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Dietary protein content and digestibility influences discrimination of amino acid nitrogen isotope values in a terrestrial omnivorous mammal

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Short Title

Influence of diet on stable nitrogen isotopes of amino acids in animals

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Abstract

RATIONALE Ecologists increasingly determine the $\delta^{15}N$ values of amino acids (AA) in animal tissue; "source" AA typically exhibit minor variation between diet and consumer, while "trophic" AA have increased $\delta^{15}N$ values in consumers. Thus, trophic-source $\delta^{15}N$ offsets (i.e., $\Delta^{15}N_{T\text{-}S}$) reflect trophic position in a food web. However, even minor variation in $\delta^{15}N_{source\ AA}$ values may influence the magnitude of offset that represents a trophic step, known as the trophic discrimination factor (i.e., $TDF_{T\text{-}S}$). Diet digestibility and protein content can influence the $\delta^{15}N$ values of bulk animal tissue, but the effects on AA $\Delta^{15}N_{T\text{-}S}$ and $TDF_{T\text{-}S}$ in mammals are unknown.

METHODS We fed captive mice (*Mus musculus*) either (A) a low-fat, high-fiber diet with low, intermediate, or high protein; or (B) a high-fat, low-fiber diet with low or intermediate protein. Mouse muscle and dietary protein were analyzed for bulk tissue $\delta^{15}N$ using elemental analyzer-isotope ratio mass spectrometry (EA-IRMS), and were also hydrolyzed into free AA that were analyzed for $\delta^{15}N$ using EA-IRMS.

RESULTS As dietary protein increased, $\Delta^{15}N_{Consumer\text{-}Diet}$ slightly declined for bulk muscle tissue in both experiments, increased for AA in the low-fat, high-fiber diet (A), and remained the same or decreased for AA in the high-fat, low-fiber diet (B). The effects of dietary protein on $\Delta^{15}N_{T\text{-}S}$ and on $TDF_{T\text{-}S}$ varied by AA but were consistent between variables.

CONCLUSIONS Diets were less digestible and included more protein in Experiment A than in Experiment B. As a result, the mice in Experiment A probably oxidized more AA, resulting in greater $\Delta^{15}N_{Consumer\text{-}Diet}$ values. However, the similar responses of $\Delta^{15}N_{T\text{-}S}$ and of $TDF_{T\text{-}S}$ to diet variation suggest that if diet samples are available, $\Delta^{15}N_{T\text{-}S}$ accurately tracks trophic position. If diet samples are not available, the patterns presented here provide a basis to interpret $\Delta^{15}N_{T\text{-}S}$ values The trophic-source offset of Pro-Lys did not vary across diets, and therefore may be more reliable for omnivores than other offsets (e.g., Glu-Phe).

Keywords

carbohydrates, compound-specific isotope analysis (CSIA), energy, fractionation

Introduction

Trophic position is a critical trait describing ecological interactions, but it is difficult to assess in wild animals. Nitrogen isotope ($\delta^{15}N$) analysis of bulk tissues is often used to estimate trophic position because consumer $\delta^{15}N$ values are systematically higher than those of their diet [1,2] (Post 2002; Vanderklift and Ponsard 2003), a difference often referred to as a trophic discrimination factor (TDF), denoted by $\Delta^{15}N_{Consumer\text{-}Diet}$. Accurately identifying trophic position from bulk tissue isotope analysis, however, requires sampling all potential diet items or constraining the food web baseline $\delta^{15}N$ value, which is often not feasible. In addition, the TDF can vary with dietary protein content and quality, tissue type, and the nitrogen excretion pathway used by the consumer [2,3,4], potentially obscuring ecological inferences about trophic position and food chain length [5].

To improve trophic position estimates, ecologists increasingly rely on $\delta^{15}N$ analysis of individual amino acids (AA), which are the building blocks of the protein-rich tissues that are often collected for isotope-based studies [6,7,8]. "Source" AA tend to retain their nitrogen atoms when they are digested and assimilated by consumers, and as a result these AA have similar $\delta^{15}N$ values across primary producers and consumers in a food web. In contrast, "trophic" AA are prone to replace their nitrogen atoms during the biochemical processing that occurs during nutrient assimilation, and these AA tend to increase in $\delta^{15}N$ value with increasing trophic position [9]. Both source and trophic AA can be broken down completely when catabolized for energy or when used to fuel metabolic pathways such as gluconeogenesis. The difference between trophic and source AA ($\Delta^{15}N_{T\text{-}S}$) in consumer tissue is therefore expected to follow a general trend: greater values of $\Delta^{15}N_{T\text{-}S}$ indicate higher trophic position within a food web.

The ability to assess trophic relationships based only on analysis of consumer tissues is a key purported advantage of this approach. The interpretation of $\Delta^{15}N_{T\text{-}S}$, however, implicitly relies upon information from the diet. This reliance is apparent in the equations that are used to quantify trophic position based on AA $\delta^{15}N$ values [7]:

$$TP = 1 + [(\delta^{15}N_T - \delta^{15}N_S - \beta) \times TDF_{T\text{-}S}^{-1}] \quad (1)$$

where TP is the trophic position, $\delta^{15}N_T$ and $\delta^{15}N_S$ are consumer tissue values for individual trophic and source AA, β is the offset between $\delta^{15}N_T$ and $\delta^{15}N_S$ in primary producer tissue at the base of the food web in which the consumer is feeding, and $TDF_{T\text{-}S}$ represents the offset in $\delta^{15}N$ values that occurs with a single trophic step [7]. To fully account for isotopic differences between consumers and their diet, which are expected to be substantial for trophic AA and minor for source AA, $TDF_{T\text{-}S}$ is defined as:

$$TDF_{T\text{-}S} = (\text{Consumer } \delta^{15}N_T - \text{Diet } \delta^{15}N_T) - (\text{Consumer } \delta^{15}N_S - \text{Diet } \delta^{15}N_S) \quad (2)$$

Several key factors influence the terms in equations 1 and 2. The most commonly used trophic-source AA pair is Glu-Phe, although Pro (trophic) and Lys (source) have been suggested as alternatives [7,8]. Similar to bulk tissue analysis, values of $TDF_{T\text{-}S}$ appear to be influenced by dietary protein quantity and quality [10,11,12], diet digestibility [12], nitrogen excretion pathway [7], physiological status [13], and consumer tissue type [7]. The $TDF_{T\text{-}S}$ generally declines as protein quality increases, probably because more dietary AA are routed into consumer tissue, while *de novo* synthesis of AA by the consumer is reduced [10]; note that protein quality is often defined as similarity in AA composition between consumer and diet. Some studies suggest that increasing dietary protein content has a similar effect as increasing protein quality and also leads to reduced $TDF_{T\text{-}S}$ [10,12]; however, higher dietary protein content could instead lead to increases in $TDF_{T\text{-}S}$, because of greater oxidation of AA for energy [14,11]. The oxidation of AA for energy can also be influenced by the non-protein components of the

diet; for example, in humans the feedback loops that regulate energy metabolism are sensitive to the relative proportions of dietary carbohydrates to lipids [15].

