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The relationship between dietary protein content, body condition, and $\Delta^{15}N$ in a mammalian omnivore

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Abstract

Seasonal reductions in food availability may cause animals to catabolize endogenous tissue and the resulting loss of lean mass can hinder their ability to forage and reproduce. While several studies have considered nitrogen isotopes ($\delta^{15}N$) as an indicator of catabolism, relationships between protein intake, body condition, and tissue $\delta^{15}N$ have not been assessed simultaneously in controlled conditions. We conducted a feeding experiment on laboratory mice (*Mus musculus*) to test the effects of low (5%) versus high (30–40%) dietary protein content on lean mass, fat mass, and tissue $\delta^{15}N$. This approach enables the distinction between use of exogenous and endogenous nitrogen, illuminating a framework of protein metabolism and tissue synthesis. As expected, lean mass and body fat were lower in mice fed low-protein diets. Nitrogen isotope discrimination ($\Delta^{15}N$) between blood plasma-diet and liver-diet did not differ between diet treatments. In contrast, $\Delta^{15}N$ for hair decreased while $\Delta^{15}N$ for muscle and RBC increased in the low-protein treatment. These patterns suggest that animals in negative nitrogen balance catabolize labile endogenous protein (e.g., muscle) to maintain vital tissues (e.g., liver) required to survive. Consequently, muscle and RBC $\delta^{15}N$ values appear to be the most useful in assessing the nutritional state of animals. Our combination of direct measurements of body condition with $\delta^{15}N$ analysis suggest how nitrogen isotopes can be better used as tracers of catabolic and anabolic activity by demonstrating connections between tissue-specific metabolic processes and $\Delta^{15}N$, thus refining the application of $\delta^{15}N$ as a tool for assessing nitrogen balance in wild animals.

Keywords Catabolism · Isotopes · Metabolism · Nitrogen · Nutrient-stress

Introduction

All animals require a continuous supply of energy to grow, maintain homeostasis, and reproduce, so when exogenous sources of energy are unavailable they must rely on endogenous stores to survive (McCue 2010). Degradation of endogenous tissue (catabolism) is particularly important for species that experience seasonal or persistent food limitation, or during reproduction for individuals that utilize capital breeding strategies (Castellini and Rea 1992; Meijer and Drent 1999; Khalilieh et al. 2012). Even species that have evolved novel strategies such as hibernation and torpor for coping with long periods of food deprivation must rely on catabolism (Balter et al. 2006; Melvin and Andrews 2009; Lee et al. 2012). Animals manage nutrient deprivation by prioritizing the use of endogenous stores as metabolic fuel, typically in the order of carbohydrates, lipids, and lastly protein (Castellini and Rea 1992; Khalilieh et al. 2012; McCue 2010). Some species can spare protein reserves during periods of minimal food intake by primarily using fat for

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This study is the first to simultaneously assess protein intake, direct measurements of body condition, and tissue $\delta^{15}N$. Results reveal differences in tissue-specific catabolic and anabolic activities during protein stress that are not only informative on physiological level, but also provide insight into the interpretation of $\delta^{15}N$ values in free-ranging animals.

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energy and keeping protein catabolism to a minimum (Cuendet et al. 1975; Karmann et al. 1994; McCue and Pollock 2013). Loss of lean mass can hinder functional performance in tasks such as foraging and reproduction, and eventually increases mortality risk (Bender et al. 2008). It is therefore important to develop tools to track catabolism of functionally important endogenous reserves (e.g., skeletal muscle or adipose tissue), particularly for application to free-ranging animals coping with resource limitation.

Hobson and Clark (1992) were the first to find a correlation between nutritional stress and tissue $\delta^{15}N$ values. Since then, nitrogen, carbon ($\delta^{13}C$), and hydrogen ($\delta^{2}H$) isotopes have been used to examine the catabolism of endogenous protein and lipid reserves (Hobson and Ewins 1997; Hobson et al. 2000; Gauthier et al. 2003; Carleton et al. 2004, 2006; Morrison and Hobson 2004; Hobson 2006; Voigt and Speakman 2007; Warner et al. 2008; Fox et al. 2009; Graves et al. 2012). The $\delta^{15}N$ values of a consumer's tissues are higher than its diet due to preferential removal of amine groups containing ^{14}N during peptide bond hydrolysis and deamination (Macko et al. 1986, 1987; Gannes et al. 1997; Balter et al. 2006). In mammals, ^{14}N is preferentially excreted in the form of urea (Steele and Daniel 1978; Sutoh et al. 1993). The offset in $\delta^{15}N$ values between a consumer's tissues and that of its diet ($\Delta^{15}N_{tissue-diet}$), commonly referred to as trophic discrimination, is influenced by several factors including dietary protein content and protein biological value, defined here as the degree to which the dietary amino acids meet the amino acid requirements of the consumer (Robbins 1993; Kelly and Martínez del Rio 2010), tissue type, growth rates, nutritional or reproductive status, and nitrogen excretion pathways (Bearhop et al. 2002; Robbins et al. 2005; Kurle 2009; Pearson et al. 2003; Vanderklift and Ponsard 2003; Fuller et al. 2005; Martínez del Rio et al. 2009; Ben-David et al. 2012). Earlier studies on arid land herbivores have also noted a variation in $\Delta^{15}N$ that could result from water stress and associated physiological adaptations to drought (Ambrose and DeNiro 1986). As $\Delta^{15}N$ can vary from 2 to 5‰ in mammals, a better understanding of the underlying physiological influences is crucial for using nitrogen isotopes to assess nutritional stress in wild animals and to further refine the use of $\delta^{15}N$ as a proxy for diet composition and trophic level in natural settings (Vanderklift and Ponsard 2003; Caut et al. 2009; Ben-David and Flaherty 2012; Martínez del Rio et al. 2009; Kurle et al. 2014).

