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PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Assimilation and discrimination of hydrogen isotopes in a terrestrial mammal

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Abstract

Stable isotope analysis has revolutionized the way ecologists study animal resource use from the individual to the community level. Recent interest has emerged in using hydrogen isotopes ($^2H/^1H$) as ecological tracers, because they integrate information from both abiotic and biotic processes. A better physiological understanding of how animals assimilate hydrogen and use it to synthesize tissues is needed to further refine this tool and broaden its use in animal ecology. We conducted a controlled-feeding experiment using laboratory mice (*Mus musculus*) in which we varied the hydrogen isotope (δ^2H) values of water and the proportions of dietary protein and carbohydrates among nine experimental treatments. For each tissue, we calculated the percent of hydrogen derived from water and the percent hydrogen derived from dietary protein versus carbohydrates using linear relationships and isotope mixing models based on accompanying carbon isotope ($\delta^{13}C$) data. The net discrimination (Δ^2H_{Net}) between mice tissues and potential water and dietary sources of hydrogen differed among tissues. Δ^2H_{Net} was positively correlated with dietary protein content in red blood cells (RBC) and muscle, but negatively correlated in liver and plasma. We also report the first estimates for hydrogen isotope discrimination factors (Δ^2H) for different sources of hydrogen (Δ^2H_{Water}, $\Delta^2H_{Protein}$, and Δ^2H_{Carbs}) available for tissue synthesis. This research provides a foundation for understanding how diet quality (e.g., protein content) influences hydrogen isotope assimilation and discrimination in different tissues of a terrestrial mammal, which is a first step towards using δ^2H as a tracer of resource use in free-ranging mammals.

Keywords Hydrogen isotope analysis · Controlled-feeding experiment · Stable isotopes analysis · Hydrogen discrimination (Δ^2H) · Tissue-specific δ^2H discrimination

Introduction

In the past two decades, stable isotope analysis has provided valuable insights into both individual and ecosystem processes in nearly every sub-discipline of ecology (Phillips et al. 2014). Specifically, hydrogen isotope (δ^2H) analysis of animal tissues has gained tremendous momentum as a tracer of animal movement and migration (Chamberlain et al. 1997; Hobson et al. 1999; Rubenstein and Hobson 2004; Bearhop et al. 2005). This approach is based on natural hydrological processes that affect δ^2H of precipitation (δ^2H_{Prec}) with varying latitude and altitude (Craig 1961; Dansgaard 1964), and the understanding that the δ^2H of animal tissues reflect δ^2H of water and food resources they consume (DeNiro et al. 1981; Bowen and Revenaugh 2003; Hobson et al. 1999). Many studies have reported a significant positive linear relationship between bird tissues (feathers) and that of local precipitation (Hobson and Wassenaar 1997; Wolf et al. 2002; Hobson 2008). These relationships, however, are often noisy with low goodness of fit (Hobson 2008), which has been attributed to differences in analytical techniques (e.g., Hobson 2008), as well as ecological (Birchall et al. 2005) and physiological mechanisms (Soto et al. 2013b; Newsome et al. 2017). Regardless, δ^2H values have been widely used to study animal migration, because external devices, such as GPS tags and telemetry antennae,

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are not feasible for studying movement of small-bodied birds and insects.

The δ^2H of local, environmental water exerts primary control on δ^2H of organisms at any trophic level, because plants use environmental water to synthesize organic compounds that fuel food webs. Lab- and field-based studies show a predictable negative isotopic discrimination between δ^2H of environmental water (e.g., precipitation and ground water) and that of primary producer tissues (Smith and Epstein 1970; Yakir 1992; Roden and Ehleringer 2000). However, this discrimination can systematically vary by 50–150‰ among plant and algae taxa (Estep and Hoering 1980; Yakir and Deniro 1990; Terwilliger et al. 2002), resulting in large differences in the δ^2H values of co-existing primary producers in marine and terrestrial ecosystems (Estep and Dabrowski 1980).

While water is a fundamental component in many biosynthetic processes (Yang and Leng 2009; Fogel et al. 2016), it only contributes a minor proportion (15–35%) of the hydrogen used by both prokaryotic and eukaryotic consumers to synthesize tissues; the large majority of hydrogen in consumer tissues is derived from food (Macko et al. 1983; Hobson et al. 1999; Ehleringer et al. 2008; Fogel et al. 2016). The fact that animals integrate hydrogen into their tissues from a variety of sources presents both challenges and opportunities for ecologists. For example, the variability in contribution of hydrogen from different sources is a challenge for movement-based studies, because it likely influences the observed noise in relationships between local precipitation and consumer tissue δ^2H values (Hobson 2008; Pietsch et al. 2011). On the other hand, the fact that δ^2H of animal tissue largely trace diet provides an opportunity to develop a new tool to study species interactions and energy flow within and among food webs (Birchall et al. 2005; Doucett et al. 2007; Solomon et al. 2009; Karlsson et al. 2012; Newsome et al. 2017).