Our understanding of $TDF_{T\text{-}S}$ values remains incomplete, despite the critical role of this variable when using $\delta^{15}N_{AA}$ values to estimate the trophic position of free-ranging animals [7,8]. In particular, there are no empirical estimates of the effect of dietary protein content on $TDF_{T\text{-}S}$ in mammals, and on the subsequent calculations of trophic position. This information is especially important for studying omnivores that consume diets which can vary widely in protein content and quality across space and time [16]. Here, in two separate controlled feeding experiments, we reared captive house mice (*Mus musculus*) on diets with protein contents ranging from 0.05 to 0.37 by mass. The protein quality was held nearly constant by consistently using the same primary protein source (casein) across treatments and experiments. The carbohydrate, lipid, and non-digestible contents also varied across experimental diets. We hypothesized that the effect of protein content on $\delta^{15}N$ values in mouse tissue would vary among AA. We predicted that the difference in AA $\delta^{15}N$ values between mouse muscle and dietary protein ($\Delta^{15}N_{Consumer\text{-}Diet}$) would remain constant for source AA, while $\Delta^{15}N_{Consumer\text{-}Diet}$ would decline with increasing dietary protein for trophic AA. These predictions reflect the biochemical mechanisms that distinguish source and trophic AA, and address the empirical basis that is currently lacking for estimating trophic position of wild terrestrial omnivorous mammals.

Methods

Experimental Design

We conducted two feeding experiments (A and B) with captive mice to assess the effects of protein content on AA $\Delta^{15}N_{Consumer\text{-}Diet}$. Weanling mice (Charles River Laboratories, Wilmington, MA, USA) were housed communally by diet treatment (see below) in 18×12-

inch plastic containers with a constant temperature of ~22˚C and a 12h light photoperiod at the University of New Mexico (UNM) Animal Research Facility (Albuquerque, NM, USA). We used cellulose as a source of undigestible fiber [17]. Water was provided *ad libitum*. Mice were euthanized via CO_2 exposure after 120 days (Experiment A) or 112 days (Experiment B) and mouse skeletal muscle (A: quadriceps femoris; B: biceps femoris) was excised and stored at -20°C; this duration was ample time for nitrogen isotope turnover in muscle tissue [18]. All animal handling and husbandry procedures were conducted with the approval of the UNM Institutional Animal Care and Use Committee (A: 13-1010131-MC; B: 16-200492-MC).

The results reported here for Experiment A represent additional analyses of samples first described in Rodriguez Curras et al [19] (2018). Specifically, thirty-four mice (each offered 10 g food day^{-1}) were divided into three diet treatments that varied in casein content: A-L for low protein, A-I for intermediate protein, and A-H for high protein (Table 1). The protein categories were based on the recommended protein content of ~0.20 by mass for mice [17]. The protein composition across these diets was mostly casein (A-L, 0.71; A-I, 0.91; A-H, 0.95), with small contributions from protein in cornmeal (A-L, 0.17; A-I, 0.05; A-H, 0.03) and protein in yeast (A-L, 0.11; A-I, 0.04; A-H, 0.02). To calculate the $\delta^{15}N$ values for the A-L, A-I, and A-H diets, we measured the $\delta^{15}N$ value of each protein source and used a linear mixing model (Table 1):

$$\delta^{15}N_{Diet} = [Casein]\times\delta^{15}N_{Casein} + [Cornmeal]\times\delta^{15}N_{Cornmeal} + [Yeast]\times\delta^{15}N_{Yeast} \quad (3)$$

We also measured the $\delta^{15}N$ value of each individual AA in each protein source, and then used a mixing model that included the concentration and $\delta^{15}N$ value of each AA in each protein source to calculate the overall dietary $\delta^{15}N_{AA}$ value:

$$\delta^{15}N_{AA} = [Cas.]\times[AA_{Cas.}]\times\delta^{15}N_{AA\text{-}Cas.} + [Corn.]\times[AA_{Corn.}]\times\delta^{15}N_{AA\text{-}Corn.} + [Y.]\times[AA_{Y.}]\times\delta^{15}N_{AA\text{-}Y.} \quad (4)$$

Body mass was recorded weekly throughout Experiment A.

The results reported here for Experiment B represent additional analyses of the samples first described in Hughes et al [4]. Fifteen mice (each offered 8–10 g food day^{-1}) were divided into two diet treatments that varied in casein content, which was the sole protein source: B-L for low protein and B-I for intermediate protein (Table 1). One week before euthanasia in Experiment B, the body condition was measured with an EchoMRI Quantitative Magnetic Resonance system (Echo Medical Systems, Houston, TX, USA). Once the system was calibrated with reference materials, an animal was restrained in a plexiglass tube and inserted for analysis. The resulting measurements of body fat and lean mass were converted to percentage body fat and lean mass [20,4].

Bulk Tissue Stable Isotope Analysis

For Experiments A and B, muscle was lipid-extracted by soaking samples for 72 hours in a 2:1 chloroform:methanol solution, replacing the solvent solution every 24 hours [4,19]. Samples were then rinsed thoroughly with deionized water and freeze-dried. Approximately 0.5–0.6 mg of muscle or diet ingredient was sealed into a 3×5-mm tin capsule and the $\delta^{15}N$ values were measured with a Costech (Valencia, CA, USA) 4010 elemental analyzer coupled to a Thermo Scientific (Waltham, MA, USA) Delta V Plus isotope ratio mass spectrometer at the University of New Mexico Center for Stable Isotopes (UNM-CSI; Albuquerque, NM, USA). Within-run analytical precision (SD) of ±0.2‰ for $\delta^{15}N$ values was determined via analysis of two protein-based internal reference materials. We also measured the weight percentage nitrogen ([N]) concentrations of each sample via analysis of organic materials with known elemental concentrations.