Nitrogen balance is one of the most important influences on $\Delta^{15}N$. Animals are in positive nitrogen balance when consuming more protein than needed for growth, maintaining homeostasis, and reproduction. Trophic discrimination factors for animals in positive nitrogen balance are shaped by a combination of mechanisms. First, excessive protein intake elevates breakdown of dietary amino acids and increases the rate of ^{14}N excretion, leading to increases in consumer tissue $\delta^{15}N$ values and associated $\Delta^{15}N$ (Pearson et al. 2003). However, this effect is countered by routing of dietary amino acids directly to tissue synthesis with little isotopic alteration or fractionation (Schwarcz and Schoeninger 1991), resulting in consumer tissue $\delta^{15}N$ resembling that of dietary protein and thus decreased $\Delta^{15}N$ (Ambrose and Norr 1993). These combined effects result in relatively low tissue $\Delta^{15}N$.

On the other hand, if animals do not consume sufficient amounts of exogenous protein and are in negative nitrogen balance, less dietary protein will be directly routed to tissue synthesis and instead endogenous nitrogen stores (e.g., skeletal muscle) will be catabolized to fuel metabolism and tissue maintenance (Hobson and Stirling 1997; O'Brien et al. 2000). However, not all sources of endogenous protein experience similar changes in catabolism and anabolism during nutritional stress. Some proteinaceous tissues must be maintained by anabolism because they are essential for basic metabolic functions. For example, the liver plays a critical role in detoxification, the metabolism of fats, carbohydrates, and proteins, as well as storage of vitamins and glycogen (Hall 2015). In times of nutritional stress, the liver may decrease in mass due to fat, glycogen, and protein depletion (Fisher and Bartlett 1957; Cotton and Harlow 2010), however, even with reduced activity it must remain functionally active and its rate of protein synthesis tends to remain much higher than tissues such as skeletal muscle (Felig et al. 1969; Yacoe 1983). Thus, because the liver is crucial for metabolism, we expect this tissue to experience sufficient anabolism to maintain functional integrity. Conversely, protein sparing of skeletal muscle tissues is less important because consequences for the organism from degraded function are not as severe. Therefore, skeletal muscle might be more susceptible to catabolism and indeed, it has been recognized as a major labile protein reserve (Swick and Benevenga 1977). Smooth muscle has also been noted as a labile pool (Yacoe 1983).

As endogenous tissues typically have higher $\delta^{15}N$ values than the diet, catabolism serves to further concentrate ^{15}N within the body nitrogen pool from which lean tissues are maintained, resulting in even greater $\Delta^{15}N$ (Gannes et al. 1997; Poupin et al. 2011; Arneson and MacAvoy 2005; MacAvoy et al. 2005). There are two proposed mechanisms for this enrichment: the 'anabolic' and the 'catabolic' models (Lee et al. 2012). The 'anabolic' model, which is more broadly supported in the literature, suggests that as tissues are catabolized and hydrolyzed into individual amino acids, they are subjected to deamination that preferentially removes ^{14}N, and that remaining ^{15}N-enriched amino acids are then incorporated into tissues during anabolism (Fuller et al. 2004; Lee et al. 2012). In contrast, the alternative 'catabolic' model proposes that there is a disproportionate loss of light amino acids during tissue catabolism, causing the $\delta^{15}N$ value of the remaining tissue to increase regardless of anabolism (Hobson et al.

1993; Gloutney et al. 1999). However, the biochemical mechanism for the catabolic model remains elusive and its existence is disputed (Lee et al. 2012).

Catabolism of endogenous resources reduces body fat and lean mass (DelGiudice 1973; Gannes et al. 1997). Accordingly, body composition is dynamic during protein stress because of the net effect catabolic versus anabolic processes. It is, therefore, informative to link direct and accurate measurements of body composition to stable isotope analysis to better understand the consequences of variation in protein metabolism for animal fitness. Directly measuring body condition is difficult, however, because most methods require euthanasia and carcass or tissue homogenization, lipid extraction, and combustion to determine protein, lipid, and mineral content (Afton and Ankney 1991); these techniques cannot be used on live animals. Techniques that can be performed on live animals such as plethysmography, hydrostatic weighing, bioelectrical impedance, and electrical conductivity are imprecise (Taicher et al. 2003) and difficult to employ in field settings. Recently, eco-physiologists have begun using proton nuclear magnetic resonance, also known as quantitative magnetic resonance (QMR), to rapidly and accurately measure lean mass and body fat in live animals (Taicher et al. 2003; Tinsley et al. 2004; Jones et al. 2009; McGuire and Guglielmo 2010; Nixon et al. 2010; Guglielmo et al. 2011).

