Research Article

Variability in the routing of dietary proteins and lipids to consumer tissues influences tissue-specific isotopic discrimination

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RATIONALE: The eco-physiological mechanisms that govern the incorporation and routing of macronutrients from dietary sources into consumer tissues determine the efficacy of stable isotope analysis (SIA) for studying animal foraging ecology. We document how changes in the relative amounts of dietary proteins and lipids affect the metabolic routing of these macronutrients and the consequent effects on tissue-specific discrimination factors in domestic mice using SIA. We also examine the effects of dietary macromolecular content on a commonly used methodological approach: lipid extraction of potential food sources.

METHODS: We used carbon (^{13}C) and nitrogen (^{15}N) isotopes to examine the routing of carbon from dietary proteins and lipids that were used by mice to biosynthesize hair, blood, muscle, and liver. Growing mice were fed one of four diet treatments in which the total dietary content of C_4-based lipids ($\delta^{13}C = -14.5‰$) and C_3-based proteins ($\delta^{13}C = -27‰$) varied inversely between 5% and 40%.

Results: The $\delta^{13}C$ values of mouse tissues increased by approximately 2–6‰ with increasing dietary lipid content. The difference in $\delta^{13}C$ values between mouse tissues and bulk diet ranged from $0.1 \pm 1.5‰$ to $2.3 \pm 0.6‰$ for all diet treatments. The mean (±SD) difference between the $\delta^{13}C$ values of mouse tissues and dietary protein varied systematically among tissues and ranged from $3.1 \pm 0.1‰$ to $4.5 \pm 0.6‰$ for low fat diets and from $5.4 \pm 0.4‰$ to $10.5 \pm 7.3‰$ for high fat diets.

CONCLUSIONS: Mice used some fraction of their dietary lipid carbon to synthesize tissue proteins, suggesting flexibility in the routing of dietary macromolecules to consumer tissues based on dietary macromolecular availability. Consequently, all constituent dietary macromolecules, not just protein, should be considered when determining the relationship between diets and consumer tissues using SIA. In addition, in cases where animals consume diets with high lipid contents, non lipid-extracted prey samples should be analyzed to estimate diets using SIA. Copyright © 2015 John Wiley & Sons, Ltd.

Stable isotope analysis (SIA) has become a ubiquitous tool in the study of animal dietary ecology. Central to the successful use of this technique is an understanding of the physiological and ecological mechanisms that result in trophic discrimination factors, which are defined as the offset between the stable isotope value of a consumer's tissue(s) and that of its diet.[1] For example, previous research has shown that trophic discrimination factors for carbon isotopes ($\delta^{13}C_{tissue-diet}$) are taxa- and tissue-specific, and can also vary with dietary macromolecular content.[1,2] It has been hypothesized that tissue-specific variations in trophic discrimination factors are driven by differences in the amino acid composition among tissues, which when coupled with variation in carbon isotope composition ($\delta^{13}C$ values) of individual amino acids within tissues, ultimately results in different bulk tissue $\delta^{13}C$ values.[1,3–6] This hypothesis, however, has not been examined in detail because few studies couple stable isotope data with tissue amino acid concentrations.

The role of tissue-specific amino acid composition is just one area in which our understanding of the mechanisms driving tissue-specific isotope discrimination is incomplete. In addition, we know little about how animals route protein versus non-protein (carbohydrates and lipids) dietary macromolecules for metabolic demands and tissue synthesis, and how this routing affects isotopic discrimination. Specifically, how does flexibility in the routing of dietary macromolecules for tissue synthesis affect isotope-based studies of animals that consume diets that vary in protein, lipid, and carbohydrate content? In principle, the concept of metabolic routing is simple. Dietary carbohydrates are preferentially used to fuel energy metabolism, proteins are used to build and repair tissues, and fatty acids are routed into consumer adipose fat tissue that serves as an onboard (endogenous) source of energy.[7,8] This scenario assumes complete (100%) routing of dietary protein; thus, the

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$\delta^{13}C_{tissue-diet}$ factor for a fully incorporated tissue would be a reflection of the differences in the $\delta^{13}C$ value of a tissue and that of dietary protein, and all other dietary macromolecules could be ignored. Note that this paradigm works well for calculating trophic discrimination factors for nitrogen isotopes ($\delta^{15}N$) because the primary source of nitrogen available to consumers is dietary protein.[4] This simple metabolic paradigm is also commonly used to calculate $\delta^{13}C_{tissue-diet}$ factors for carnivorous species.[9] If, however, the carbon skeletons of non-protein dietary macromolecules, such as lipids, are used to synthesize non-essential amino acids, the trophic discrimination factors for $\delta^{13}C$ should be calculated as the difference between the $\delta^{13}C$ value of the consumer tissue and that of the bulk diet, not simply the dietary protein.

The effects of metabolic routing on $\delta^{13}C_{tissue-diet}$ factors are relatively well understood for herbivores and omnivores consuming varying levels of protein and carbohydrates. Previous studies have indicated that varying the dietary protein (or carbohydrates) results in flexibility in the routing of these macromolecules within consumers' bodies, and, consequently, in the type of macromolecule used to supply the energetic and structural needs of the consumer.[10–12] Fewer studies have addressed the effects of varying dietary protein and lipid contents. For example, in carnivores that consume very low (if any) levels of carbohydrates, proteins and lipids are both used as fuels for oxidative respiration and tissue synthesis.[13,14] Two studies have addressed the effects of limiting dietary inputs of protein or lipids on metabolic routing in carnivores.[14,15] These studies show that dietary limitations probably result in plasticity in the routing of proteins and lipids in a similar fashion to that observed for carbohydrates and proteins. Ben-David et al.[14] showed that captive mink (Neovision vision) fed a high-lipid (~50%) diet used lipids preferentially to fuel metabolism, while dietary protein sources were routed directly to tissue synthesis. In contrast, individuals in the low-lipid (~5%) but high-protein diet treatment used protein for both metabolic and structural needs. Cherry et al.[15] also observed that proteinaceous tissues contained some amount of lipid carbon in free-ranging polar bears (Ursus maritimus). Unfortunately, neither of these studies characterized the relationship between dietary protein and lipid contents and metabolic routing; nor did they describe pathways by which dietary lipids can be used to synthesize proteinaceous structural tissues, which ultimately influence $\delta^{13}C_{tissue-diet}$ discrimination factors.