To better utilize δ^2H as a tracer of food use, ecologists, and eco-physiologists need to conduct more controlled-feeding experiments to better understand how hydrogen isotopes are assimilated during physiological processes, and what influences isotope discrimination between δ^2H of consumer tissue and their dietary and water sources. A few natural and controlled-feeding experiments have estimated hydrogen isotope incorporation rates (Podlesak et al. 2008, Storm-Suke et al. 2012a, b; Wolf et al. 2012) and assimilation of hydrogen from a variety of sources in birds (Hobson et al. 1999; Wolf et al. 2012, 2013), humans (Ehleringer et al. 2008), fish (Soto et al. 2013b, Newsome et al. 2017), invertebrates (Solomon et al. 2009; Wang et al. 2009, Finlay et al. 2010; Nielson and Bowen 2010; Soto et al. 2013a), and bacteria (Fogel et al. 2016). These authors have suggested that hydrogen isotope discrimination is complex and is likely influenced by diet composition (Doucett et al. 2007; Finlay et al. 2010), trophic position (Birchall et al. 2005; Lott and Smith 2006; Solomon et al. 2009), and physiology (Soto et al. 2013b; Newsome et al. 2017). However, like carbon isotopes (Caut et al. 2009; Wolf et al. 2015; Ben-David et al. 2012), hydrogen discrimination is likely tissue-specific and driven by differences in amino acid concentrations among tissues (Fogel et al. 2016; Newsome et al. 2017). These experiments show that there is opportunity to use δ^2H values to trace energy within and among food webs; however, we know little about how hydrogen isotope discrimination varies with diet quality (e.g., protein content), which is an important source of variation in observed discrimination of carbon and nitrogen isotopes within and among taxa (O'Brien et al. 2005; Poupin et al. 2011; Ben-David et al. 2012; Wolf et al. 2015).

We conducted a controlled-feeding experiment on a terrestrial omnivorous mammal (*Mus musculus*) in which both the δ^2H values of (drinking/food) water and the relative proportions of dietary protein and carbohydrates were varied. The goals of this study were to (1) quantify the proportion of hydrogen in animal tissues that is derived from food and water, (2) estimate the net hydrogen isotope discrimination factors (Δ^2H_{Net}) between mouse tissues and sources of hydrogen (water and food) available for tissue synthesis, (3) estimate hydrogen isotope discrimination (Δ^2H) between mouse tissues and specific (macro)molecules, including water (Δ^2H_{Water}), protein ($\Delta^2H_{Protein}$), and carbohydrates (Δ^2H_{Carbs}), and (4) investigate how diet quality (protein content) affects tissue δ^2H, Δ^2H_{Net}, and (macro)molecule-specific Δ^2H. Our experiment highlights some of the ecological (i.e., diet quality) and physiological (e.g., protein routing) processes that influence hydrogen isotope discrimination in a terrestrial mammal, which we anticipate will aid in future studies that include δ^2H as a tracer of diet to study interactions of animals within food webs.

Methods and materials

Experimental design and tissue collection

Sixty-three weanling house mice (*Mus musculus*) were purchased from Charles River Laboratories (Inc.) and raised in 18 × 12 inch plastic containers with a constant temperature of ~ 22 °C and a photoregimen of 12 h days and 12 h nights at the UNM Animal Research Facility. Access to food and water was ad libitum for the entire experiment, which were replenished daily. Our experiment was composed of a 3 × 3 block design in which seven mice were randomly assigned to one of nine treatment groups. Each mouse was weighed and fitted with an electronic pit tag (BioMark LPT8) for identification. Mice were weighed once a week to monitor their health. After 120 days, mice were sacrificed by CO_2

asphyxiation, the mice were dissected, and tissues were immediately collected for stable isotope analysis. Blood was collected using heparinized micro-capillary tubes within 2 min of death and stored on ice. Blood was subsequently separated within 6 hof collection into plasma and red blood cells (RBC) via centrifugation at 10 k rpm for 10 min. The quadriceps femoris muscle and liver were also collected and stored frozen in plastic micro-centrifuge tubes at − 20 °C. All animal handling and husbandry were conducted with the approval and under the guidelines for care and use of animals of the University of New Mexico Institutional Animal Care and Use Committee (13-1010131-MC).

