{A}	−24.0 (1.2)^{A}
Phenylalanine (Phe)	Essential	−27.4 (0.1)	−28.2 (0.5)^{A}	−27.1 (0.3)^{A}	−28.0 (1.0)^{A}	−26.8 (0.5)^{A}
Bulk muscle	–	–	−23.1 (0.7)^{A}	−22.8 (0.3)^{A}	−21.9 (0.6)^{B}	−20.4 (0.4)^{C}
$\Delta^{13}C_{Muscle–Bulk\ Diet}$	–	–	1.7 (0.7)	0.5 (0.7)	0.2 (1.1)	−1.1 (1.5)
$\Delta^{13}C_{Muscle–Diet\ Protein}$	–	–	3.8 (0.7)	4.1 (0.4)	5.0 (0.6)	6.5 (0.4)

Notes: Also reported are $\delta^{13}C$ values of bulk mouse muscle. Numbers in parentheses adjacent to $\delta^{13}C$ values represent standard error; numbers in parentheses adjacent to dietary lipid contents represent the number of mice analyzed from each treatment. Groups not connected by the same superscript letter are significantly different ($P < 0.05$). Amino-acid pathways are labeled as described in the text. Also presented are calculated $\delta^{13}C$ trophic-discrimination factors between (lipid-extracted) mouse muscle and bulk diet that was not lipid-extracted ($\Delta^{13}C_{Muscle–Bulk\ Diet}$) and dietary protein ($\Delta^{13}C_{Muscle–Diet\ Protein}$).

synthesized *de novo* by eukaryotes, it is considered to be a conditionally essential amino acid in rapidly growing animals like the mice examined in our study (Ball et al. 1986; Chung and Baker 1993). The milk casein used as a dietary protein source in our experiment contains a high concentration of Proline (McKeekin et al. 1949). Thus, the high availability of Proline in our high protein (25–40%) and low lipid (5–15%) diets could result in direct routing of dietary Proline sourced from casein into mouse muscle rather than *de novo* synthesis from Glutamate. Proline concentrations in mouse muscle (~5%), however, were relatively low in comparison to Glutamate and Aspartate and thus play a smaller role in driving the pattern observed among muscle $\delta^{13}C$ values and the associated variation in trophic-discrimination factors among diet-treatments.

Results for Glycine and Serine, two non-essential glucogenic amino acids that are synthesized only from intermediaries in glycolysis, show that this pathway is less sensitive to dietary lipid content in comparison to amino acids solely derived from the Krebs cycle (Glutamate and Aspartate). Glycine is synthesized from Serine, which is in turn derived from 3-phosphoglycerate. Significant effects of dietary lipid content on the $\delta^{13}C$ value of Glycine and Serine were not observed until the 40% lipid diet-treatment, but we observed a large effect as $\delta^{13}C$ values of these two amino acids increased by 3–4‰ between 25% and 40% dietary lipid content (Table 2). Mouse muscle contains a high concentration of Glycine (11%), but less Serine (6%). Thus, the large difference of approximately 1.5‰ in muscle $\delta^{13}C$ trophic-discrimination factors between the treatments containing 25% and 40% lipid content (Table 2) may be largely driven by the large (3–4‰) increases in Glycine and Serine $\delta^{13}C$ values observed between these two diets.

Alanine is synthesized in one step by transamination from glutamate onto pyruvate, an intermediary in glycolysis. Alanine $\delta^{13}C$ values did not significantly increase until the 25% lipid diet-treatment, and values in mouse muscle from the 15% lipid treatment were similar to those in both the 5% and 25% lipid diet-treatments. As observed in Glycine and Serine, results for Alanine suggest that glucogenic amino acids are less sensitive to dietary lipid content in comparison to amino acids derived from the Krebs cycle (Glutamate and Aspartate). Similar to many other glucogenic and ketogenic non-essential amino acids, Alanine is an important component of mouse muscle (9%).