Amino Acid $\delta^{15}N$ Analysis

Samples of diet ingredients (casein, cornmeal, brewer's yeast) and mouse muscle were analyzed for bulk and AA-specific $\delta^{15}N$ values. Measurements of AA-specific $\delta^{15}N$ values for mouse muscle in Experiment A were conducted at the University of California (UC) Life and Environmental Sciences Unit, School of Natural Sciences, (Merced, CA, USA). All other measurements were conducted at the UNM-CSI. The analytical methods (e.g., derivatization protocols), instrumentation, and internal reference materials were identical in both laboratories. For all $\delta^{15}N_{AA}$ analyses, 5–20mg of each sample was hydrolyzed in 1 mL of 6N HCl for 20 hours at 110°C. During hydrolysis, glutamine was converted to glutamic acid and asparagine was converted to aspartic acid. For cornmeal, the hydrolysate was then passed through a cation exchange column containing DOWEX 50WX8 100-200 mesh resin to isolate AA from carbohydrates [21] (Amelung and Zhang 2001). The reference materials were a custom solution of pure powdered amino acids (Sigma Aldrich, St. Louis, MO, USA) that had previously been individually measured for $\delta^{15}N$ using a Costech 4010 elemental analyzer coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer. This reference material was used in both the UC and the UNM laboratories. All hydrolyzed samples, and the internal reference materials, were dried under a stream of N_2 gas then derivatized to N-trifluoroacetic acid isopropyl esters and resuspended in dichloromethane [22,8]. Aliquots (1µL) of derivatized samples were injected in triplicate into a Thermo Scientific Trace 1310 gas chromatograph containing a 60m BPx5 column (0.32mm ID, 1.0µm film thickness; SGE Analytical Science, Ringwood, Australia). The separated AA were then reduced to N_2 in a Thermo Scientific GC Isolink II and analyzed on a Thermo Scientific Delta V Plus isotope ratio mass spectrometer.

This method yields $\delta^{15}N$ values of seven trophic AA (Ala, Asp, Glu, Ile, Leu, Pro, Val), three source AA (Phe, Tyr, Lys), and three AA which do not fall into either category (Gly, Ser, Thr). The AA $\delta^{15}N$ values were corrected with the equation:

$$\delta^{15}N_{sample.underiv} = (\delta^{15}N_{sample.deriv} + (\delta^{15}N_{ref.deriv} - \delta^{15}N_{ref.underiv}) \quad (5)$$

where $\delta^{15}N_{sample.underiv}$ is the corrected value of the amino acid, $\delta^{15}N_{sample.deriv}$ is the measured value of the derivatized amino acid; $\delta^{15}N_{ref.deriv}$ is the measured value of the derivatized amino acid in the reference material; and $\delta^{15}N_{ref.underiv}$ is the measured value of the un-derivatized amino acid in the reference material. For AA $\delta^{15}N$ measurements conducted at both analytical facilities, the SD for multiple injections of the same sample or of the same reference material averaged < 1.0‰ (range 0.1–1.9‰).

Statistical Analysis

Program R was used for most statistical analyses [23], including the *boot* package [24]. Within experiments we compared the change in body mass (and in Experiment B only, body fat content) during the experiment (i.e., final mass × initial mass^{-1}) between treatment groups using a Welch's t-test. Across both experiments, we used a bootstrapping procedure (N = 10,000) to estimate the mean (±SD) $\Delta^{15}N_{Consumer–Diet}$ and $TDF_{T–S}$ for both bulk and AA-specific measurements which included measurement error. We explicitly included the bootstrapping approach to account for propagation error when calculating each estimate. For $\Delta^{15}N_{Consumer–Diet}$, we also incorporated the mean (±SD) of $\delta^{15}N_{Diet}$ into the bootstrap procedure. We compared $\Delta^{15}N_{Consumer–Diet}$ among treatments, and with a condition of zero discrimination (i.e., $\Delta^{15}N = 0‰$), using a bootstrap t-test. We used a linear regression to test the effect of protein content on $\Delta^{15}N_{T-S}$, using values from consumer tissue alone, and TDF_{T-S}, which is $\Delta^{15}N_{T-S}$ corrected for the diet $\delta^{15}N$ value (see equation 2). Using $\Delta^{15}N_{T-S}$ is the

most likely scenario for most applications of AA isotope data to wild animals, in which diet is unknown; in contrast, $TDF_{T\text{-}S}$ represents a scenario in which diet is known. If protein content affects $\Delta^{15}N_{T\text{-}S}$ (i.e., a calculation based on consumer tissue alone) and $TDF_{T\text{-}S}$ (i.e., a calculation based on diet and consumer tissue) in the same manner, this would provide empirical justification for the practice of interpreting $\Delta^{15}N_{T\text{-}S}$ in wild animals when diet is unknown.

Results

Body Mass

At the end of Experiment A, mice consuming the low-protein diet had gained more mass (A-L; 165% ± 15%) than mice consuming the high-protein diet (A-H; 150% ± 8%; P = 0.02, t = 2.59; Figure S1, supporting information). Mice fed the intermediate-protein diet (A-I; 156% ± 18%) gained a similar amount of mass to those fed both the A-L and the A-H diet (P = 0.23, t = 1.22 and P = 0.36, t = 0.94, respectively). In contrast, at the end of Experiment B, mice fed the low-protein diet (B-L) had gained far less body mass (64% ± 35%) and had relatively low body fat 3% (±1%) in comparison with mice fed the intermediate-protein diet (B-I; mass gain 167% ± 44%; body fat 23% ± 5%; Figure S1, supporting information).