Experimental waters and diets

Table 1 displays the proportions (weight percent) and isotopic composition of ingredients used to make the three synthetic diets used in our experiment. Throughout the experiment, the mice were supplied with water of consistent δ^2H values, with a total of three drinking water treatments. We used Albuquerque, NM tap water (− 95 ± 2‰) for one treatment and for the other two tap water was mixed with di-deuterium oxide (D_2O) to create drinking water that had δ^2H values of − 50 and + 5‰. ^2H-enriched water treatments were mixed at the beginning of the experiment and stored in 20L carboys. Aliquots of each water treatment were analyzed weekly to ensure their δ^2H values remained constant throughout the experiment. δ^2H values of drinking water collected throughout the experiment were measured to be − 95 ± 2, − 50 ± 2, and + 5 ± 2‰ for the three treatments.

The relative proportions of protein (casein) and carbohydrates (sucrose) were varied among diet treatments, and the proportions of all other ingredients were kept constant (Table 1, diets formulated in accordance with Rowe et al. 1974). All dry ingredients (Table 1) were homogenized with ~ 4 L of the treatment-specific water assigned to them, so that water available in food would have similar δ^2H values as drinking water. Diets were homogenized with waters in individual tubs and stored frozen (− 20 °C) to minimize evaporation and to prevent microbial contamination. Each treatment was given ~ 10 g of food per mouse daily throughout the experiment.

Stable isotope analysis

Muscle and liver were lipid extracted via immersion in a 2:1 chloroform:methanol solvent solution for 72 h; a fresh aliquot of solvent solution was replaced every 24 h. The tissues were then rinsed five times with DI water to remove residual solvent and subsequently freeze dried for ~ 24 h. Blood plasma was directly pipetted into pre-weighed tin or silver capsules, dried in an oven at 50 °C to remove water,

Table 1 Macromolecular components of each dietary treatment

Ingredient	Macromolecule	Diet 1	Diet 2	Diet 3	δ^2H (SD)	[H] (SD)	$\delta^{13}C$ (SD)	[C] (SD)	$\delta^{15}N$ (SD)	[N] (SD)
Casein	Protein	0.05	0.20	0.35	− 107 ± 4.2	6.0 ± 0.02	− 24.8 ± 0.2	48.4 ± 0.32	5.5 ± 0.3	14.1 ± 0.35
Sucrose	Carbohydrates	0.45	0.30	0.15	− 14 ± 4.1	6.3 ± 0.04	− 11.7 ± 0.8	41.9 ± 0.15	–	–
Corn Meal	Carbohydrates	0.15	0.15	0.15	− 18 ± 4.4	5.8 ± 0.05	− 10.8 ± 0.2	47.2 ± 0.05	1.5 ± 0.8	0.8 ± 0.05
Corn Oil	Lipids	0.02	0.02	0.02	− 154 ± 4.6	9.6 ± 0.21	− 15.5 ± 0.2	73.9 ± 2.65	–	–
Cellulose	Binder	0.25	0.25	0.25	− 25 ± 4.0	5.8 ± 0.02	− 25.6 ± 0.2	41.9 ± 0.41	–	–
Fortified Salt	Salt	0.04	0.04	0.04	–	–	–	–	–	–
Brewer's Yeast	Yeast	0.02	0.02	0.02	− 66 ± 4.1	5.8 ± 0.05	− 21.4 ± 0.3	44.0 ± 0.30	3.2 ± 0.2	7.3 ± 0.15
Vitamin Mix	Vitamins	0.01	0.01	0.01	1 ± 4.4	6.3 ± 0.05	− 12.9 ± 0.3	39.9 ± 0.38	–	–
	Protein:Carb Ratio	5:60	20:45	35:30						
	Bulk δ^2H	− 26.0 ± 3.0	− 41.0 ± 3.0	− 56.0 ± 3.0						
	Bulk $\delta^{13}C$	− 16.2 ± 0.2	− 18.3 ± 0.2	− 20.3 ± 0.2						
	Bulk $\delta^{15}N$	2.6 ± 0.7	3.8 ± 0.6	4.3 ± 0.5						

The proportion, δ^2H, $\delta^{13}C$, $\delta^{15}N$, and dry mass fraction of hydrogen, carbon, and nitrogen (i.e., [H], [C], and [N]) of each ingredient that was used to synthesize the diets are reported. Across diets (Diets 1, 2, and 3) only Casein and Sucrose change in proportion, changing the protein:carbohydrate ratio and the bulk δ^2H and $\delta^{13}C$ (reported for each diet)

and then re-reweighed to estimate sample weight prior to isotope analysis; RBC was simply freeze dried.

For δ^2H analysis, ~ 0.1–0.2 mg of dried mouse tissue or diet ingredient was sealed in 3 × 5 mm silver capsules and along with internal reference materials was placed in a drying oven (50 °C) under a steady stream of N_2 to for at least 2 weeks prior to analysis to ensure that samples remained dry and were subjected to the same ambient water vapor (Bowen et al. 2005). For $\delta^{13}C$ and $\delta^{15}N$ analysis, ~ 0.5–1.0 mg of dried mouse tissue or diet ingredient was sealed in 3 × 5 mm tin capsules.