Here, we present the results of an experiment that utilizes carbon (^{13}C) and nitrogen (^{15}N) isotope analysis and tissue amino acid concentrations to investigate the routing of dietary proteins and lipids in mice, and the consequent effects of this phenomenon on $\delta^{13}C_{tissue-diet}$ and $\delta^{15}N_{tissue-diet}$ factors. This work is an extension of that presented by Newsome et al.[16] and examines the effects of the metabolic routing of proteins and lipids on trophic discrimination factors ($\delta^{13}C_{tissue-diet}$ and $\delta^{15}N_{tissue-diet}$) of bulk tissues, rather than the effects on individual amino acids as described by Newsome et al.[16] By feeding growing mice one of four diet treatments in which the total dietary content of C_4-based lipids ($\delta^{13}C = -14.5‰$) and C_3-based proteins ($\delta^{13}C = -27‰$) varied inversely between 5% and 40%, while carbohydrates were kept at a constant level (35%), we were able to examine how dietary proteins and lipids were used by mice to synthesize structural tissues largely made of protein, including hair, blood, muscle, and liver. If dietary protein is routed directly to structural tissues, and lipids are used solely to fuel metabolism, we would expect the isotopic discrimination between dietary protein and consumer tissues to be consistent among diets. If routing is more flexible, and consumers can use carbon from dietary lipids to build structural tissues, we would expect the isotopic discrimination between dietary protein and consumer tissues to vary with decreasing dietary protein content. We also expected to see a strong relationship between observed variation in $\delta^{13}C_{tissue-diet}$ factors among tissues and tissue-specific variation in the concentration of non-essential amino acids, especially glucogenic forms that are known to have high $\delta^{13}C$ values relative to other amino acids. The final goal of our experiment was to examine the effect of lipid extraction of potential food sources, a commonly used approach in stable isotope ecology, on $\delta^{13}C_{tissue-diet}$ factors.

EXPERIMENTAL

Experimental design

All work was conducted with the approval of the University of Wyoming Animal Care and Use Committee (#A-3216-01). Thirty-eight recently weaned mice (Mus musculus) approximately 3 weeks old (mean starting M_b = 13.9 ± 1.7 g; Charles River Laboratories International, Wilmington, MA, USA) were housed at the University of Wyoming Animal Care Facility. The mice were randomly divided into four diet 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 (28 cm × 42 cm × 22 cm) at 25°C with a 12L:12D photoperiod. We chose to use mice because they thrive in captivity and are capable of eating a wide variety of synthetic diets. In addition, because the mice were recently weaned rapidly growing juveniles, they incorporated dietary components at a much faster rate than adults.[1] This rapid incorporation allowed for a shorter experimental period, without affecting our results. Each of the treatment groups received one of four diets that varied in protein/lipid content and isotopic composition (Table 1). The total dietary content of C_4-based lipids ($\delta^{13}C = -14.5‰$) and C_3-based proteins ($\delta^{13}C = -27‰$) varied inversely between 5% and 40%. The mice were fed ad libitum. Food and water were replaced daily. The mice were also weighed weekly to monitor growth and as a general indicator of health.

Sample collection and stable isotope analysis

The mice were kept on the four diet treatments for 4 months. We chose this time based on the carbon and nitrogen incorporation rates presented by MacAvoy et al.,[17] who determined that the average half-lives of $\delta^{13}C$ and $\delta^{15}N$ in mouse whole blood were 17.3 and 15.4 days, respectively. Consequently, we are confident that the mouse tissues sampled in our experiment had fully incorporated after 4 months. In addition, because the mice were growing

Table 1. Composition (weight percent) and $\delta^{13}C$ values (‰) of experimental diets and dietary components for four experimental diets of varying protein/lipid (p/f) ratios

Ingredient	Treatment #1 4.8L:40.4P	Treatment #2 15.1L:30.1P	Treatment #3 25.1L:20.4P	Treatment #4 39.8L:5.4P	Ingredient $\delta^{13}C$ (±SD)
Brewer's yeast	2.4	2.4	2.4	2.4	−23.5±0.8
USP salt	4.2	4.2	4.2	4.2	−
Vitamin 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	

juveniles, we expected their isotopic incorporation rate to be faster than that for fully grown adults.[1] Following this period, the mice were euthanized using CO_2 asphyxiation, and hair, blood, muscle, liver, and adipose tissue samples were collected. Tissue samples were stored at −20°C until processing. All tissue samples except hair, adipose tissue, and a sample of the diet from each treatment were dried in an oven at 50°C and ground into a fine powder using a mortar and pestle. Hair samples were cut into small segments using surgical scissors. The adipose tissue was freeze-dried (Labconco Freezone 4.5L benchtop freeze dry system; Labconco Kansas City, MO, USA) and ground to a fine powder using a mortar and pestle. A sub-sample of each muscle and liver tissue sample and each diet was soaked in three 24-h treatments of petroleum ether to remove lipids.[7] The weight percent C/N ratios of the lipid-extracted samples were indicative of pure protein.[7] Aliquots (approximately 1 mg) of each processed sample were loaded into tin capsules (Costech Analytical Technologies Inc., Valencia, CA, USA) for $\delta^{13}C$ and $\delta^{15}N$ analysis. The $\delta^{13}C$ and $\delta^{15}N$ values were determined using a Costech 4010 elemental analyzer interfaced with a Delta^{PLUS} XP continuous-flow stable isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the University of Wyoming Stable Isotope Facility (Laramie, WY, USA). The analytical precision (SD) for both $\delta^{13}C$ and $\delta^{15}N$ values was ≤0.2‰ through analysis of beef blood and acetanilide reference materials. Because the lipid material that makes up the main component of adipose tissue contains no nitrogen, adipose tissues were not analyzed for $\delta^{15}N$ values.