$\delta^{15}N$ Values

The three diets in Experiment A exhibited slight variation in $\delta^{15}N$ values of bulk tissue (Figure 1A), trophic AA (Figure 1A), and source AA (Figure 2A), because of small differences in contributions from different protein sources (Table 1). In contrast, the two diets in Experiment B had identical $\delta^{15}N$ values for bulk measurements and for individual AA (Figures 1 and 2) because each diet contained the same sole protein source, casein. For both

experiments, mouse muscle exhibited variable $\delta^{15}N$ values, with the highest values generally occurring for mice on the high-protein diet in Experiment A (A-H).

For both experiments, the $\Delta^{15}N_{Consumer\text{-}Diet}$ for bulk tissue fell from ~4.3‰ for mice on low-protein diets to ~3.5‰ for mice on intermediate- and high-protein diets (Figure 1B). However, for most AA in Experiment A, the $\Delta^{15}N_{Consumer\text{-}Diet}$ exhibited the opposite pattern and increased with dietary protein content (Figures 1B and 2B). Relative to A-L mice (i.e., those fed the low protein diet in Experiment A), $\Delta^{15}N_{Consumer\text{-}Diet}$ was greater for A-I mice for five AA (Ala, Gly, Ile, Ser, Val) and greater for A-H mice for nine AA (Asp, Gly, Ile, Leu, Phe, Pro, Ser, Thr, Val). In contrast, as dietary protein increased in Experiment B, the $\Delta^{15}N_{Consumer\text{-}Diet}$ remained mostly steady, changing for only two AA: for Asp, $\Delta^{15}N_{Consumer\text{-}Diet}$ was smaller in B-I than in B-L; and for Thr, $\Delta^{15}N_{Consumer\text{-}Diet}$ was greater in B-I than in B-L. For most AA in both experiments, $\Delta^{15}N_{Consumer\text{-}Diet}$ differed from zero, with exceptions of Lys across all diet groups in both experiments, Phe in B-L, and Tyr in B-I (Figures 1B and 2B).

While the dietary protein contents were similar between experiments (i.e., between A-L and B-L, and between A-I and B-I), the $\Delta^{15}N_{Consumer\text{-}Diet}$ for AA tended to be smaller in Experiment B than in A (Figures 1B and 2B). Compared with that for A-L mice, the B-L mice $\Delta^{15}N_{Consumer\text{-}Diet}$ value was smaller for four AA (Glu, Lys, Phe, Thr) and greater for one AA (Val). Compared with that for A-I mice, the B-I mice $\Delta^{15}N_{Consumer\text{-}Diet}$ was smaller for eight AA (Ala, Asp, Glu, Gly, Leu, Phe, Pro, Val). As a high-protein diet was only offered in Experiment A, a comparison of $\Delta^{15}N_{Consumer\text{-}Diet}$ with Experiment B was not possible.

Pooling data across Experiments A and B, dietary protein content had variable effects on AA $\delta^{15}N$ values. $\Delta^{15}N_{T\text{-}S}$ in mouse muscle was substantially higher if the source AA was Lys (6–14‰) than if the source AA was Phe (-1–5‰; Figure 3A). As dietary protein increased, $\Delta^{15}N_{T\text{-}S}$ declined for Glu-Phe and Pro-Phe, but slightly increased for Glu-Lys and did not change for Pro-Lys (Figure 3A; Table 2). $TDF_{T\text{-}S}$, which is corrected for $\delta^{15}N_{diet}$ (see equation

2), exhibited similar trends to $\Delta^{15}N_{T\text{-}S}$ (Figure 3B). Values of $TDF_{T\text{-}S}$ were greater when the source AA was Lys than if it was Phe, and, as dietary protein increased, $TDF_{T\text{-}S}$ declined for Glu-Phe and Pro-Phe, but did not change for Glu-Lys and Pro-Lys (Figure 3B; Table 2).

Discussion

We found that, as expected, $\Delta^{15}N_{Consumer\text{-}Diet}$ was generally greater for trophic AA than for source AA. As dietary protein content increased, the $\Delta^{15}N_{Consumer\text{-}Diet}$ of bulk muscle tissue slightly declined. However, simultaneously, increased dietary protein caused the $\Delta^{15}N_{Consumer\text{-}Diet}$ of individual AA to generally increase in Experiment A, but to remain the same or decrease in Experiment B. In the following sections, we interpret these results as reflecting differences in diet quality, especially digestibility and lipid content, between experiments. Relative to Experiment B, diets in Experiment A had more indigestible matter (cellulose, salt, and vitamins) and lower lipid content (Table 1), the latter of which is an important non-protein source of energy. We suggest that the macromolecular composition of the diets in Experiment A caused mice to oxidize dietary protein to a greater degree than in Experiment B, enhancing fractionation of dietary AA and ultimately increasing $\Delta^{15}N_{Consumer\text{-}Diet}$ for individual AA. Variation in $\Delta^{15}N_{Consumer\text{-}Diet}$ affected the estimated relative trophic position, which was dependent on the identity of the AA pair. As dietary protein increased, $\Delta^{15}N_{T\text{-}S}$ declined for Glu-Phe and Pro-Phe, but increased for Glu-Lys, and did not change for Pro-Lys (Figure 3). These results were similar when using data from consumer tissue alone or when correcting consumer tissue with diet data, so they provide a robust framework for applying $\Delta^{15}N_{T\text{-}S}$ measurements to estimate the trophic position of wild omnivorous terrestrial mammals.

Trophic Discrimination and Protein Oxidation

In Experiment A, increased dietary protein content generally led to greater $\Delta^{15}N_{Consumer\text{-}Diet}$ for individual AA. Relative to those for mice fed the low-protein (A-L) diet, values of $\Delta^{15}N_{Consumer\text{-}Diet}$ were higher for those fed the intermediate-protein (A-I) diet for five AA (Ala, Gly, Ile, Ser, Val), and for those on the high-protein (A-H) diet for nine AA (Asp, Gly, Ile, Leu, Phe, Pro, Ser, Thr, Val). We suggest that this pattern reflects catabolism-driven isotopic fractionation that occurs when AA are deaminated and their carbon skeletons are oxidized to CO_2 for energy. Specifically, AA must be deaminated prior to oxidation. This step probably favors AA containing isotopically-light ^{14}N atoms that are then incorporated into urea and excreted, resulting in ^{15}N-enrichment of the remaining AA pool used for tissue synthesis. Oxidation of dietary AA is surprisingly common for animals getting enough protein, even for essential AA. For example, in piglets fed milk and in humans fed mixed meals (composition of kJ: 0.18 protein, 0.32 lipid, 0.50 carbohydrate), 45–58% of the dietary Phe was deaminated and fully oxidized immediately after absorption in the GI tract [25].