Our study employs $\delta^{15}N$ analysis to assess the use of exogenous (i.e. diet derived) versus endogenous nitrogen and thus illuminate the framework of protein metabolism, catabolism, and tissue synthesis in an omnivorous mammal. We conducted a controlled feeding experiment on laboratory mice (*Mus musculus*) to evaluate the effects of dietary protein content (low 5%, high 30–40%), encompassing the seasonal range expected for wild mammalian omnivores (Leirs et al. 1994; Felton et al. 2009), on body condition and $\Delta^{15}N$ discrimination. While $\delta^{15}N$ is potentially a useful tool for examining nitrogen balance in wild animals and several feeding experiments have examined the effects of dietary protein levels on tissue $\delta^{15}N$ (Karmann et al. 1994; Hilderbrand et al. 1996; Felicetti et al. 2015; Robbins et al. 2005), previous studies have not included direct measurements of body composition. We directly measured mice lean mass and body fat via QMR to track the effects of anabolic and catabolic processes on body condition and the $\delta^{15}N$ values of several tissues that are commonly analyzed by animal ecologists. We expected that mice consuming low-protein diets would experience negative nitrogen balance resulting in catabolism of labile protein reserves to maintain critical tissues, and overall have lowered body fat and lean mass. Our experiment is valuable for developing more accurate discrimination factors during periods of nutritional stress, improving the application of $\delta^{15}N$ as a tool for tracing nitrogen balance in wild animals.

Materials and methods

Experimental design and tissue collection

Sixty-four female weanling laboratory mice (*Mus musculus*) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the University of New Mexico Animal Research Facility (Albuquerque, NM). The mice were co-housed by diet treatment ($n = 8$ per treatment) in 18 × 12 inch plastic bins at a temperature of ~ 23 (± 2) °C and a 12-h photoperiod. This temperature may be below their thermoneutral temperature (~ 31 °C; Hudson and Scott 1979; although see Speakman and Keijer 2013). Our experiment consisted of eight diet treatments in which we systematically altered the weight percent of protein (casein), carbohydrates (sucrose), and fat (lard) according to Table 1. Each diet was equally enriched with a fortified salt (4%) and vitamin mixture (1%). All dry ingredients were homogenized with ~ 4 L of water and stored frozen (− 20 °C). Mice in diet H1 were fed 12 g of food per day per mouse and mice in diets H2–5 and L1–3 were fed 8 g per day per mouse. Access to water was ad libitum, with food and water being replenished daily. At the beginning of the experiment, each mouse was weighed, subcutaneously injected with an electronic pit tag (BioMark LPT8; Boise, ID) for identification, then randomly assigned to one of the eight diet treatments. Each treatment group contained eight mice.

After 112 days, the mice were euthanized via exposure to CO_2 and tissues were immediately collected for stable isotope analysis. Blood was collected via cardiac puncture and transferred into heparinized micro-capillary tubes and stored on ice until centrifugation. Within 6 h of collection, blood was separated into plasma and red blood cells (RBC) via centrifugation at 10,000 rpm for ~ 10 min. Liver and

Table 1 Weight percent proportions of protein (casein), carbohydrates (sucrose), and fat (lard) for the low- and high-protein diet treatments

Diet	Protein	Fat	Carbohydrates	Cellulose
Low protein				
L1	0.05	0.35	0.35	0.20
L2	0.05	0.40	0.20	0.30
L3	0.05	0.05	0.75	0.10
High protein				
H1	0.30	0.30	0.30	0.05
H2	0.30	0.30	0.30	0.05
H3	0.35	0.35	0.20	0.05
H4	0.35	0.05	0.35	0.20
H5	0.40	0.05	0.20	0.30

Each diet was equally enriched with a fortified salt (4%) and vitamin mixture (1%)

biceps femoris muscle tissues were dissected and stored frozen in plastic micro-centrifuge tubes at − 20 °C. A patch of hair near the base of the tail was shaved 2 months preceding euthanasia, then hair that had regrown in this area was collected at the end of the experiment, thus ensuring that it had equilibrated with diet. All animal handling and husbandry was conducted with the approval of the University of New Mexico Institutional Animal Care and Use Committee (16-200492-MC).

Body composition

We weighed mice weekly throughout the experiment to track growth. One week prior to euthanasia, body fat and lean mass were directly measured in triplicate for each mouse using an EchoMRI QMR system (Echo Medical Systems, Houston, TX). After the QMR was calibrated with reference materials, each animal was restrained in a Plexiglass tube and inserted into the magnet for analysis. The QMR system produced rapid (~ 180 s) and precise (± 0.1 g) measurements of body fat and lean mass, which were converted to percent body fat and lean mass using measurements of body mass (Jones et al. 2009; McGuire and Guglielmo 2010; Nixon et al. 2010). This technique has been validated on live un-anaesthetized small mammals, passerine birds, and bats (Tinsley et al. 2004; McGuire and Guglielmo 2010; Guglielmo et al. 2011).