Amino acid concentrations

Tissue samples were prepared from two randomly selected mice per diet treatment (8 mice total) for amino acid concentration analysis. Amino acid concentrations were determined by hydrolyzing ~1.0 mg of lipid-extracted tissue in 6 N hydrochloric acid (HCl) at 110°C for 20 h. Then 100 μL of the amino acid hydrolysate was dried in a centrifugal evaporator (Labconco micro centrivap IR) and combined with 100 μL 20 mM HCl. The 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; Eppendorf North America, Hauppauge, NY, USA). Volumes of 10 μL of the redissolved samples were combined with 70 μL of borate buffer (100 mM sodium tetraborate, pH 8.8), and 20 μL of Waters AccQ-Tag reagent (~10 mM 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate in acetonitrile; Waters Corp., Milford, MA, USA) was added. The reactions were mixed, incubated at room temperature for 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 to 100 pmol/μL in the final reaction volume. Derivatized amino acids were analyzed on an Acquity UPLC system (Waters Corp.). 1 μL aliquots were injected on a Waters AccQ-Tag Ultra C18 column (2.1 × 100 mm), run at 0.7 mL/min. at 55°C. The amino acid derivatives were detected at 260 nm in an Acquity system photodiode array detector with a 500 nL flow cell. Peaks were identified, integrated, and quantified with the Waters Empower software package.

Statistical analysis and stable isotope mixing models

Differences in the stable isotope values of like tissues between diet treatments were examined using a two-way analysis of variance (ANOVA) with either the $\delta^{13}C$ value or the $\delta^{15}N$ value as the dependent variable and the diet treatment group as the independent variable. To assess differences in the relationships between the stable isotope values of consumer tissues and those of lipid-extracted and non lipid-extracted diets, we used linear regression F tests with the consumer tissue stable isotope value as the dependent variable and the dietary stable isotope value as the independent variable. Tissue to diet trophic discrimination factors for carbon ($\Delta^{13}C_{tissue-diet}$) and nitrogen ($\Delta^{15}N_{tissue-diet}$) were calculated by subtracting the $\delta^{13}C$ or $\delta^{15}N$ value of the dietary protein or the bulk diet from that of tissues. Significant differences in $\Delta^{13}C_{tissue-diet}$ or $\Delta^{15}N_{tissue-diet}$ factors between tissues were examined using two-way ANOVA and Tukey's HSD with $\Delta^{13}C_{tissue-diet}$ or $\Delta^{15}N_{tissue-diet}$ as the dependent variable and tissue type and diet treatment as independent variables. All statistical analyses were performed using Program JMP 10.0 (The SAS Institute, Cary, NC, USA).

A concentration-dependent mixing model[18] was used to quantify the percentage of carbon in mouse muscle tissue derived from protein and non-protein dietary sources. Model inputs included casein protein ($\delta^{13}C = -27‰$) and non-protein dietary components ($\delta^{13}C = -13‰$). The carbon

isotope value of the non-protein components was estimated by averaging the $\delta^{13}C$ values of dietary carbohydrates (sucrose, $\delta^{13}C = -11.5‰$) and dietary lipids ($\delta^{13}C = -14.5‰$). The model takes the form:

$$\delta^{13}C_{muscle} = \left(\left(f_{protein}*[C]_{protein}\right)\left(\delta^{13}C_{protein} + \Delta^{13}C_{muscle-diet}\right)\right)$$
$$+\left(\left(1-f_{protein}*[C]_{non-protein}\right)\left(\delta^{13}C_{non-protein} + \Delta^{13}C_{muscle-diet}\right)\right),$$

where $f_{protein}$ and $1 - f_{protein}$ are the fractional contributions of protein and non-protein components to mouse muscle, $[C]_{protein}$ and $[C]_{non-protein}$ are the concentrations of carbon in the protein and non-protein dietary sources, and $\Delta^{13}C_{muscle-diet}$ is the trophic discrimination factor, or the difference in the $\delta^{13}C$ values between mouse muscle and diet, which we assumed was 1‰.[2] $[C]_{non-protein}$ was estimated for each diet treatment using a weighted average based on the relative proportions of lipids and carbohydrates in each diet treatment.

RESULTS

During the experimental period, the mice grew an average of 15.30 g ± 8.84 g. The high (diet treatment #1) and low (diet treatment #4) protein diet treatments had the smallest overall gains in mass (8.1 ± 3.6 g and 10.6 ± 9.6 g, respectively). Mice fed diet treatments #2 and #3 had the highest overall mass gains of 21.9 ± 4.3 g and 21.5 ± 6.7 g respectively.