Hydrogen isotope (δ^2H) values of mice tissues and dietary ingredients were measured with a Thermo Scientific thermal conversion elemental analyzer (TCEA) coupled to a Thermo Scientific Delta V isotope ratio mass spectrometer following pyrolysis at 1400 °C and reduction with glassy carbon. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values were measured on a Costech 4010 elemental analyzer coupled to a Thermo Scientific Delta V isotope ratio mass spectrometer. All isotope measurements were conducted at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). Stable isotope data are expressed as δ values using the equation $\delta X = (R_{Sample}/R_{Standard}) - 1$, where X is any isotope system of interest (e.g., H, C, or N) and R_{Sample} and $R_{Standard}$ are the ratios of the heavy to light isotope (e.g., $^2H/^1H$, $^{13}C/^{12}C$) for each sample and standard, respectively. The internationally accepted standards are Vienna Standard Mean Ocean Water (V-SMOW) for δ^2H, Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$, and atmospheric nitrogen for $\delta^{15}N$; units are expressed as parts per thousand, or per mil (‰).

Mean within-run analytical precision (± SD) for δ^2H measurements of organic materials was determined by analysis of external (USGS-KHS and USGS-CBS) and internal reference materials described below and measured to be ± 2–3‰ (SD). Analytical precision for $\delta^{13}C$ and $\delta^{15}N$ was determined via analysis of protein- and carbohydrate-based internal reference materials and measured to be ± 0.2‰ (SD). We also measured the weight percentage hydrogen ([H]), carbon ([C]), and nitrogen ([N]) concentrations of each sample via analysis of organic materials with known elemental concentrations. Precision of drinking water δ^2H values was measured with a Picarro WS-CRDS-based analyzer and normalized to the V-SMOW-SLAP scale via repeated analysis of three laboratory reference waters (− 42, − 68, and − 95‰) that were included in each run. Average within-run analytical precision (± SD) was ± 1‰ and measured tank water δ^2H data were reduced using a three-point linear calibration of internal reference waters. We collected and analyzed drinking water bi-weekly to assure that there was no change in water δ^2H over the course of the experiment.

Approximately 5–20% of the organically bound hydrogen in proteins and carbohydrates can exchange freely with hydrogen in (ambient) atmospheric water vapor (Wassenaar and Hobson 2000; Bowen et al. 2005; Sauer et al. 2009; Meier-Augenstein et al. 2011; Qi and Coplen 2011; Coplen and Qi 2012); however, lipids contain negligible amounts of exchangeable hydrogen, because nearly, all of the hydrogens are bound to carbon. To correct for exchangeable hydrogen in organic tissues, we made a series of lipid-extracted protein (keratin, muscle, and liver) and carbohydrate (sucrose and leaves) internal reference materials for which the δ^2H composition of the non-exchangeable fraction of hydrogen ($\delta^2H_{non\text{-}ex}$) was determined via equilibration experiments (Bowen et al. 2005) followed by verification at two other stable isotope facilities: Carnegie Institution for Science (Washington, DC) and the University of Wyoming (Laramie, WY). Detailed information about these exchange experiments is provided in the supporting online material. Internal reference materials were normalized to the V-SMOW scale and their $\delta^2H_{non\text{-}ex}$ values bracket that of most of our unknown samples; keratins: − 55, − 95, − 165‰; muscle: − 70 and − 150‰; liver: − 50 and − 135‰; sucrose: − 15‰; α-cellulose: − 45‰; and leaves: − 82‰. We also used a series of non-exchangeable oil internal reference materials (− 115, − 175, and − 230‰) and USGS NBS-22 (− 117‰) scale normalized to V-SMOW to measure dietary lipid δ^2H values. Finally, we used external (published) keratin standards USGS-KHS and USGS-CBS as an independent check on our internal (proteinaceous) reference materials. While published δ^2H standards are not available for most of the tissues we analyzed in our study, the proportion of exchangeable hydrogen in muscle, liver, and blood (~ 17–20%) is very similar to the range reported for keratins (Schimmelmann 1991; Wassenaar and Hobson 2000).