Results for the essential amino acids Leucine and Phenylalanine conformed to expectations; $\delta^{13}C$ values were similar to that of dietary casein and did not change with increasing dietary lipid

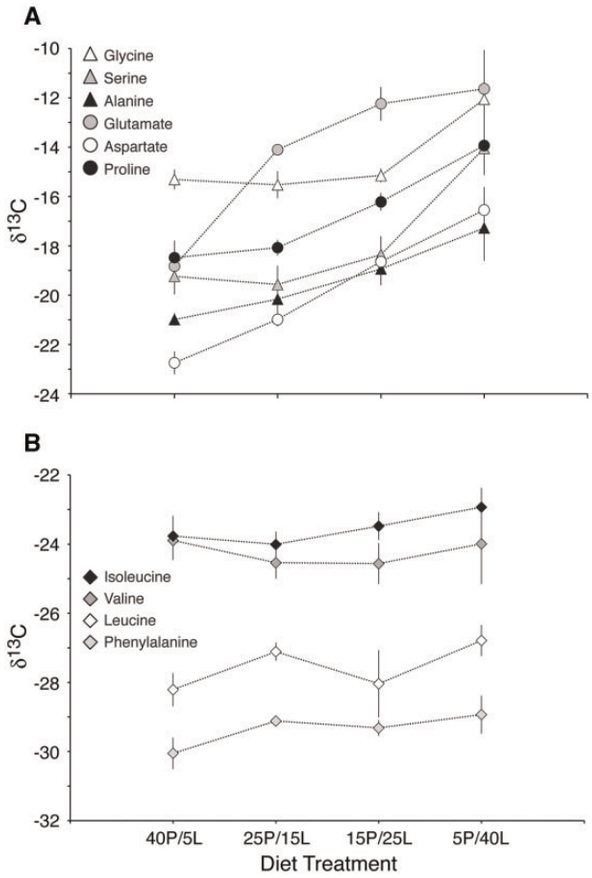

Fig. 1 Mean $\delta^{13}C$ values of non-essential (**A**) and essential (**B**) amino acids among the four diet-treatments; error bars represent standard error. The relative proportions (5–40%) of protein (P) and lipids (L) are shown for each diet. Note difference in the scale of $\delta^{13}C$ on the *y*-axis in panels A (14‰) and B (10‰).

content (Table 2 and Fig. 1). This pattern suggests that these essential amino acids are being directly routed from dietary protein (casein), even in the low protein (5%), high lipid (40%) treatment. The $\delta^{13}C$ values of Isoleucine and Valine in mouse muscle did not significantly change with increasing dietary lipid content; however, their $\delta^{13}C$ value was higher than that of the respective amino acid in dietary casein (Table 2). With the exception of Leucine (9%), most of these essential amino acids are found in mouse muscle at relatively low concentrations (4–6%) in comparison to non-essential amino acids (Fig. 2).

As expected, the concentration of amino acids in mouse muscle varied predictably between non-essential and essential forms (Fig. 2). Of the 15 major amino acids found at more than 1% (weight percent) in mouse muscle, the eight non-essentials accounted for approximately 61% of the total composition, and the seven essentials accounted for the remaining approximately 39%.

Values of $\delta^{13}C$ in bulk (lipid-extracted) muscle of all mice in our experiment are presented in Table 2; values were not statistically different between the diet-treatments with 5% and 15% lipid content, but significantly increased in the 25% and 40% treatments. Furthermore, mouse muscle from the diet-treatment containing 40% lipids had significantly higher mean $\delta^{13}C$ values than did the 25% lipid treatment.

Finally, growth rates for mice on the high (40%) protein and high (40%) lipid diets were lower than for mice on the two intermediate diet-treatments. Despite observed differences in growth rates among treatments, we assume that the mouse muscle tissue had equilibrated with diet when mice were sacrificed after 4 months. MacAvoy et al. (2006) found complete isotopic incorporation for whole blood in mice to be approximately 80–90 days, and in other endotherms whole blood has similar incorporation rates as muscle (Hobson and Clark 1992). Although isotopic incorporation rates likely scale with growth rate in rapidly growing endotherms and ectotherms (Reich et al. 2007; Carleton and Martinez del Rio 2010), there is no evidence that $\delta^{13}C$ trophic-discrimination factors are impacted by growth rates.

Discussion

Our results show that mice can use carbon sourced from non-protein dietary macromolecules (carbohydrates and lipids) to synthesize non-essential amino acids. Our study also shows that this phenomenon can have a significant impact on the $\delta^{13}C$ value of bulk muscle tissue when the non-protein (lipid and carbohydrate) macromolecular content of diet exceeds 50%. This finding has implications for the calculation of accurate trophic-discrimination factors and application of SIA to reconstruct the diet composition of omnivores and carnivores that consume a variety of dietary macromolecules that vary in carbon-isotope ($\delta^{13}C$) composition.