The $\delta^{13}C$ values of mouse tissues increased ~2–6‰ with increasing dietary lipid content (Fig. 1, Table 2). The $\delta^{15}N$ values, in contrast, only increased by ~0.3–0.8‰ with increasing dietary lipid content (Table 2). We detected a significant difference in the $\delta^{13}C$ values of like tissues between treatment groups for all five of our sample tissues (hair $F_{3,25} = 61.76$, $p < 0.001$; blood $F_{3,26} = 113.60$, $p < 0.001$; muscle $F_{3,26} = 37.88$, $p < 0.001$; liver $F_{3,25} = 72.27$, $p < 0.001$; adipose tissue $F_{3,25} = 180.81$, $p < 0.001$ (Fig. 1)). The Tukey's HSD test results are shown in Table 2. We also found significant differences in the $\delta^{15}N$ values of like tissues between treatment groups for three of the four sample tissues analyzed: hair, blood, and muscle (hair $F_{3,26} = 4.60$, $p = 0.01$; blood $F_{3,26} = 5.80$, $p = 0.003$; muscle $F_{3,26} = 4.05$, $p = 0.01$; liver $F_{3,26} = 2.59$, $p = 0.07$). The Tukey's HSD test results are shown in Table 2.

We found a strong significant relationship between the $\delta^{13}C$ value of the bulk diet and that of tissue for all five of our sample tissues (hair $F_{1,27} = 180.57$, $p < 0.0001$; blood $F_{1,28} = 358.16$, $p < 0.0001$; muscle $F_{1,28} = 104.26$, $p < 0.0001$; liver $F_{1,27} = 232.22$, $p < 0.0001$; adipose tissue $F_{1,27} = 46.61$, $p < 0.0001$). In contrast, there was not a significant relationship between the $\delta^{13}C$ value of the lipid-extracted diet

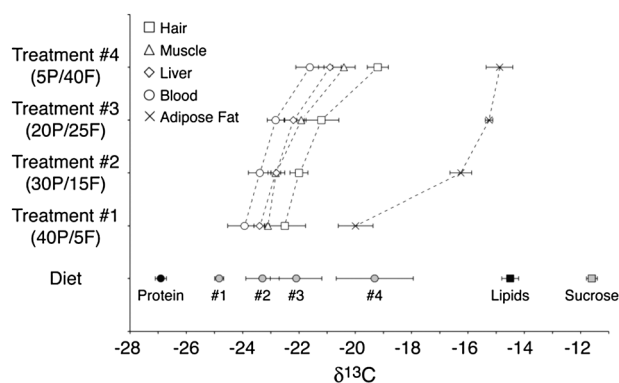**Figure 1.** Mean $\delta^{13}C$ (‰) values of mouse hair, muscle, liver, blood, adipose tissue, and bulk diets (grey circles) and dietary components for the four diet treatments; error bars represent standard error.

Table 2. Diet lipid/protein (L/P) ratio (weight percent), mean $\delta^{13}C$ and $\delta^{15}N$ values (‰) of mouse tissues, and tissue-bulk diet discrimination (‰) values for the four diet treatments

	Treatment #1 4.8L:40.4P	Treatment #2 15.1L:30.1P	Treatment #3 25.1L:20.4P	Treatment #4 39.8L:5.4P
Diet $\delta^{13}C$ ± SD	−24.8 ± 0.2	−23.3 ± 0.6	−22.1 ± 0.9	−19.3 ± 1.4
Hair $\delta^{13}C$ ± SD	−22.5 ± 0.6[A]	−22.0 ± 0.4[AB]	−21.2 ± 0.3[B]	−19.2 ± 0.5[C]
Hair $\Delta^{13}C_{tissue-diet}$	2.3 ± 0.6	1.3 ± 0.7	0.9 ± 0.9	0.1 ± 1.5
Muscle $\delta^{13}C$ ± SD	−23.1 ± 0.7[A]	−22.8 ± 0.3[AB]	−21.9 ± 0.6[B]	−20.4 ± 0.4[C]
Muscle $\Delta^{13}C_{tissue-diet}$	1.7 ± 0.7	0.5 ± 0.7	0.2 ± 1.1	−1.1 ±1.5
Blood $\delta^{13}C$ ± SD	−23.9 ± 0.1[A]	−23.4 ± 0.2[B]	−22.8 ± 0.2[C]	−21.6 ± 0.4[D]
Blood $\Delta^{13}C_{tissue-diet}$	0.9 ±0.2	−0.1 ± 0.6	−0.7 ± 0.9	−2.3 ± 1.5
Liver $\delta^{13}C$ ± SD	−23.4 ± 0.2[A]	−22.8 ± 0.3[B]	−22.2 ± 0.3[B]	−20.9 ± 0.4[C]
Liver $\Delta^{13}C_{tissue-diet}$	1.4 ± 0.3	0.5 ± 0.7	−0.1 ± 0.9	−1.6 ± 1.5
Diet $\delta^{15}N$ ± SD	5.7 ± 0.2	5.6 ± 0.0	5.6 ± 0.2	5.6 ± 0.2
Hair $\delta^{15}N$ ± SD	9.5 ± 0.4[A]	8.9 ± 0.3[AB]	9.1 ± 0.3[AB]	8.7 ± 0.4[B]
Hair $\Delta^{15}N_{tissue-diet}$	3.8 ± 0.4	3.3 ± 0.3	3.5 ± 0.4	3.1 ± 0.4
Muscle $\delta^{15}N$ ± SD	8.9 ± 0.3[A]	9.3 ± 0.4[AB]	9.5 ± 0.3[AB]	9.6 ± 0.4[B]
Muscle $\Delta^{15}N_{tissue-diet}$	3.3 ± 0.4	3.7 ± 0.4	3.9 ± 0.4	4.0 ± 0.4
Blood $\delta^{15}N$ ± SD	8.5 ± 0.1[A]	8.6 ± 0.2[AB]	8.9 ± 0.3[AB]	9.1 ± 0.5[B]
Blood $\Delta^{15}N_{tissue-diet}$	2.8 ± 0.2	3.0 ± 0.2	3.3 ± 0.4	3.4 ± 0.5
Liver $\delta^{15}N$ ± SD	9.9 ± 0.2[A]	9.7 ± 0.2[A]	10.0 ± 0.5[A]	10.0 ± 0.4[A]
Liver $\Delta^{15}N_{tissue-diet}$	4.2 ± 0.3	4.1 ± 0.2	4.4 ± 0.5	4.4 ± 0.4