Extensive use of AA as an oxidative substrate could lead to a tradeoff between energy demand and protein synthesis. In Experiment A, mice fed the high-protein diet gained the least amount of body mass, indicating that their synthesis of new tissue may have been hindered by oxidizing dietary AA for energy rather than routing these AA to tissue production. Animal nutrition guidelines indicate that growing mice require a dietary protein content of ~0.20 by mass [17]. Therefore, the intermediate-protein (0.22 protein by mass; 0.25 by digestible kJ) and high-protein (0.37 protein by mass; 0.41 protein by digestible kJ) treatments in Experiment A provided adequate and excess AA, respectively. However, Experiment A diets also had high contents of indigestible material, as 0.30 of the diet was a mixture of cellulose, salt, and vitamins. The remaining digestible component was notably low in lipid content (0.02 by mass; 0.09–0.12 by digestible kJ), the most energy-dense

macronutrient, and as a result the abundance of dietary protein may have been used for fueling oxidation and meeting energy demands rather than for tissue growth.

As an alternative to AA catabolism, the increase in AA $\Delta^{15}N_{Consumer\text{-}Diet}$ with greater dietary protein content could have instead been driven by greater *de novo* synthesis of AA. This scenario is unlikely, however, because (1) the need for *de novo* synthesis should decline as the availability of dietary AA increases, and (2) $\Delta^{15}N_{Consumer\text{-}Diet}$ values were large for essential AA (Ile, Val, Phe) which cannot be synthesized *de novo* by mice. The AA $\delta^{15}N$ values could have also been influenced by the contribution of microbially-synthesized AA [26,27,28], although the reliance on this alternative AA source is also expected to decline as dietary protein increases.

Intriguingly, the $\Delta^{15}N_{Consumer\text{-}Diet}$ for bulk muscle tissue slightly but significantly declined with increasing dietary protein content in both Experiment A and Experiment B (Figure 1). Although the magnitude of this decline was relatively small (~0.9‰), it was the opposite direction of the trend that we observed for most individual AA in Experiment A. This discrepancy of $\Delta^{15}N_{Consumer\text{-}Diet}$ patterns in bulk tissue versus those of individual AA could be caused by the presence of AA in bulk tissue that we did not measure. Our derivatization and AA separation techniques do not yield measurements of arginine, histidine, tryptophan, and methionine, which combined provide ~21% of the nitrogen in mammalian skeletal muscle [29] (Beach *et al.* 1943). In addition, the measurement of bulk muscle tissue probably also included waste nitrogen that had recently been removed from AA that were being oxidized for energy. In the model of catabolism-driven fractionation described above, this waste nitrogen pool would have included a high proportion of ^{14}N, contributing to the relatively low $\delta^{15}N$ values of bulk muscle tissue. In muscle tissue, waste nitrogen is generally bonded to pyruvate to synthesize Ala which is subsequently exported to the liver via the glucose-alanine cycle [30]. In support of this potential mechanism, the Ala $\delta^{15}N$ value in muscle tissue was

lower for mice fed high-protein than intermediate-protein diets, as expected when Ala is being used to shuttle a large flux of waste ^{14}N.

Trophic Discrimination and Oxidation of Non-Protein Nutrients

In contrast to Experiment A, values of AA $\Delta^{15}N_{Consumer\text{-}Diet}$ in Experiment B were similar for mice consuming the low- (B-L) and intermediate-protein (B-I) diets, with only two exceptions: mice fed the intermediate-protein diet had a smaller offset for Asp and a more negative offset for Thr. The decline in $\Delta^{15}N_{Consumer\text{-}Diet}$ for Asp may indicate that mice responded to the greater dietary availability of this AA by directly routing more of it from the diet into their tissues. In contrast, the greater negative offset for Thr suggests increased fractionation of this amino acid, which could be explained by catabolism, as suggested above for many AA in Experiment A. However, Thr did not show this pattern in Experiment A. These inconsistent responses of Thr between experiments are difficult to interpret. The fundamental mechanism that causes negative fractionation for this essential AA likewise remains unclear [31].

In Experiment B, dietary protein content had little effect on $\Delta^{15}N_{Consumer\text{-}Diet}$, and $\Delta^{15}N_{Consumer\text{-}Diet}$ was greater than zero for most AA. Together, these results suggest that mice on both the low- and the intermediate-protein diets had achieved a similar balance between routing some AA directly from diet into tissue, and catabolizing and oxidizing other AA for energy. The consistent routing of some AA into tissue, as observed in Experiment B, contrasts strongly with the extensive oxidation of dietary AA and resulting fractionation that appeared to occur in Experiment A. In agreement with the prediction that routing yields lower $\Delta^{15}N_{Consumer\text{-}Diet}$ for individual AA, $\Delta^{15}N_{Consumer\text{-}Diet}$ was smaller for mice from Experiment A than for those from Experiment B for four AA (Glu, Phe, Lys, Thr) among the

low-protein groups, and for seven AA (Ala, Asp, Glu, Leu, Pro, Val, Gly, Phe) among the intermediate-protein groups.

While Experiment A had diets with reduced availability of non-protein oxidative substrates, Experiment B had highly digestible diets that were rich in non-protein macronutrients. Only 0.10–0.25 of the diets were indigestible in Experiment B, and energy-dense lipids provided 0.56–0.68 of the digestible, non-protein kJ; in Experiment A, the diet included 0.30 indigestible components, and lipids provided 0.09-0.12 of the digestible, non-protein kJ. The high abundance of energy available from lipids in Experiment B probably reduced the need for oxidation of dietary AA, instead allowing them to be directly routed to tissue synthesis, even for mice fed the low-protein diet.

Despite the apparent routing of dietary AA to tissue synthesis in Experiment B, mice on the low-protein diet gained less mass than mice on the intermediate-protein diet. Much of the mass gain by mice fed the intermediate-protein diet was the accumulation of lipid reserves (adipose tissue), as indicated by their higher body fat (23%) than mice on the low-protein diet (3%). Lipid reserves can be increased by directly routing dietary lipids to adipose tissue, or by synthesizing new lipids from dietary carbohydrates, and both processes are compatible with routing of dietary AA into endogenous tissues.