Mixing models

We used an isotopic mass balance equation to distinguish among different sources of hydrogen available for tissue synthesis (Eq. 1):

$$\begin{aligned}\delta^2H_{Tissue} = {} & p_{Water}\left(\delta^2H_{Water} + \Delta^2H_{Water}\right) \\ & + p_{Protein}\left(\delta^2H_{Protein} + \Delta^2H_{Protein}\right) \\ & + p_{Carbs}\left(\delta^2H_{Carbs} + \Delta^2H_{Carbs}\right),\end{aligned} \quad (1)$$

where δ^2H_X is the isotopic composition of X, p_S is the proportion of hydrogen derived from source (S), and Δ_X is the isotope discrimination factor associated with X in the models. Lipids were not considered in models, because they were minor components of diet (2%) and the previous studies (e.g., Newsome et al. 2017) have shown that they make minimal contributions to tissue synthesis at low

dietary proportions. Furthermore, we have excluded cellulose, salt, Brewer's yeast, and the vitamin mix from our mixing models, because these are all dietary components that are either not broken down by the body (e.g., cellulose and vitamin mix), do not have hydrogen (e.g., salt), or are in such low quantities that incorporation is minimal (e.g., Brewer's yeast). Ultimately, the total percent of hydrogen that a consumer can incorporate must be a total of the available hydrogen from food and water (Eq. 2):

$$p_{\text{Water}} + p_{\text{Protein}} + p_{\text{Carbs}} = 1. \tag{2}$$

By rearranging Eq. 1 and extracting the Δ terms with their associated p terms, we can solve for Δ^2H_{Net}, the net effective trophic discrimination factor of hydrogen of the different sources that mice had available to them (Eq. 3):

$$\Delta^2H_{\text{Net}} = p_{\text{Water}}\left(\Delta^2H_{\text{Water}}\right) + p_{\text{Protein}}\left(\Delta^2H_{\text{Protein}}\right) + p_{\text{Carbs}}\left(\Delta^2H_{\text{Carbs}}\right). \tag{3}$$

Δ^2H_{Water}, $\Delta^2H_{\text{Protein}}$, and Δ^2H_{Carbs}

Once we estimated the percent of hydrogen derived from water, protein, and carbohydrates, we solved a system of equations (regression analysis) to estimate the discrimination factors of each of those sources. Ultimately, we had three different equations (averaged across diet treatment) associated with each tissue, so we mathematically solved for discrimination factors by solving a system the equations with the three unknown variables being the discrimination factors of water, protein, and carbohydrates following Cramer's rule (Eq. 4):

$$\begin{bmatrix} p_{X_{\text{Water}}}\left(\Delta_{X_{\text{Water}}}\right) + p_{X_{\text{Protein}}}\left(\Delta_{X_{\text{Protein}}}\right) + p_{X_{\text{Carbs}}}\left(\Delta_{X_{\text{Carbs}}}\right) = \delta^2\Delta_{X\text{Net}} \\ p_{Y_{\text{Water}}}\left(\Delta_{Y_{\text{Water}}}\right) + p_{Y_{\text{Protein}}}\left(\Delta_{Y_{\text{Protein}}}\right) + p_{Y_{\text{Carbs}}}\left(\Delta_{Y_{\text{Carbs}}}\right) = \delta^2\Delta_{Y\text{Net}} \\ p_{Z_{\text{Water}}}\left(\Delta_{Z_{\text{Water}}}\right) + p_{Z_{\text{Protein}}}\left(\Delta_{Z_{\text{Protein}}}\right) + p_{Z_{\text{Carbs}}}\left(\Delta_{Z_{\text{Carbs}}}\right) = \delta^2\Delta_{Z\text{Net}} \end{bmatrix}, \tag{4}$$

where X is low (5%), Y is medium (20%), and Z is high (35%) protein content, δ^2H_{X-Z} is the isotopic composition of the sources that vary in δ^2H composition (water, protein, and carbohydrates) p_{X-Z} are the percent of hydrogen derived from these sources, and Δ_{X-Z} are the discrimination factor for each source in each of the treatments.

Statistical analysis

We used a standard least-squares linear regression to calculate the percent of hydrogen derived from water (Ehleringer et al. 2008) using the following equation (Eq. 5):

$$\delta^2H_{\text{Tissue}} = p_{\text{Water}}\left(\delta^2H_{\text{Water}}\right) + \beta, \tag{5}$$

where $\delta^2H_{\text{Tissue}}$ is the isotopic composition of tissues, p_{Water} is the slope of the regression which is equal to the percent of hydrogen derived from water, δ^2H_{Water} is the isotopic composition of the different water treatments, and β is the y-intercept received from the model.