Mean values with different superscripted letters are significantly different.

and those of any of the sample tissues except adipose tissue (hair $F_{1,28} = 1.29$, $p = 0.26$; blood $F_{1,28} = 2.31$, $p = 0.14$; muscle $F_{1,28} = 1.63$, $p = 0.21$; liver $F_{1,28} = 2.49$, $p = 0.13$; adipose tissue $F_{1,27} = 19.86$, $p = 0.0001$). Significant relationships also existed between the $\delta^{15}N$ values of the bulk diet and those of tissue for three of the four tissues analyzed (hair $F_{1,28} = 4.76$, $p = 0.04$; blood $F_{1,28} = 13.93$, $p = 0.0009$; muscle $F_{1,28} = 11.96$, $p = 0.002$; liver $F_{1,28} = 2.26$, $p = 0.14$). There was a significant relationship between the $\delta^{15}N$ values of the lipid-extracted diet and those of hair but not for any other tissue (hair $F_{1,28} = 9.21$, $p = 0.005$; blood $F_{1,28} = 0.17$, $p = 0.68$; muscle $F_{1,28} = 1.69$, $p = 0.002$; liver $F_{1,28} = 1.33$, $p = 0.26$).

For comparisons between tissues and bulk diets, the $\Delta^{13}C_{tissue-diet}$ factors differed between both tissues and diet treatments (Fig. 2). In general, hair had the largest mean $\Delta^{13}C_{tissue-diet}$ factor, followed by muscle, liver, and blood (Table 2). The $\Delta^{13}C_{tissue-diet}$ factors were far greater and displayed greater variation between tissues and diet treatments for comparisons made directly with lipid-extracted diets (i.e. dietary protein) than with bulk diets (Fig. 2, Table 2). There were significant differences in the tissue-specific $\Delta^{13}C_{tissue-diet}$ factor when it was calculated using lipid-extracted diet $\delta^{13}C$ values versus bulk diet $\delta^{13}C$ values for all diet treatment groups (paired $T_3 = 0.03$ for all tissues). The $\Delta^{15}N_{tissue-diet}$ factor for bulk diets also differed between both tissues and diet treatments; however, these differences were much smaller than those observed for $\Delta^{13}C_{tissue-diet}$. Liver had the largest mean ($\pm$SD) $\Delta^{15}N_{tissue-diet}$ factor, followed by muscle, hair, and blood (Table 2). Since lipids contain negligible amounts of nitrogen and the major source of dietary nitrogen is in the form of protein, the $\Delta^{15}N_{tissue-diet}$ factors were similar when calculated with dietary protein versus bulk diet $\delta^{15}N$ values.

For mouse hair, muscle, liver, and blood, the concentrations of alanine, glycine, serine, glutamic acid, aspartic acid, arginine, proline, tyrosine, leucine, isoleucine, threonine, phenylalanine, lysine, and histidine differed between tissue types (Fig. 3). For all tissues, ~56–70% of the amino acids were non-essential while the other amino acids in these tissues were essential (Fig. 3).
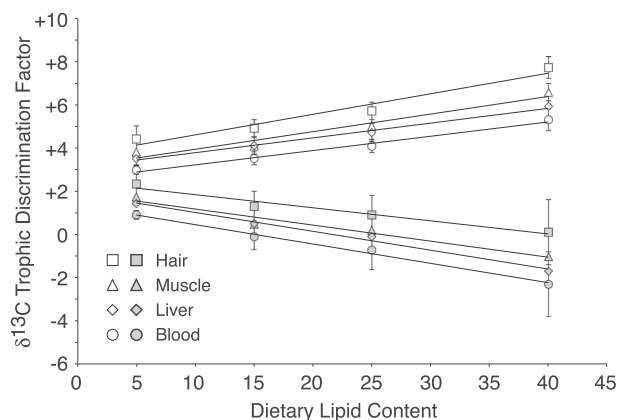

Figure 2. $\delta^{13}C$ trophic discrimination factors ($\Delta^{13}C_{diet-tissue}$) for comparisons between mouse tissues and dietary protein (open symbols) or bulk diet (shaded symbols); error bars represent standard error.

DISCUSSION

The differences in $\Delta^{13}C_{tissue-diet}$ factors that we observed among diet treatments with different protein/lipid ratios suggest that mice can use both dietary protein and lipids for tissue synthesis (Fig. 2). If protein components were routed directly to structural tissues and lipids were used solely to fuel metabolism, we would expect $\delta^{13}C$ discrimination between dietary protein and consumer tissues to remain constant between diet treatments with different protein/lipid contents. Instead, the $\delta^{13}C$ offset between mouse tissues and that of dietary protein ($\Delta^{13}C_{tissue-protein}$) increased with increasing dietary lipid content (open symbols, Fig. 2). In contrast, the $\delta^{13}C$ offset between mouse tissues and the bulk diet ($\Delta^{13}C_{tissue-bulk\ diet}$) decreased with increasing dietary lipid content (shaded symbols, Fig. 2). Furthermore, the shift in the $\delta^{13}C$ values with decreasing dietary protein suggests that mice fed low (5%) and intermediately low (15%) protein diets used some amount of lipid-derived carbon to synthesize proteinaceous tissues.