Stable isotope analysis

Blood plasma was pipetted into pre-weighed tin capsules and dried for isotope analysis. Liver samples were lipid-extracted in a 2:1 chloroform:methanol solvent solution for 72 h, replacing the solvent solution every 24 h. The tissues were then rinsed five times with deionized water to remove residual solvent. Similarly, muscle samples were lipid-extracted using petroleum ether. Surface contaminants were removed from hair via rinsing in a 2:1 chloroform:methanol solvent solution. Liver, RBC, muscle, hair, and dietary casein were freeze-dried for 24 h and then weighed into tin capsules for isotope analysis. Nitrogen isotope ($\delta^{15}N$) values were measured using a Costech 4010 elemental analyzer coupled to a Thermo Scientific Delta V isotope ratio mass spectrometer at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). Stable isotope data are expressed as δ values using the equation $\delta^{15}N = [(R_{Sample} - R_{Standard})/R_{Standard}] \times 1000$, where R_{Sample} and $R_{Standard}$ are the ratios of $^{15}N/^{14}N$ for each sample and standard. The internationally accepted standard for $\delta^{15}N$ is atmospheric N_2 and the units are expressed as permil (‰). Internal lab reference materials included four protein-based materials: soy protein, whey protein, casein, and tuna muscle with mean $\delta^{15}N$ values (± SD) of 1.1 ± 0.2, 5.9 ± 0.2, 6.5 ± 0.2 and 13.2 ± 0.2, respectively. Analytical precision was estimated via repeated (within-run) measurements of these reference materials calibrated to internationally accepted standards; within-run standard deviation for all reference materials was ≤ 0.2‰ for $\delta^{15}N$. We also measured the weight percent carbon and nitrogen concentrations of each sample; all tissue samples had [C]:[N] that were similar to the theoretical ratio of 3.2–3.5 for unaltered protein (Ambrose 1992).

Statistical analysis

Triplicate QMR results for each mouse were averaged. For the purpose of this study, diets were binned according to the proportion of protein (Table 1), with the low-protein group including diets containing 5% protein and the high-protein group including diets containing 30–40% protein. Significant differences in body composition and tissue-specific $\delta^{15}N$ and $\Delta^{15}N$ between the two protein groups were evaluated with one-way analysis of variance (ANOVA). Significant differences among mean tissue isotope values within low- and high-protein groups were assessed using Kruskal–Wallis rank sum tests followed by post hoc pairwise Tukey honest significant difference (HSD) tests. Additionally, a heatmap visualizing correlations between dietary protein content, body composition data, and $\Delta^{15}N$ was created using the R package corrplot (Wei and Simko 2016).

Results

Body composition

Mice fed low-protein diets had significantly less body fat (ANOVA $F_{1,62} = 42.6$, $P < 0.001$) and lean mass (ANOVA $F_{1,62} = 88.8$, $P < 0.001$), lower fat:lean mass ratios (ANOVA $F_{1,62} = 30.9$, $P < 0.001$), weighed less (ANOVA $F_{1,62} = 61.8$, $P < 0.001$), and had a lower percent weight gain throughout the trial (ANOVA $F_{1,62} = 58.7$, $P < 0.001$) than mice fed high-protein diets (Table 2, Online Resource 1).

$\delta^{15}N$ trophic discrimination factors

Since casein ($\delta^{15}N = 6$‰) was the only source of nitrogen in every diet treatment, nitrogen isotope trophic discrimination factors ($\Delta^{15}N$) were calculated as $\delta^{15}N_{tissue} - \delta^{15}N_{casein}$. Tissue-specific mean (± SD) $\delta^{15}N$ values are reported in Table 2 and $\Delta^{15}N$ values are illustrated in Fig. 1. $\Delta^{15}N$ was significantly higher in the low-protein diets for RBC (ANOVA $F_{1,57} = 189$, $P < 0.001$) and muscle (ANOVA $F_{1,61} = 82.1$, $P < 0.001$), but significantly lower for plasma (ANOVA $F_{1,55} = 7.6$, $P = 0.008$) and hair (ANOVA $F_{1,58} = 10.9$, $P = 0.002$). $\Delta^{15}N$ for liver did not differ significantly between the low- and high-protein diet treatments

Table 2 Mean (± SD) body composition data and tissue $\delta^{15}N$ for low- and high-protein diet treatments

	Low protein	High protein	*P* value	*F* value
Body composition				
Final weight	24.3 ± 5.1	41.3 ± 10.6	< 0.001	61.78
Total percent gain	24.3 ± 25.6	112.0 ± 52.3	< 0.001	58.67
Lean mass percent	14.0 ± 3.9	22.3 ± 3.0	< 0.001	42.58
Body fat percent	5.0 ± 2.2	15.7 ± 7.7	< 0.001	88.82
Fat:lean mass	0.3 ± 0.1	0.7 ± 0.3	< 0.001	30.86
$\delta^{15}N$				
Liver	10.3 ± 0.3^{a}	10.2 ± 0.3^{a}	NS	0.982
Plasma	10.4 ± 0.3^{a}	10.6 ± 0.2^{b}	0.008	7.626
RBC	9.6 ± 0.2^{b}	8.8 ± 0.2^{c}	< 0.001	189
Muscle	10.2 ± 0.4^{a}	9.4 ± 0.3^{d}	< 0.001	82.1
Hair	8.6 ± 0.8^{c}	9.2 ± 0.6^{d}	0.002	10.85

Significant differences in body composition and tissue isotope values between low- and high-protein diet treatments were assessed with a one-way ANOVA. Significant differences in $\delta^{15}N$ among tissues within each protein treatment were assessed using post hoc Tukey HSD tests. Tissues that share the same letter superscript did not have significantly different ($P < 0.05$) $\delta^{15}N$ values within each protein treatment