All linear models, t tests, one-way ANOVA's, Wilcoxon tests, and Mixing Models were processed using R statistical packages (e.g., stats, BSDA, car, and SIAR; R Core Team 2016). We used a modified Welch two-sample t tests to test for statistical differences among slopes of each tissue across dietary treatments. To examine the differences between mouse tissues across dietary and water treatments, we used a one-way ANOVA combined with a post hoc Tukey honest significant difference (HSD) test. We used a non-parametric Wilcoxon–Mann–Whitney two-sample test, with a null hypothesis mean difference setting equal to 0, to assess differences in the net effective trophic discrimination factor of hydrogen (Δ^2H_{Net}) among tissues and diet treatments. To estimate the percent of hydrogen derived from dietary protein (p_{Protein}) and carbohydrates (p_{Carbs}) that had distinct $\delta^{13}C$ values (Table 1), we used a Bayesian isotope mixing model Stable Isotope Analysis in R (SIAR, Parnell et al. 2010); more details about this model are available in Supplementary material: Using $\delta^{13}C$ to Estimate Proportion of Hydrogen Derived from Protein and Carbohydrates. This approach assumes that carbon and hydrogen are routed to similar degrees from dietary macromolecules to tissues, which we believe is a robust first-order approximation based on both theoretical considerations and empirical data (see "Discussion").

Results

Mice in all treatments gained weight over the course of the experiment (SM Fig. 4), suggesting that even the low-protein diet (5% protein) was sufficient to maintain positive protein balance.

p_{Water}

Linear correlations between δ^2H of drinking water and δ^2H of tissues were all found to be positive and significant (Fig. 2); summary and statistical output are reported in SM Tables 1 and 3, respectively. The mean proportions of hydrogen derived from water (p_{Water}) in each tissue ranged from 15.3 ± 2 to 26.2 ± 3% (SM Table 1). In plasma and liver, estimates of p_{Water} for diet treatments with low-protein content were higher than medium and high protein treatments (SM Table 1). These values ranged from 15.3 to 24.2% and 18.7 to 26.2%, respectively. In contrast, estimates of mean p_{Water} for red blood cells (RBC) and muscle tissue were similar

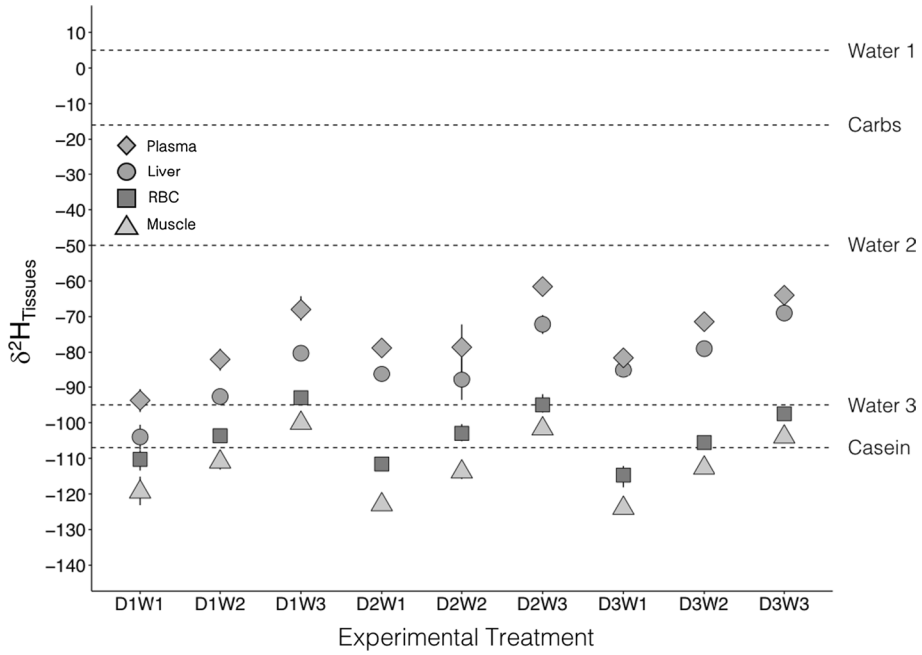

Fig. 1 Tissue δ^2H values across experimental diet (*D*) and water (*W*) treatments for plasma (diamonds), liver (circles), RBC (squares), and muscle (triangles). Dashed lines represent δ^2H values of water, dietary protein (casein), and dietary carbohydrates (carbs); error bars (± SD) not shown are smaller than symbols