Carbon from the fatty acids in dietary lipids can be used to synthesize amino acids, which are the molecular building blocks of structural tissues, via beta-oxidation.[19] In this process, fatty acids from lipids are broken down into acetyl-CoA that can enter the Krebs cycle and serve as a precursor for the synthesis of ketogenic non-essential amino acids including aspartate, glutamate, arginine, and proline. A second metabolic pathway in which lipid-derived carbon can be used to synthesize non-essential amino acids is gluconeogenesis, specifically the conversion of glycerol into glucose. Glucose is the first intermediary of glycolysis, a process that produces several intermediaries (3-phosphoglycerate and pyruvate) from which the glucogenic amino acids, alanine, glycine, and serine, are derived. Several of these ketogenic and glucogenic non-essential amino acids are important components in structural tissues such as muscle, hair, and liver (Fig. 3). Obviously, tissues with a larger proportion of non-essential amino acids have a greater potential to include carbon that is originally derived from dietary lipid or carbohydrate sources. Non-essential amino acids represent the majority (53–71%) of the total amino acid content of the tissues analyzed here (Fig. 3), and in most ecological studies[20] diets that contain a high proportion of non-protein macromolecules are expected to affect consumer tissue $\delta^{13}C$ values and the corresponding $\Delta^{13}C_{tissue-diet}$ factors.

We recognize that the carbohydrates (sucrose) included in our diet treatments were also a potential source of carbon used to synthesize mouse tissues. Because of the similarity in the $\delta^{13}C$ values of carbohydrates and lipids in our diet treatments, it is impossible to quantify the relative contribution of carbon from lipids versus that from carbohydrates to mouse tissues. However, our concentration-dependent mixing model provides an estimate. For the low-lipid (5%) diet treatment, our mixing model shows that ~20% of the carbon in mouse muscle is derived from non-protein dietary sources. Given that carbohydrates represent the majority of the non-protein component in the low-lipid diet, we expect a significant portion of the non-protein-derived carbon in tissues to be sourced from dietary carbohydrates. Conversely, in the high-lipid (40%) diet treatment, the mixing model estimates that ~60% of the carbon in mouse muscle tissue is derived from non-protein sources. In
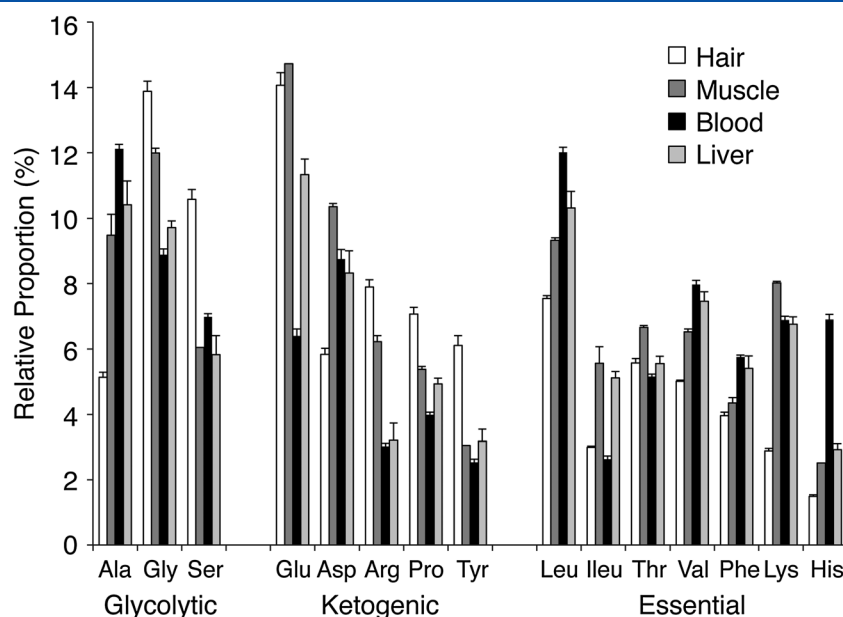
Figure 3. Relative mean proportions of alanine, glycine, serine, glutamine, aspartic acid, proline, tyrosine, leucine, isoleucine, threonine, valine, phenylalanine, lysine, and histidine in mouse hair, muscle, liver, and blood. Relative mean proportions represent tissues from eight randomly selected mice (two mice per diet treatment); error bars represent standard error.

this diet treatment, lipids account for ~55% (by weight) of the non-protein dietary carbon. Consequently, we expect most of the non-protein-derived carbon in the tissues of mice in the high-lipid diet treatment to be sourced from dietary lipids. In addition, lipids have a much higher concentration of carbon than carbohydrates – 75% and 40%, respectively; thus, lipid carbon may be more available for the synthesis of non-essential amino acids via beta-oxidation and gluconeogenesis. Lastly, amino acid $\delta^{13}C$ analysis of mouse muscle from our experiment showed that ketogenic amino acids were more sensitive to dietary lipid content than glucogenic forms, suggesting that the breakdown of lipids into acetyl-CoA via beta-oxidation was the primary pathway for the incorporation of lipid-derived carbon into non-essential amino acids in mouse muscle;[16] we expect that other mouse tissues will show similar patterns. While we recognize that some portion of the non-protein-derived carbon in mouse tissues was probably derived from dietary carbohydrates, we do not believe that this negates our interpretation of the routing of dietary lipid carbon into the structural tissues of mice.