Protein routing is an often cited but poorly understood mechanism, and it has serious implications for how studies utilizing stable isotopes are designed, samples are processed, and data are interpreted. Previous studies that analyzed isotopes of individual amino acids have confirmed that routing is not 100% complete and that eukaryotic organisms, from butterflies to pigs, synthesize amino acids from a variety of non-protein dietary macromolecules, including sugars (O'Brien et al. 2002) and other carbohydrates (Hare et al. 1991; Howland

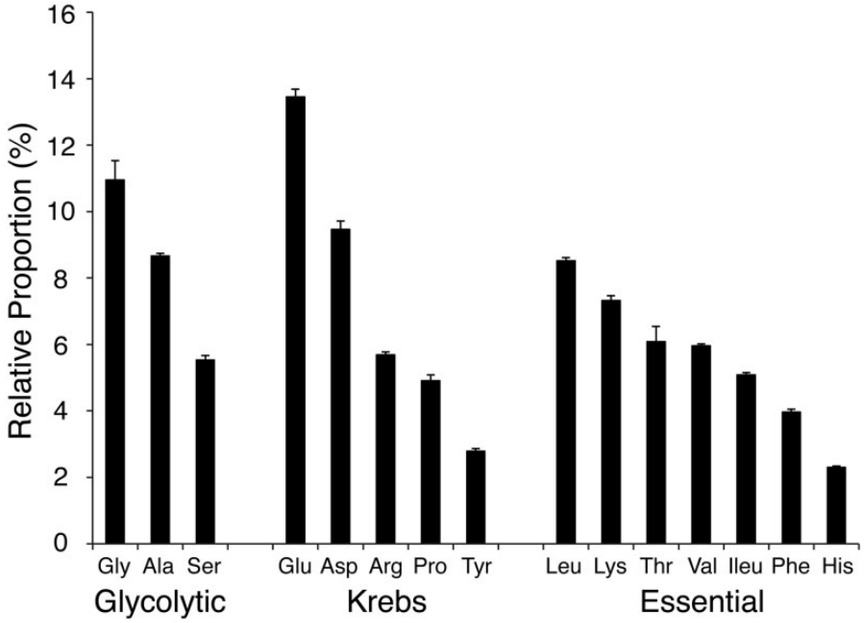

Fig. 2 Mean relative proportions of amino acids in mouse muscle ($n = 8$); error bars represent standard error. Amino acids are grouped by relative abundance within each biosynthetic pathway.

et al. 2003; Jim et al. 2006). Our study expands these findings to show that carbon from dietary lipids also can contribute to the synthesis of non-essential amino acids.

Unlike the synthetic diets used in our experiment with laboratory mice, lipids in food that wild animals consume have lower $\delta^{13}C$ values than do associated protein (Podlesak and McWilliams 2007; Newsome et al. 2010; Cherry et al. 2011). The basic pathway of lipid synthesis is similar in all organisms (even plants), and results in a kinetic carbon-isotope fractionation during the conversion of pyruvate to acetyl CoA by pyruvate dehydrogenase (DeNiro and Epstein 1977; Podlesak and McWilliams 2007). Further ^{13}C depletion may occur during the conversion of acetyl CoA to malonyl CoA, which requires the addition of carbon dioxide that is likely in the form of $^{12}CO_2$ sourced from decarboxylation reactions in glycolysis and/or the Krebs cycle. Variation in the magnitude of the ^{13}C depletion in lipids could be taxon-specific as it primarily depends on the concentration of pyruvate, which is largely controlled by the composition of the diet.

Since dietary lipids have significantly lower $\delta^{13}C$ values than do associated proteins, animals that consume lipid-rich foods are anticipated to have lower than expected $\delta^{13}C$ trophic-discrimination factors, which are commonly calculated as the difference between the consumer tissue of interest and the lipid-extracted (protein) portion of the diet ($\Delta^{13}C_{\text{Muscle–Diet Protein}}$). In our experiment, the calculation of trophic-discrimination factors using $\delta^{13}C$ values of bulk diet that had *not* been lipid-extracted yielded values ranging from 0.2‰ to 1.7‰ (Table 2), which are similar to published estimates for mammalian muscle (Kelly 2000; Roth and Hobson 2000; Caut et al. 2009; Tyrrell et al. 2013). In contrast, the comparison of mouse muscle's $\delta^{13}C$ values to that of dietary protein (casein) yielded trophic-discrimination factors that were significantly higher (3.8–6.4‰) than our expectations, which were based on previous work (Kelly 2000; Caut et al. 2009).