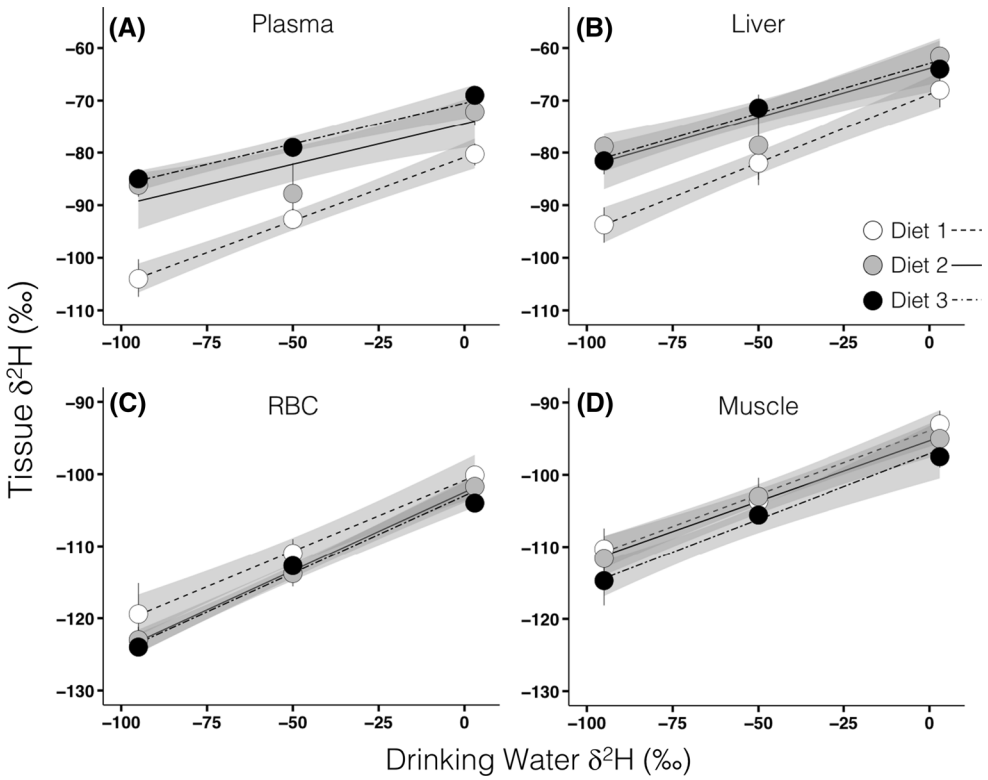

Fig. 2 Linear model (with 95% confidence intervals) of δ^2H values of drinking water versus plasma (**a**), liver (**b**), RBC (**c**), and muscle (**d**) separated by diet treatment; error bars (± SD) not shown are smaller than symbols

among diet treatments and ranged from 19.7 to 21.9% and 16.8 to 18.4%, respectively (SM Table 1).

For plasma and liver, estimates of p_{Water} were found to be significantly higher for the low-protein treatment than the medium and high treatments (Fig. 2), see SM Table 3 for statistical output. For RBC and muscle, estimates of p_{Water} were not significantly different across treatments (SM Table 3).

$p_{Protein}$ and p_{Carbs}

Mean estimates of $p_{Protein}$ and p_{Carbs} for each tissue from each diet and water treatment are reported in Fig. 3. Dietary protein content was positively and significantly correlated with $p_{Protein}$ in each tissue (see SM Fig. 1, SM Table 5). Dietary protein content had the largest effect

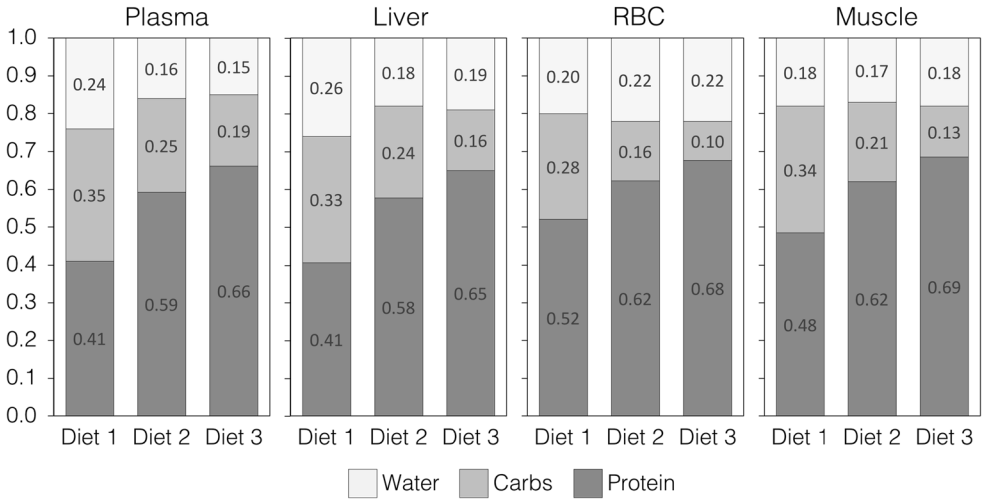

Fig. 3 Summary of the mean proportion of hydrogen derived from water (p_{Water}), carbohydrates (p_{Carbs}), and protein ($p_{Protein}$) for each treatment

on $p_{Protein}$ of plasma (slope: 0.86 ± 0.04) and liver (slope: 0.85 ± 0.03), which was higher than slopes of this relationship for RBC (slope: 0.53 ± 0.02) or muscle (slope: 0.67 ± 0.03; SM Table 6).