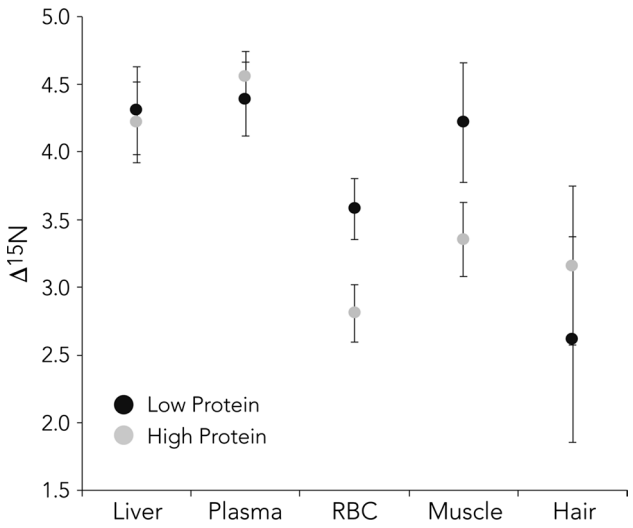

Fig. 1 Mean $\Delta^{15}N$ of tissues for low- and high-protein diet treatments; error bars represent standard deviation

(ANOVA $F_{1,63} = 1.0$, $P = 0.32$). Within the low-protein group, tissue-specific differences were noted (Kruskal–Wallis rank sum test $\chi^2 = 80.4$, $df = 4$, $P = 0.002$), however, pairwise analysis revealed that $\Delta^{15}N$ did not differ among liver, plasma, and muscle (Tukey's HSD test $P > 0.05$). Tissue-specific differences were also noted within the high-protein group (Kruskal–Wallis rank sum test $\chi^2 = 143.6$, $df = 4$, $P < 0.001$), although $\Delta^{15}N$ of hair and muscle were indistinguishable (Tukey's HSD test $P > 0.05$).

Relationship between body condition and tissue $\Delta^{15}N$

Correlation analysis (Fig. 2, Online Resource 2) revealed significant positive correlations between dietary protein content and body condition metrics (total weight gain, lean mass, and body fat percentages) and hair $\Delta^{15}N$ values, but significantly negative correlations to muscle and RBC $\Delta^{15}N$ values ($P < 0.05$). Dietary protein content was not correlated to plasma or liver $\Delta^{15}N$ values. Body condition metrics were positively correlated to hair and plasma $\Delta^{15}N$ values and negatively correlated to liver, muscle, and RBC $\Delta^{15}N$ values; the only exception being no significant correlation between body fat percent and hair $\Delta^{15}N$.

Discussion

By combining direct measurements of body condition with stable isotope analysis of multiple tissues, our study illustrates the influence of nutritional state on tissue-specific anabolic and catabolic processes that affect nitrogen isotope discrimination. Proteinaceous tissues are synthesized from the free amino acid pool of blood plasma (Waterlow et al. 1978), thus their $\delta^{15}N$ values and associated $\Delta^{15}N$ reflect changes in the isotopic composition of that pool. Differences in $\Delta^{15}N$ among tissues in mice fed with the same diet may also be due to differing amino acid compositions, as amino acids can vary in $\delta^{15}N$ by ~ 20‰ (Hare et al. 1991; McClelland and Montoya 2002). In the following sections, we describe why the influence of dietary protein content on nitrogen isotope discrimination differed among tissues, and how this relationship could help researchers characterize the body condition and nutritional status of wild free-ranging animals. To aid our explanation, we use a conceptual model (Fig. 3) which emphasizes the availability of dietary protein and its impact on tissue-specific anabolism and catabolism that ultimately influences $\Delta^{15}N$.

Diet-independent effects

Regardless of protein intake, plasma and liver $\Delta^{15}N$ values were higher than other tissue types, consistent with previous observations (Arneson and MacAvoy 2005; Kurle et al. 2014). Plasma likely had high $\Delta^{15}N$ values because it represents amino acids that have been retained after the selective excretion of ^{14}N in urea, the most abundant nitrogenous waste product in mammalian urine (Poupin et al. 2011; Arneson and MacAvoy 2005; MacAvoy et al. 2005). Liver tissue performs many critical physiological functions, and as one of the most metabolically active tissues in the body it requires constant replacement (anabolism) of its proteins (Tieszen et al. 1983). Such proteins