The coupling of isotopic data and concentration data for individual amino acids also helps illuminate how non-protein dietary sources can impact the $\delta^{13}C$ values of bulk tissues. Non-essential amino acids represent approximately 60% of the total amino-acid composition of mouse muscle (Fig. 2). The non-essential amino acids that were most sensitive to dietary lipid content, Glutamate and Aspartate, accounted for 22% of the total amino-acids. Two other non-essential amino acids sensitive to dietary lipid content, Alanine and Proline, contributed an additional 9% and 5%, respectively. Finally, mouse muscle contains 11% and 6% of Glycine and Serine, respectively, two glucogenic amino acids that were sensitive to dietary lipid contents exceeding 25%. In total, these six non-essential amino acids account for approximately 53% of the total amino-acid

content of mouse muscle tissue, which is more than 10% larger than the total contribution of essential amino acids (Fig. 2). Two other ketogenic amino acids (Arginine and Tyrosine), which we could not analyze for $\delta^{13}C$, account for another 9% of the total amino-acid composition in mouse muscle. Thus, if the majority of proteins in tissues are constructed from non-essential amino acids that can be synthesized from carbon derived from dietary lipids (or carbohydrates), dietary lipid content could potentially have a significant effect on bulk tissue $\delta^{13}C$ values, and thus on trophic-discrimination factors used to reconstruct diet composition.

It should be noted that the difference in $\delta^{13}C$ between the protein (casein) and lipid components in our experimental diets was approximately 12‰, which is approximately twice the magnitude observed in nature. In other words, our experiments overestimate the isotopic effect a lipid-rich diet may have on the composition of consumers' tissues and associated trophic-discrimination factors; however, the magnitude of the effect we observed was large enough that it likely has an impact on the interpretation of $\delta^{13}C$ data from natural settings. Trophic-discrimination factors varied by more than 7‰ across diet-treatments, whether $\delta^{13}C$ values from bulk diets or lipid-extracted diets were used to calculate them (Table 2). Smaller differences in $\delta^{13}C$ between protein and lipids (3–8‰) observed in natural settings also likely have a significant effect on trophic-discrimination factors for animals that frequently consume lipid-rich foods.

As we expected, the $\delta^{13}C$ value of Isoleucine and Valine did not significantly change with increasing dietary lipid content (Table 2), but we were surprised to find that the $\delta^{13}C$ values of these essential amino acids in mouse muscle were significantly higher than that of the corresponding essential amino acids in dietary protein (casein) for all diet-treatments. Since dietary lipids and carbohydrates in our diet-treatments had higher $\delta^{13}C$ values than did Isoleucine and Valine available in dietary protein (Table 2), it is possible that a portion of these essential amino acids in mouse muscle was synthesized *de novo* by gut microflora from non-protein dietary sources (Metges 2000; Karasov and Carey 2008; Newsome et al. 2011; Arthur et al. 2014). Prokaryotic provisioning of essential amino acids has been previously identified with amino acid $\delta^{13}C$ analysis (Newsome et al. 2011; Arthur et al. 2014). For example, Arthur et al. (2014) used an amino-acid fingerprinting approach (Larsen et al. 2009) to show that essential amino acids in tissues of herbivorous green sea turtles (*Chelonia mydas*) were synthesized by gut microbial symbionts. Newsome et al. (2011) suggested that the prokaryotic microflora of the gut synthesized a significant percentage (~50%) of the essential amino acids in tilapia muscle tissue from dietary carbohydrates when the fish were fed diets with low protein contents (<5%). In rapidly growing animals such as mice, it is possible that the amount of essential amino acids required for growth and maintenance exceeds that available from the diet. The concentration of Isoleucine and Valine in casein (5–6%) (McMeekin et al. 1949) is similar to the concentrations in mouse muscle (Fig. 2); however, the dietary source of these essential amino acids may be limiting when on diets with low protein contents. More work is needed to examine the role of microbial assemblages in the gut in supplying essential and non-essential amino acids to their hosts, but we suspect that during periods when dietary protein sources are limiting and do not meet growth or homeostatic requirements, gut microflora contribute significantly to the budgets of essential amino acid of their eukaryotic hosts.