Lastly, we observed an increase in the $\delta^{13}C$ offset between dietary lipids and mouse adipose tissue with increasing dietary protein content (Fig. 1). We expected mice to use carbon from dietary carbohydrates to synthesize fatty acids found in adipose fat tissue. Contrary to this expectation, our results show a decrease in adipose fat $\delta^{13}C$ values with increasing dietary protein content, suggesting that mice were using carbon from dietary proteins to synthesize fatty acids when fed diets high in protein content (40%). While carbon from protein could be used for lipogenesis when ketogenic or glucogenic amino acids are converted into acetyl CoA, a precursor for fatty acid synthesis, this phenomenon is not well understood and deserves further investigation.

Effects of tissue amino acid composition on consumer $\delta^{13}C$ values

Since the $\delta^{13}C$ values of individual amino acids in a single tissue can vary by over 20‰,[16,21–24] variation in the concentration of amino acids among tissues[25] probably influences the bulk tissue $\delta^{13}C$ values, and consequently drives variation in $\delta^{13}C$ trophic discrimination factors ($\Delta^{13}C_{tissue-diet}$) among tissues. Based on the hypothesis that amino acid composition is, at least in part, responsible for a tissue's isotopic composition, we would expect differences in the $\delta^{13}C$ values of tissues to be consistent among diet treatments in our experiment – a prediction supported by our results (Fig. 1). Correspondingly, differences in the $\delta^{13}C$ value and consequent $\Delta^{13}C_{tissue-diet}$ of the same tissues in varying diet treatments are the result of differences in the macromolecular origin of the dietary carbon in the non-essential amino acids of mouse tissues. Unfortunately, current analytical limitations preclude us from measuring a $\delta^{13}C$ value for every amino acid in a tissue, and thus prohibit the use of a mixing model to better quantify these predictions.

To our knowledge, no one has systematically examined the relationship between tissue amino acid composition and $\Delta^{13}C_{tissue-diet}$ factors for mammals; however, several studies have reported patterns in the amino acid $\delta^{13}C$ values that suggest a link between tissue amino acid composition and bulk tissue $\delta^{13}C$ values.[16,21–24] Specifically, tissues with larger concentrations of serine and glycine, two non-essential glucogenic amino acids, tend to have larger $\Delta^{13}C_{tissue-diet}$ factors than tissues with smaller concentrations of these forms. In experiments examining the differences in the $\delta^{13}C$ values of individual amino acids in the diet and bone collagen of pigs (*Sus scrofa*), both Hare *et al.*[21] and Howland *et al.*[23] found that serine and glycine had higher $\delta^{13}C$ values

thanany other non-essential amino acid analyzed. Our results are in agreement with this pattern. Mouse hair had significantly higher amounts of serine and glycine and correspondingly larger $\Delta^{13}C_{tissue-diet}$ factors (Fig. 3) than any other tissue that we examined (Table 2).

Implications for isotopic studies of animal diets

The flexibility in the metabolic routing of dietary macromolecules observed in our experiment has important implications for nutrient assimilation in animals and for the utility of stable isotope analysis to study foraging ecology. Because animals frequently choose dietary resources based on nutritional needs,[26–28] the ability to use a variety of dietary macromolecules for fueling energetic demands and constructing structural tissues may translate into increased flexibility in the types of resources that animals can consume. This increased flexibility might allow consumers to survive on diets with lower macromolecular diversity during periods of decreased resource availability, such as winter or during migration.[29] The ability to consume a more limited diversity of dietary items may also help to explain how strictly carnivorous animals, particularly those that consume diets with high lipid/protein ratios such as marine mammals and seabirds, are able meet metabolic demands and maintain/grow tissues while consuming lipid-rich diets that contain little to no carbohydrates.[28,30–33] For example, many species of forage fish, such as herring (*Clupea* spp.) and eulachon (*Thaleichthys pacificus*) that are important food resources to marine predators, contain 25–50% lipids.[33–36] Many terrestrial animals also make use of lipid-rich dietary resources. For example, some commonly consumed insect larvae, such as tebo worms (*Chilecomadia moorei*), contain almost twice as much lipid as crude protein.[35] Likewise, several types of fruits frequently eaten by migrating passerines are comprised of 24–50% lipids.[36] Darimont *et al.*[37] noted that gray wolves (*Canis lupus*) in British Columbia, Canada, preferentially consumed the lipid-rich head portions of spawning salmon (*Oncorhunchus* spp.). Similarly, Mayntz *et al.*[38] found that, when provided with two types of food with varying protein and lipid contents, mink (*Mustela vision*) selectively foraged on the food with a high lipid/protein ratio of ~1.5:1.

The increased flexibility in the metabolic routing of dietary macromolecules demonstrated by our study suggests that animal ecologists who use stable isotope analysis to examine diet should consider both (a) the isotopic composition of all the major macromolecular constituents (protein, lipids, or carbohydrates) of potential food sources, not simply those of dietary protein, and (b) that variation in the metabolic routing of protein versus lipids and carbohydrates can shift with changes in dietary macromolecular content. Ignoring the variation in metabolic routing and macromolecular composition of an animal's diet may result in inaccurate estimates of $\Delta^{13}C_{tissue-diet}$ factors and, consequently, quantification of relative resource use via mixing models. Newsome *et al.*[39] noted that this was the case in California sea otters (*Enhydra lutris nereis*), for which the $\Delta^{13}C_{tissue-diet}$ factors decreased with increased consumption of lipid-rich and ^{13}C-depleted red sea urchins (*Strongylocentrotus fransciscanus*). Likewise, Cherry *et al.*[15] observed that smaller prey (ringed seals) characterized by higher lipid/protein contents made a disproportionate contribution to the $\delta^{13}C$ values

of polar bear tissue proteins compared with larger prey sources that had lower amounts of lipid relative to protein. While considerations of the metabolic routing of protein versus carbohydrates in omnivorous animals have been at the forefront of stable isotope based investigations of foraging ecology,[40,41] our research demonstrates that similar considerations should be given to animals that routinely consume lipid-rich diets.