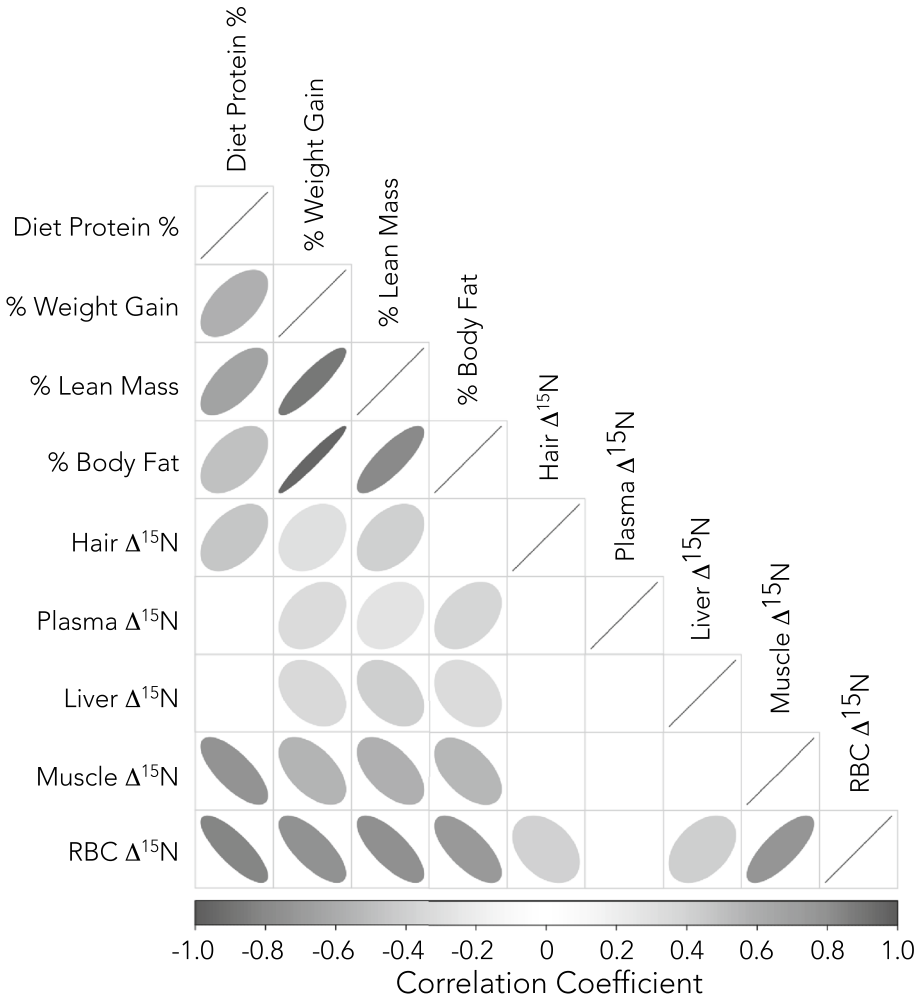

Fig. 2 Heatmap created from Pearson correlation matrix of dietary protein content, body condition scores and $\Delta^{15}N$. Shaded ellipses are significant (P value < 0.05); slant angle indicates negative (right) or positive (left) correlations while ellipse width and hue indicate magnitude of correlation; darker and narrower are more strongly correlated

are synthesized from the free amino acid pool in blood plasma, and therefore the rapid shuttling of amino acids from plasma into liver proteins (Fig. 3) likely explains their similar $\Delta^{15}N$ within and among diet treatments in our experiment. In comparison to liver and plasma, RBC and muscle are less metabolically active and exhibit a lower rate of protein replacement during normal physiological maintenance (Arneson and MacAvoy 2005; Kurle 2009). As a result, these tissues had $\Delta^{15}N$ values that were different than that of the plasma amino acid pool, but the magnitude of discrimination varied depending on dietary protein content (see below).

Although plasma $\Delta^{15}N$ was significantly different between high-protein versus low-protein diets, the mean difference of ~ 0.2‰ is not biologically meaningful and is similar in magnitude to analytical precision for $\delta^{15}N$ analysis of organic substrates via EA-IRMS. The similarity in plasma and liver $\Delta^{15}N$ between high- and low-protein diet treatments implies that the enhanced metabolic activity inherent to these tissues influences their $\delta^{15}N$ value more than variation in dietary protein content or nitrogen balance.

High-protein diet

$\Delta^{15}N$ for muscle and RBC were lower in mice fed high-protein diets than low-protein diets. This pattern meets our expectations because high-protein diets are associated with increased protein routing, which causes the $\delta^{15}N$ value of tissues to more closely resemble that of dietary protein and thus leads to decreased $\Delta^{15}N$ (Schwarcz and Schoeninger 1991; Ambrose and Norr 1993). Mice fed high-protein diets also exhibited greater weight gain and lean mass percentages (Table 2, Online Resource 1), indicative of higher rates of anabolism (Fig. 3a). As anabolic processes draw upon the plasma amino acid pool, it follows that the isotopic composition of muscle should mirror that of the plasma. However, the observed patterns of relatively low muscle $\Delta^{15}N$ and disequilibrium between liver and muscle $\delta^{15}N$ are expected if direct routing of dietary protein to muscle synthesis overwhelmed de novo amino acid synthesis (Fig. 3a).

The positive correlation between increasing hair $\Delta^{15}N$ values and dietary protein content is consistent with previous studies on humans (Petzke et al. 2005) and ungulates