Implications for $\delta^{15}N_{AA}$-Based Estimates of Trophic Position

Our data indicate that dietary protein content, digestibility, and availability of non-protein sources of energy (kJ) influence the $\Delta^{15}N_{\text{Consumer-Diet values}}$ of individual AAs. Importantly, studies using $\Delta^{15}N_{T\text{-}S}$ to estimate the trophic position of wild animals typically only measure $\delta^{15}N$ values of consumer tissues, because a purported strength of this approach is not needing to analyze potential diet items [7,8]. Although such studies often assume that the $\delta^{15}N$ values of canonical source AA (Phe and Lys) remain constant among trophic levels, our data show that

the $\delta^{15}N$ values of both source and trophic AA can vary substantially between diet and consumer. Encouragingly, however, this variation was similar between source and trophic AA for several trophic-source pairs, and was strongly correlated with dietary protein content (Figure 3). As a result, values of the offset between trophic and source AA in consumer tissue ($\Delta^{15}N_{T\text{-}S}$), and values of $TDF_{T\text{-}S}$ which account for AA $\delta^{15}N$ values in the diet, were similarly affected by protein content. This consistency between $\Delta^{15}N_{T\text{-}S}$ and $TDF_{T\text{-}S}$ suggests that while $\Delta^{15}N_{T\text{-}S}$ may vary with protein content and with the trophic-source AA pair used to calculate these metrics, the magnitude of the $\delta^{15}N$ offset that represents a trophic step will vary in a similar fashion, providing empirical support for using $\Delta^{15}N_{T\text{-}S}$ to estimate trophic position.

It is important to acknowledge that ecologists rarely have the benefit of knowing the precise magnitude of the $\delta^{15}N$ offset that represents a trophic step ($TDF_{T\text{-}S}$), because this knowledge requires equation 2, which relies on data from diet samples. Our data therefore provide a basis for ecologists to understand how variation in protein intake and diet digestibility may influence AA $\delta^{15}N$ results, when $TDF_{T\text{-}S}$ is unknown. In tissue samples from mammalian omnivores, individuals with higher dietary protein intake may have smaller $\Delta^{15}N_{Glu\text{-}Phe}$ and $\Delta^{15}N_{Pro\text{-}Phe}$, and slightly higher $\Delta^{15}N_{Glu\text{-}Lys}$. Unless diet samples are available to allow simultaneous calculation of $TDF_{T\text{-}S}$, this could cause erroneous inferences regarding trophic position. This problem may be avoided by using $\Delta^{15}N_{Pro\text{-}Lys}$ as the basis for inferring trophic position, because in our experiments both the magnitude of a trophic step for this pairing (i.e., $TDF_{T\text{-}S}$), and the magnitude of $\Delta^{15}N_{Pro\text{-}Lys}$ in consumer tissue, were unaffected by variation in dietary protein content from 0.05 to 0.37 (by mass). Overall, the variable influence of dietary protein on $\Delta^{15}N_{T\text{-}S}$ reinforces that relative comparisons of trophic position for consumers in a single food web are probably more reliable than estimating a precise number for trophic position.

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Table 1. Diets offered to house mice in two different experiments (A and B). Acronyms for diet treatments refer to protein content as low (L), intermediate (I), or high (H). Diet composition is described by ingredients ($g \times g^{-1}$ diet), macronutrient content ($g \times g^{-1}$ macronutrients), and by sources of digestible energy ($kJ \times kJ^{-1}$ macronutrients).

	Experiment A			Experiment B	
Diet Acronyms	A-L	A-I	A-H	B-L	B-I
Mice (N)	10	13	11	4	10
Diet Ingredients					
Casein	0.05	0.20	0.35	0.05	0.30
Sucrose	0.45	0.30	0.15	0.35	0.30
Corn Meal	0.15	0.15	0.15	0.00	0.00
Corn Oil	0.02	0.02	0.02	0.00	0.00
Lard	0.00	0.00	0.00	0.35	0.30
Cellulose	0.25	0.25	0.25	0.20	0.05
Fortified Salt	0.04	0.04	0.04	0.04	0.04
Brewer's Yeast	0.02	0.02	0.02	0.00	0.00
Vitamin Mix	0.01	0.01	0.01	0.01	0.01
Macronutrients*					
Protein	0.07	0.22	0.37	0.05	0.30
Carbohydrate	0.60	0.45	0.30	0.35	0.30
Lipid	0.02	0.02	0.02	0.35	0.30
Other	0.30	0.30	0.30	0.25	0.10
Digestible KJ					
Protein	0.08	0.25	0.41	0.03	0.18
Carbohydrate	0.83	0.65	0.47	0.29	0.26
Lipid	0.09	0.10	0.12	0.68	0.56

*Columns sum to 0.99 because of rounding in individual categories.

Table 2. Regression statistics (± SD) of the relationship between the predictor of dietary protein content and the response variable of either $\Delta^{15}N_{T-S}$ (offset between trophic-source amino acids in consumer tissue) or TDF_{T-S} (offset that represents a single trophic step), for selected pairs of amino acids. See Figure 5 for regressions.