It is important to note, however, that the influence of lipid content on metabolic routing is likely to be less dramatic in wild animals than in our experiment. The $\delta^{13}C$ values of the protein and lipids in our experimental diets differed by ~13‰, a difference larger than has been observed between these dietary macromolecules in wild diets. For example, Cherry *et al.*[15] found a mean difference of ~7‰ between the $\delta^{13}C$ values of lean muscle (protein) and blubber (lipids) from pinnipeds and cetaceans consumed by polar bears; other studies have reported similar offsets in $\delta^{13}C$ values between protein and lipids.[39,42] Despite the smaller offset in $\delta^{13}C$ values between dietary protein and lipid components in wild settings, the impact of lipid content on metabolic routing can still be significant in wild populations. For example, using $\delta^{13}C$ analysis, Cherry *et al.*[15] found that ringed seals contributed 13–16% to the diet of male polar bears when only considering dietary protein as a carbon source. The range of this estimate increased to 15–44% when considering both dietary protein and lipids as potential carbon sources to polar bear tissues. Furthermore, this study concluded that failure to consider both protein and lipid dietary sources to polar bears could result in overestimating the importance of some food sources and underestimating or entirely excluding other potentially important foods.

We also found significant differences in $\Delta^{13}C_{tissue-diet}$ factors for all diet treatments when using dietary protein versus bulk diet as the source of dietary carbon (Fig. 2 and Table 2). While the low-lipid dietary treatment only contained 5% fat, we observed a ~2.1‰ difference in the $\Delta^{13}C_{tissue-diet}$ factors calculated using dietary protein versus bulk diet $\delta^{13}C$ values (Table 2). Consequently, we advocate that future studies using stable isotope analysis to investigate animal foraging ecology should not automatically remove lipids from prey samples, but instead assess the lipid content of potential prey types, which can be qualitatively accomplished through the analysis of weight percent carbon/nitrogen concentrations ([C]/[N]). Increases in $\Delta^{13}C_{tissue-diet}$ factors that are estimated when using prey that has been lipid-extracted will result in unreliable estimates of diet composition for wild animals that consume lipid-rich diets. In addition, because bulk diets have a higher [C]/[N] ratio than diets from which the lipids have been extracted, concentration-dependent mixing models populated with [C] and [N] values from bulk prey items will result in more accurate estimates of dietary inputs for animals with high-lipid-content diets than will models that have been populated with values from lipid-extracted diet sources.[1]

Variation in $\Delta^{15}N_{tissue-diet}$ factors among tissues and diet treatment

The variations in the $\Delta^{15}N_{tissue-diet}$ factors for liver, muscle, blood, and hair tissues were small, but statistically significant. These discrimination factors might reflect the metabolism of nitrogen and amino acids under protein-limited to protein-

replete conditions. In all four of our diet treatments, the liver tissue $\delta^{15}N$ values were invariant, but liver had the greatest $\Delta^{15}N_{tissue-diet}$ factor of any of the measured tissues. This is probably the result of the function of a vertebrate's liver as an excretory organ where the urea cycle occurs. The $\Delta^{15}N_{tissue-diet}$ of blood was the lowest of any of the measured tissues across all four treatments. The blood $\Delta^{15}N_{tissue-diet}$ factors increased as lipids became the predominant carbon and energy source, and proteins became limited. In diet treatments containing high lipid contents, amino acids synthesized from lipids are probably aminated from a nitrogen supply increasingly affected by excretion. Patterns in the $\Delta^{15}N_{tissue-diet}$ factors of muscle among diet treatments were similar to that of blood. Conversely, the hair $\Delta^{15}N_{tissue-diet}$ factors decreased with lipid content in the diet. We suspect that this is the result of the large concentrations of glycine and serine in hair, two 'source' amino acids with $\delta^{15}N$ values similar to those of their dietary counterparts.[43] In addition, because mice needed to synthesize more of their non-essential (for carbon) and trophic (for nitrogen) amino acids in diet treatments with low protein and high lipid contents, the $\delta^{15}N$ values of trophic amino acids may not have the characteristic elevated values previously measured in most vertebrates.[44–46] The interplay of nitrogen cycling and carbon sources for animal physiology measured in this experiment highlights the need for further experimentation in this field.

It should be noted that rapidly growing individuals tend to have lower $\Delta^{15}N_{tissue-diet}$ factors than non-growing individuals.[10] This decrease in nitrogen isotope discrimination is the result of the amplified demand for nitrogen to satisfy the requirements for tissue synthesis in growing animals.[10] No similar phenomenon has been observed for carbon. Because we used growing juvenile mice in our study, one might expect that the $\Delta^{15}N_{tissue-diet}$ factors for mice in our experiment would be lower than those for adult mice. The $\Delta^{15}N_{tissue-diet}$ factors estimated from our experiment, however, are within the typical range (3–4‰) previously estimated for non-growing adult mammals.[47,48]

CONCLUSIONS

Our results provide further evidence for the influence of dietary macromolecular composition on metabolic routing. Thus it is important that dietary composition be considered when designing experiments or interpreting results from isotope-based studies of foraging ecology in wild animals, particularly those focused on carnivorous and omnivorous species that routinely consume lipid-rich diets. Our results also show significant differences in $\Delta^{13}C_{diet-tissue}$ factors when using dietary protein $\delta^{13}C$ values versus that of bulk diet in all our diet treatments (Fig. 2). Consequently, we advocate that future studies using stable isotope analysis to investigate animal foraging ecology should not automatically remove lipids from prey items, but, instead, assess the lipid content of different prey types and use concentration-dependent mixing models that account for variation in both the isotopic composition and the stoichiometry of potential sources of food.

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