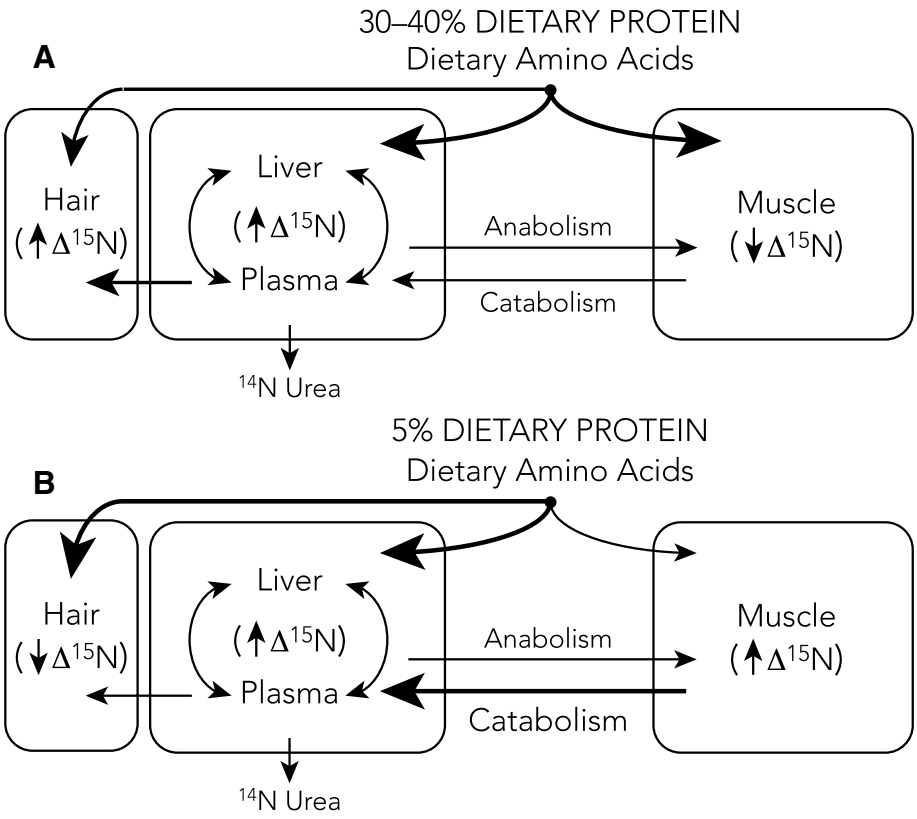

Fig. 3 Conceptual figure describing the relative contribution of anabolic versus catabolic processes as related to dietary protein content on tissue $\Delta^{15}N$. Consequences of **a** high-protein diets are higher anabolism, lower catabolism, and a higher degree of dietary protein routing. The effects of **b** low-protein diets are lower anabolism, higher catabolism, and lower availability of dietary protein for routing. Thickness of arrows represents relative flux through each pathway

(Sponheimer et al. 2003). We hypothesize that for mice fed high-protein diets, muscle and hair were in isotopic equilibrium because they were built from similar proportions of amino acids that were directly routed from diet versus those synthesized de novo from non-protein dietary macromolecules (Newsome et al. 2014) (Fig. 3a).

Low-protein diet

Muscle and RBC $\Delta^{15}N$ values were high for mice fed low-protein diets, suggesting negative nitrogen balance and extensive catabolism (Fig. 3b). We contend that the 'anabolic' model is the most likely mechanism for the increase in tissue $\delta^{15}N$ values during inadequate protein intake, suggesting that amino acids used to rebuild and maintain tissues are derived from the breakdown of endogenous proteins (Lee et al. 2012). The catabolism of these endogenous proteins leads to repeated recycling of nitrogen, yielding greater fractionation, loss of ^{14}N, and a corresponding increase in ^{15}N in the amino acid pool (Hobson et al. 1993). Thus, ^{15}N-enriched amino acids are retained in the body and used to maintain tissues, which explains the increases in $\delta^{15}N$ values observed in the tissues from mice fed low-protein versus high-protein diets (Fig. 3; Sick et al. 1997). Similar increases in muscle $\Delta^{15}N$ during nutritional stress have been observed in captive feeding experiments on American crows (*Corvus brachyrhynchos*), Japanese quail (*Coturnix japonica*), and Ross' Geese (*Chen rossii*) (Hobson and Clark 1992; Hobson et al. 1993). Lee et al. (2012) suggested that the anabolic model should lead to increases in the $\delta^{15}N$ values of liver but not muscle, however, our results indicate that muscle is being maintained by a ^{15}N-enriched amino acid pool in mice fed low-protein diet. This maintenance is likely because the mice in our experiment were neither hibernating nor fasting like the adult Arctic ground squirrels (*Urocitellus parryii*) studied by Lee et al. (2012). Instead, mice in our experiment were growing juveniles, physically active, fed daily, and all maintained or gained weight throughout the trial; although, the mice fed low-protein diets did not gain as much weight and had less lean mass as a proportion of total body weight than mice fed high-protein diets.

The $\delta^{15}N$ values of liver, plasma, and muscle of mice fed low-protein diets were not significantly different from one another, which suggests that catabolic and anabolic processes were acting in tandem to shuttle nitrogen among these tissues, resulting in isotopic equilibrium. In low-protein diets, there was less exogenous protein available for direct routing to tissue synthesis, so the routing of protein directly from diet likely did not overwhelm the de novo synthesis of non-essential amino acids from non-protein dietary macromolecules (Fig. 3b).