AA Pair	Intercept	Slope	*P*	Adjusted R^2
$\Delta^{15}N_{T-S}$				
Glu-Phe	4.0 ± 0.3	-6.6 ± 1.1	< 0.01	0.43
Pro-Phe	3.2 ± 0.4	-9.5 ± 1.5	< 0.01	0.46
Glu-Lys	9.2 ± 0.5	4.6 ± 2.0	0.03	0.08
Pro-Lys	8.4 ± 0.4	1.8 ± 1.7	0.30	0.00
TDF_{T-S}				
Glu-Phe	4.6 ± 0.3	-6.2 ± 1.2	< 0.01	0.36
Pro-Phe	3.6 ± 0.4	-6.7 ± 1.6	< 0.01	0.22
Glu-Lys	5.9 ± 0.5	3.2 ± 1.9	0.10	0.04
Pro-Lys	4.7 ± 0.4	3.0 ± 1.5	0.06	0.06

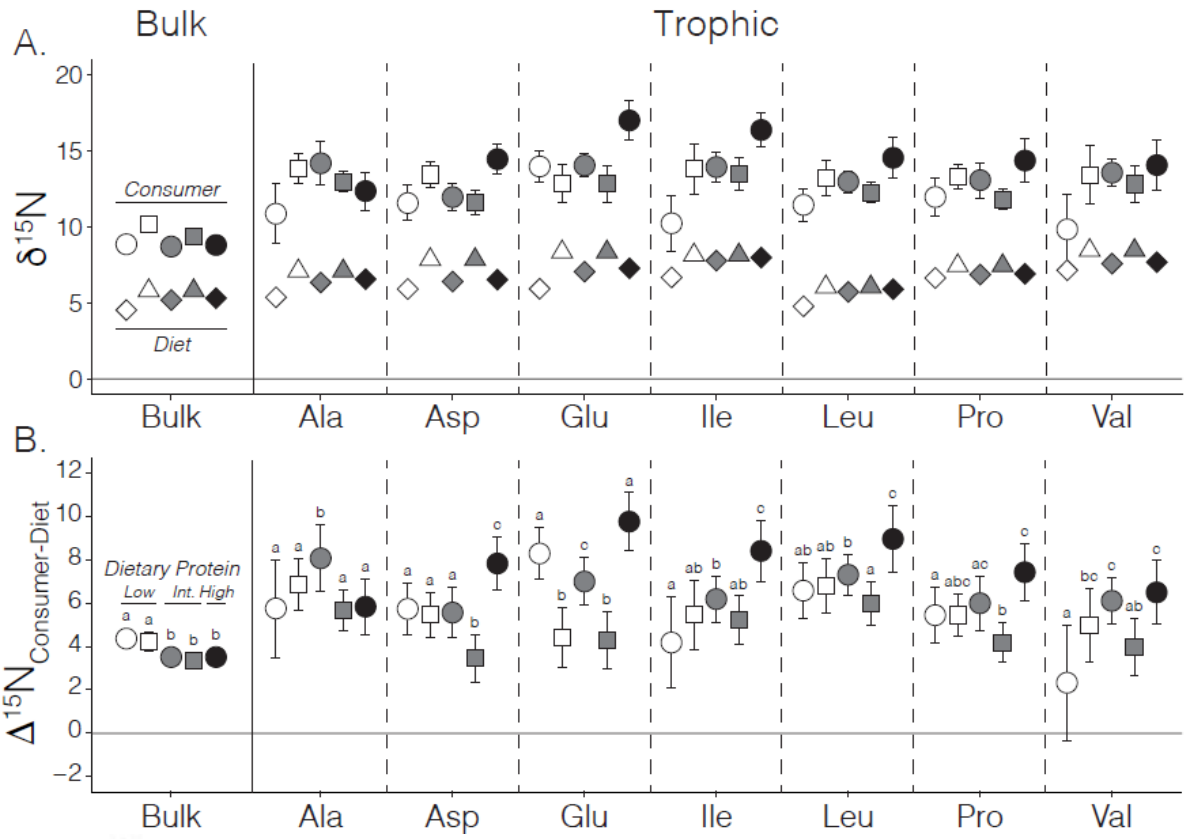

Figure 1. (A) Bootstrap mean (± SD) $\delta^{15}N$ values of bulk samples and individual amino acids in mouse skeletal muscle for Experiments A and B. Experiment A is indicated by circles (muscle) and corresponding diamonds (diet), and Experiment B is indicated by squares (muscle) and corresponding triangles (diet). In both panels, the protein concentration of the diet treatment is indicated by color: white is low protein, gray is intermediate protein, and black is high protein. (B) Bootstrap mean (± SD) of trophic discrimination factors ($\Delta^{15}N_{Consumer\text{-}Diet}$) for bulk tissue and trophic amino acids in mouse skeletal muscle from Experiments A (circles) and B (squares). Lower case letters indicate significant differences for specific AAs among diet treatments.