The relative influence of lipids and carbohydrates on the biosynthesis of mouse muscle

Since dietary carbohydrates and lipids had similar $\delta^{13}C$ values in all diet-treatments, it is impossible to deconvolve the relative contribution of these non-protein dietary sources of carbon to the synthesis of non-essential amino acids in mouse muscle. Dietary carbohydrates likely contribute to non-essential amino-acid synthesis in all diet-treatments. A linear mixing model shows that approximately 20% of the carbon in mouse muscle is derived from (C_4) non-protein dietary sources when mice were fed a low-lipid (5%) diet. A significant portion of the non-protein C_4 component is likely derived from carbohydrates since it represents 35% of 40% (weight percent) of the non-protein portion of diet in the low-lipid diets. At the other end of the dietary spectrum, where lipids represent the majority (40% of 75%) of (C_4) non-protein carbon available for the synthesis of tissue, the mixing model predicts that approximately 40% of carbon in mouse muscle is derived from non-protein dietary sources. Also note that stoichiometric considerations show that lipid carbon is nearly two times more abundant than carbohydrate carbon because these two macromolecules are approximately 75% and approximately 40% carbon (weight percent), respectively. So while lipids account for just over 50% (40/75) of non-protein carbon by mass for mice fed a

high lipid diet, lipid carbon is much more available than carbohydrate carbon for the synthesis of amino acids and tissues.

In addition, consideration of the various pathways that animals use to synthesize non-essential amino acids provides a way to evaluate the relative importance of dietary carbohydrates and lipids as sources of the non-protein carbon mice use to synthesize muscle. Dietary carbohydrates are predicted to have a greater influence on the synthesis of glucogenic amino acids since they are synthesized from two intermediaries in glycolysis: 3-phosphoglyerate (Glycine and Serine) and pyruvate (Alanine). In agreement with this prediction, $\delta^{13}C$ values of Glycine and Serine in mouse muscle did not significantly increase except for mice on the high-lipid (40%) diet (Table 2). $\delta^{13}C$ values of Alanine were significantly higher in muscle for mice fed a diet containing 25% and 40% lipids relative to diets containing 5% and 15% lipids. In contrast to glucogenic amino acids, dietary lipids are predicted to have a greater influence on the synthesis of ketogenic amino acids because beta-oxidation of fatty acids produces acetyl CoA, an upstream precursor to oxaloacetate and α-ketoglutarate in the Krebs cycle. Aspartate is synthesized from oxaloacetate, whereas Glutamate, Arginine, and Proline are synthesized from α-ketoglutarate. In agreement with this prediction, the $\delta^{13}C$ values Aspartate and Glutamate in mouse muscle increased significantly between mice on a 5% lipid diet and those on a 15% lipid diet (Table 2); we observed further significant increases in the $\delta^{13}C$ values of these two amino acids for mice fed diets with 25% and 40% lipid. Since the proportion of dietary carbohydrates was constant among treatments, greater utilization of lipids from diets containing higher lipid content is assumed to be the primary driver behind observed increases in the $\delta^{13}C$ value of ketogenic amino acids. In contrast, the $\delta^{13}C$ of glucogenic amino acids are more sensitive to dietary carbohydrate content, and thus did not significantly change until dietary lipid content exceeded 25% (Table 2).

Ecological implications

The large degree of variation in $\delta^{13}C$ values that we observed (1) in the major non-essential amino-acid components of mouse muscle and (2) in the calculated trophic-discrimination factors among diet-treatments shows that the extraction of dietary lipids could influence interpretations gleaned from stable-isotope data in studies of omnivores and carnivores. Several studies provide empirical evidence that this pathway may be important for mammalian consumers, especially carnivores that frequently consume lipid-rich diets. Fox-Dobbs et al. (2007) found that hair (keratin) from captive wolves (*C. lupus*) fed a lipid-rich (25% wet mass) diet largely recorded the fat fraction rather than the protein fraction of diet. This pattern mirrored that found in vibrissae of wild southern sea otters (Newsome et al. 2010) feeding largely on lipid-rich red sea urchins (*Strongylocentrotus franciscanus*) that had a mean (±SD) lipid content of 17% (±1.5) dry mass; in plasma of polar bear cubs (Polischuk et al. 2001); and in bone collagen of California sea lion pups (*Z. californianus*; Newsome et al. 2006) that consumed lipid-rich milk.