Hair $\Delta^{15}N$ was low in mice fed low-protein diets, contradicting some previous studies of humans that consumed low-protein diets; e.g., patients suffering from anorexic

nervosa (Mekota et al. 2006) and pregnant women experiencing weight loss caused by morning sickness (Fuller et al. 2004). Although these two studies found an inverse relationship between hair $\Delta^{15}N$ values and dietary protein content, this may be explained by temporal shifts in dietary nitrogen sources as diet content could not be controlled. In our study, dietary protein $\delta^{15}N$ was constant since casein ($\delta^{15}N = 6‰$) was the sole protein source. The positive relationship we observed between hair $\delta^{15}N$ and protein intake agrees with other studies that used animal- or plant-derived protein (Sponheimer et al. 2003; Petzke et al. 2005). We suggest that mice consuming low-protein diets had lower hair $\Delta^{15}N$ for two possible reasons. First, hair growth was stimulated by shaving a small region of skin. Because thermoregulation is critical to mammalian health and function (Terrien et al. 2011), mice may have prioritized hair regrowth and its associated protein requirements over the maintenance of other tissues. Therefore, these mice could have experienced greater protein routing from exogenous sources into hair to meet these demands (Fig. 3b). Second, the low-protein diets may have altered protein composition of the hair (Noer and Garrigues 1956; Robbins 1993), potentially decreasing the relative abundance of certain amino acids (cysteine, arginine, and methionine; Noer and Garrigues 1956; Koyanagi and Takanohaski 1961; Friedman and Orraca-Tetteh 1978; Robbins 1993), resulting in the observed decreased discrimination. We are currently testing this second explanation via amino acid $\delta^{15}N$ and concentration analyses of mouse tissues from our experiment.

Use of $\delta^{15}N$ to assess body condition and nutritional stress

Our results provide a better understanding of how $\Delta^{15}N$ varies in response to dietary protein content at a tissue-specific level, providing a framework for the use of nitrogen isotopes as tracers of catabolic and anabolic activity during periods of nutritional stress. Specifically, our data provide expectations for the $\Delta^{15}N$ of multiple tissues of small, non-hibernating omnivorous rodents that can be used to study wild populations of this diverse and ubiquitous group of mammals. Our results also show that phenotypic plasticity of mice in response to dietary protein deficiency generates variation in tissue $\delta^{15}N$ that may confound isotope-based estimates of diet composition and trophic level.

The observed pattern in body condition and $\Delta^{15}N$ among tissues suggests that mice fed low-protein diets were in negative nitrogen balance, which resulted in catabolism of labile endogenous protein stores (e.g., skeletal muscle) to maintain vital tissues (e.g., liver) required to survive in a nutritionally compromised state. Therefore, given each tissues' propensity towards catabolic and anabolic processes based on their importance to maintaining bodily function, we propose that certain tissues are more informative for assessing the physiological responses to nutritional stress. We suggest that ecologists interested in minimally invasive assessments of the nutritional state of free-ranging animals should analyze the $\delta^{15}N$ values of muscle, RBCs, and plasma. $\Delta^{15}N$ of muscle and RBC provided the most reliable information regarding body condition and catabolism of endogenous resources, as these metrics closely correlated with dietary protein, weight gain, lean mass, and body fat (Fig. 2). RBC samples are routinely collected from live animals, however, it is also possible to collect non-lethal muscle biopsies from a wide range of larger taxa (Sponheimer et al. 2006; Tilley et al. 2013; Henderson et al. 2016). Our data indicate that blood plasma $\delta^{15}N$ values are not sensitive to nutritional stress, and thus may be a more faithful proxy for assessing diet composition and trophic level than catabolic activity. And because plasma is in isotopic equilibrium with the liver, it is not necessary to sacrifice animals to collect liver for isotope analysis. Although not examined here, measuring the $\delta^{15}N$ value of urine could also prove insightful in assessing nitrogen balance as it is expected to become progressively enriched if the animal is not at steady state (McCue and Pollock 2008), thus reflecting enrichment of the body nitrogen pool. Moreover, the anabolic model requires the preferential excretion of ^{14}N via urea, so measuring the $\delta^{15}N$ values of urine would provide useful information to complement tissue $\delta^{15}N$ data. And because incorporation rates differ among tissues (Martínez del Rio et al. 2009), it is important to remember that shifts over time in the nitrogen isotope values of dietary inputs could cause changes in $\Delta^{15}N$ similar in magnitude and direction as those observed in response to nutritional stress (1–2‰, Fig. 1). Lastly, tissue $\delta^{15}N$ data should be paired with direct data on body condition, such as that generated by field-portable QMR systems like the one used in our study, to create a comprehensive understanding of the physiological pathways by which animals were likely gaining or losing lean mass at the time of sampling.

Researchers should also consider the emerging technique of amino acid $\delta^{15}N$ analysis, which could provide an additional context for interpreting nitrogen dynamics during anabolic and catabolic activity. For example, "trophic" amino acids are easily transaminated and therefore their $\delta^{15}N$ increases with trophic level (O'Connell 2017). Some trophic amino acids are non-essential (e.g., glutamic acid, aspartic acid) and are likely synthesized de novo by animals eating protein-deficient diets. In contrast, other trophic amino acids are essential (e.g., isoleucine, valine, leucine) and cannot be synthesized de novo. It is thus possible that the $\delta^{15}N$ values of non-essential, trophic amino acids increase during negative nitrogen balance, while the $\delta^{15}N$ of essential, trophic amino acids remains constant. We are currently testing this hypothesis via amino acid $\delta^{15}N$ analysis of the mouse tissues collected in this experiment and we encourage other

researchers to examine such patterns with additional controlled feeding experiments.

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Author contribution statement KLH and SDN conceived the ideas and designed methodology, KLH performed analytical measurements, all authors analyzed the data, and KLH led the writing of the manuscript.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. Animal handling and husbandry was conducted with the approval of the UNM Institutional Animal Care and Use Committee (16-200492-MC).

Data accessibility All body condition and tissue $\delta^{15}N$ values are present in the manuscript.

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