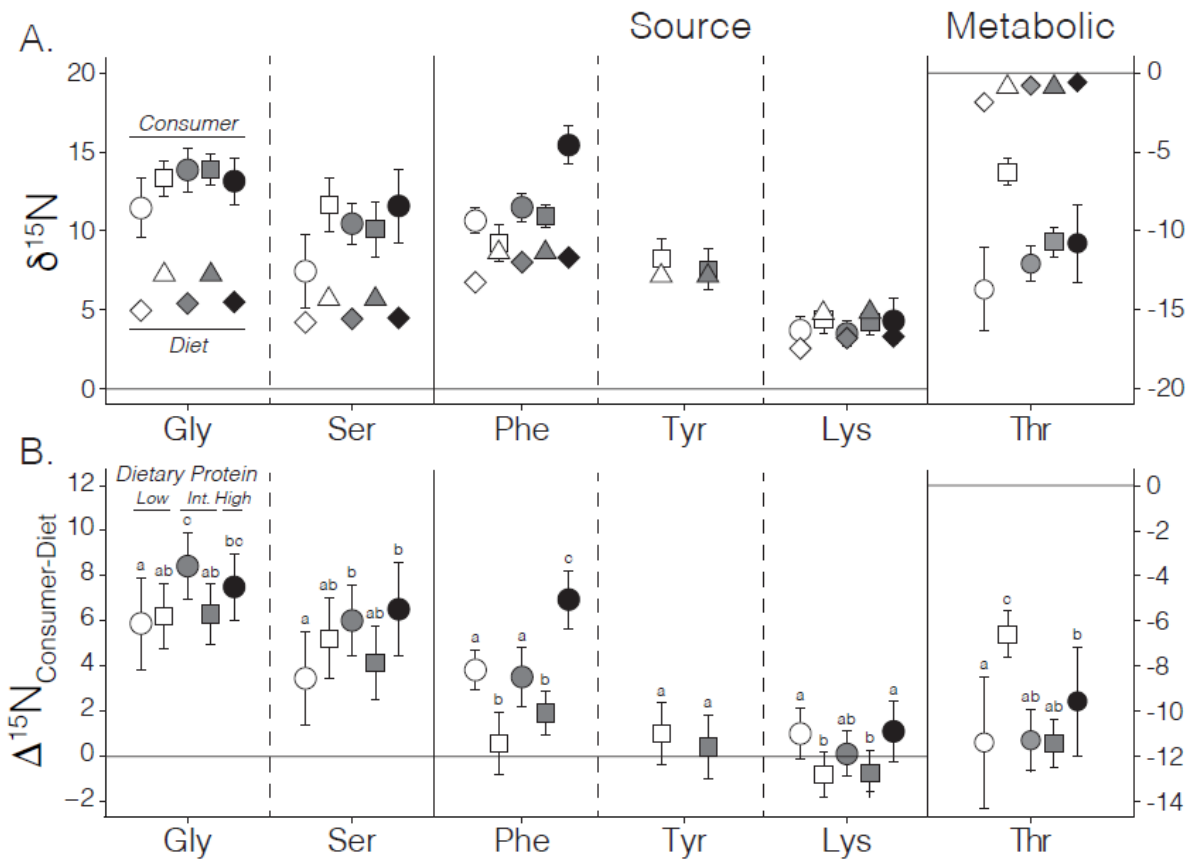

Figure 2. (A) Bootstrap mean (± SD) $\delta^{15}N$ values of amino acids in mouse skeletal muscle and their diets for Experiment A and B. Experiment A is indicated by circles (muscle) and corresponding diamonds (diet), and Experiment B is indicated by squares (muscle) and corresponding triangles (diet). In both panels, protein concentration of the diet treatment is indicated by color: white is low protein, gray is intermediate protein, and black is high protein. (B) Bootstrap mean (± SD) of trophic discrimination factors ($\Delta^{15}N_{Consumer\text{-}Diet}$) for amino acids in mouse skeletal muscle from Experiment A (circles) and B (squares). Tyr $\delta^{15}N$ data for Experiment A were not available because of co-elution of chromatogram peaks with Lys. Lower case letters indicate significant differences for specific AAs among diet treatments.

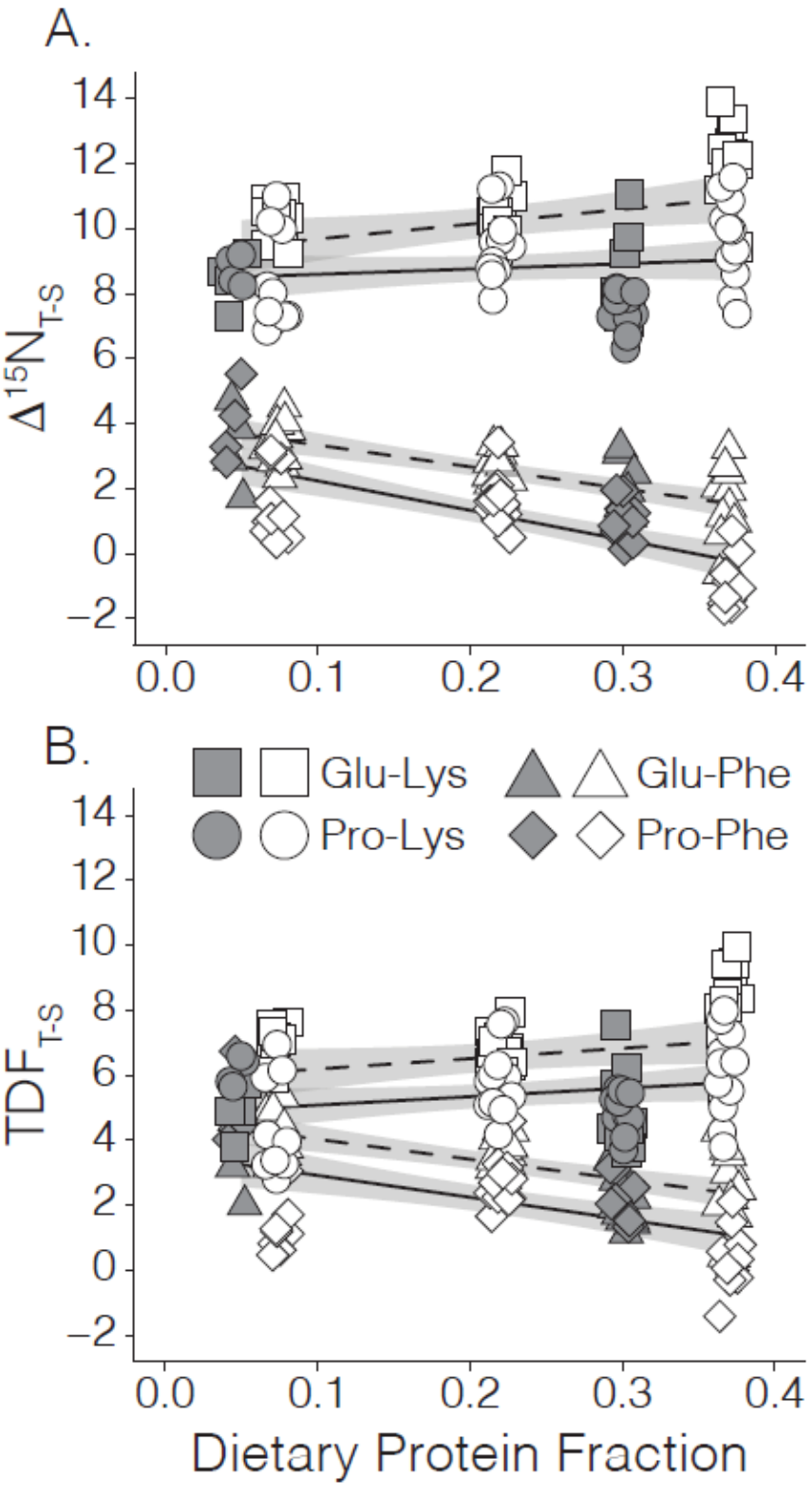

Figure 3. Regressions for selected pairs of amino acids between dietary protein content and (A) the offset between trophic-source amino acids in consumer tissue, and (B) the offset that represents a single trophic step, corrected for dietary $\delta^{15}N$. Regressions include data pooled from Experiment A (white symbols) and Experiment B (gray symbols). The gray shading represents the 95% confidence interval of the regression lines for each AA pair.