The utilization of lipid-derived carbon to synthesize non-essential amino acids may be especially pertinent for marine animals that consistently consume lipid-rich prey (Rosen and Trites 2000; Copeman and Laurel 2010; Cherry et al. 2011; Whiteman et al. 2012; Parrish 2013). For example, many species of marine forage fish—herring, capelin, sand lance, and eulachon—that provide an abundant and energy-dense resource for marine mammals, seabirds, and fish in many coastal and pelagic ecosystems may contain 25–50% lipids (Hislop et al. 1991; Lawson et al. 1998; Robards et al. 1999; Anthony et al. 2000; Wanless et al. 2005). For marine consumers, lipid content of prey frequently has been linked to reproductive success and fitness. Some have attributed the decline of the western population of Steller sea lions (*Eumetopias jubatus*) in the North Pacific to a lack of lipid-rich herring (*Clupea pallasii*), and sand lance (*Ammodytes hexapterus*) in their diet (Merrick et al. 1997; Rosen and Trites 2000). Likewise, short-term decreases in the energy density of important prey, which is largely dependent on lipid content, has been shown to be a probable cause of reproductive failure in seabirds (e.g., Wanless et al. 2005).

Our results also may be important for terrestrial consumers that ingest lipids in amounts that could impact the assumptions for protein routing implicit in SIA-based reconstructions of dietary composition. For example, when salmon are abundant, brown bears (*Ursus arctos*) primarily consume the lipid-rich skin, roe, and organs and discard the protein (muscle) portion. Like vertebrates, the lipid content of insects varies with reproductive condition, and many larvae contain 25–50% lipids, which can exceed the percentage of crude protein (Finke 2008). Omnivores that consume high proportions of seasonal fruits also may utilize dietary lipids to synthesize structural tissues. Smith et al. (2007) reported that several fruits frequently consumed by

migratory passerines contained 24–50% lipids (dry mass). Ingested lipids are metabolized as a readily available source of energy; however, at times when ingestion exceeds energy requirements, lipid-derived carbon may be converted to glucose through gluconeogenesis or to acetyl CoA through the fatty acid spiral and become available for the synthesis of non-essential amino acids.

Preparation protocols that call for the extraction of dietary lipids prior to SIA may not lead to accurate representation of the full suite of dietary macromolecules animals use to synthesize proteinaceous tissues. As such, use of $\Delta^{13}C_{tissue\text{-}diet}$ trophic-discrimination factors calculated through comparison of consumers' tissues to protein of potential prey will lead to erroneous estimates of the composition of diets. We suggest that samples of potential prey initially be analyzed in bulk without eliminating lipids through standard extraction methods (Folch et al. 1957; Bligh and Dyer 1959; Dobush et al. 1985). If potential sources of prey contain significant amounts of lipids—something that can be quickly assessed by examining carbon-to-nitrogen concentrations (or C:N ratios) produced during routine $\delta^{13}C$ and $\delta^{15}N$ analysis—then a sub-sample of such prey should also be lipid-extracted to assess how lipid content affects $\delta^{13}C$ values. Since the difference in $\delta^{13}C$ value between lipids and associated protein can range from 3‰ to 8‰, the effects of lipid content on $\delta^{13}C$ likely vary on a situational basis. For this reason, we suggest that normalization is *not* a preferred method for correcting $\delta^{13}C$ data (Sweeting et al. 2006; Post et al. 2007) unless normalization curves have been established for the taxon of interest (Logan et al. 2008).

Our experiment highlights previously unconsidered flexibility in how omnivorous animals can use carbon derived from dietary lipids to build proteinaceous tissues. Our study also has implications for the application of SIA to study animal ecology and physiology. Specifically, our results suggest that the assumptions of complete (100%) routing of dietary protein or the complete mixing of dietary macromolecules (i.e., the ''scrambled-egg hypothesis'') do not provide an accurate picture of tissue synthesis. Distinctive isotopic labeling of all macromolecular components in an animal's diet, even lipids, should be considered when using SIA to characterize or quantify consumers' diets using trophic-discrimination factors and mixing models. Not only do lipids and carbohydrates provide a calorie-rich energy resource for consumers, they are also a large source of carbon atoms that can contribute to the synthesis of non-essential amino acids through glycolysis and/or the Krebs cycle. The exclusion of non-protein dietary macromolecules from the metabolic pathways that synthesize amino acids, which are the building blocks of the proteinaceous tissues frequently analyzed by ecologists for their isotopic composition, ignores a process that likely contributes to variation in $\delta^{13}C$ trophic-discrimination among organisms. Further understanding of the factors that control variation in trophic-discrimination factors, like those presented here, will better refine the capabilities and expose the limitations of SIA for studying animals' use of resources and habitats.

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