Effect of diet quality on tissue isotope composition

Our experimental treatments varied the protein-to-carbohydrate content within mouse diets, which also changed the bulk δ^2H and $\delta^{13}C$ composition of each diet treatment. The δ^2H value of dietary protein (δ^2H: $-107‰$) was much lower than that of carbohydrates (δ^2H: $-16‰$); therefore, an increase in protein content within diets caused a decrease in the bulk δ^2H of diet. Plasma and liver had consistently higher δ^2H values than RBC and muscle (Fig. 1). We found significant linear relationships between the isotopic composition of our tissues analyzed ($\delta^2H_{Tissues}$) and the percent protein in diet (%); however, the relationships were found to be positive for plasma and liver and negative for RBC and muscle (SM Fig. 2). Surprisingly, δ^2H of plasma and liver did not vary in accordance with an increase in protein (lower δ^2H of bulk diet), even though $p_{Protein}$ was positively correlated with the percent of protein in diet.

Net hydrogen isotope discrimination (Δ^2H_{Net})

Figure 4 represents the estimates of Δ^2H_{Net} for each tissue grouped by dietary and water treatment (SM Table 2). Varying the isotopic composition of water did not change Δ^2H_{Net} across diet treatments (SM Table 2; Fig. 4). We found that only four comparisons of Δ^2H_{Net} values across treatments significantly differed (Plasma: D2W2–D2W3, $p = 0.05$, Liver: D2W1–D2W2 and D2W2–D2W3,

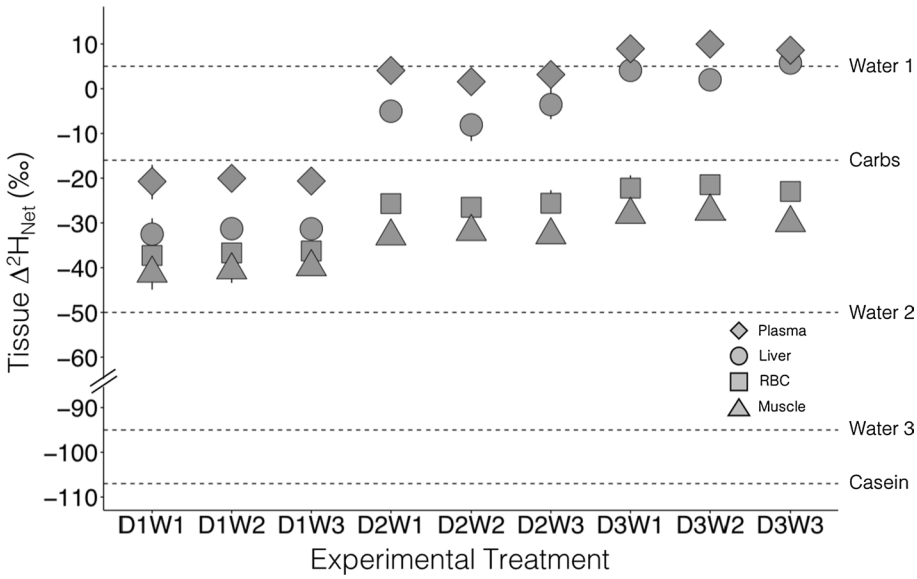

Fig. 4 Δ^2H_{Net} across the experimental diet (*D*) and water (*W*) treatments for plasma (diamonds), liver (circles), RBC (squares), and muscle (triangles). Dashed lines represent δ^2H values of water, dietary protein (casein), and dietary carbohydrates (carbs); error bars ($\pm$ SD) not shown are smaller than symbol

$p = 0.02$ and 0.05, respectively, and Red Blood Cells: D3W2–D3W3, $p = 0.02$). However, these differences are likely due to a single individual that was an outlier within D2W2 and D3W2. Because of our sample sizes, statistical power was given to individuals that diverged greatly from the mean. We contend that varying the isotopic composition of water did not change Δ^2H_{Net} across diet treatments, comparing the overall mean of each treatment to the treatment themselves (SM Table 2; Fig. 4). Δ^2H_{Net} of all tissues analyzed was positively and significantly correlated with dietary protein content (see SM Fig. 3, $p < 0.001$). The effect of changing protein content more strongly influenced Δ^2H_{Net} and was more similar between plasma and liver (1.19 ± 0.07 and 1.01 ± 0.06, respectively), compared to red blood cells and muscle (0.42 ± 0.03 and 0.50 ± 0.04, respectively) (see SM Fig. 3, Table 5).

Δ^2H_{Water}, $\Delta^2H_{Protein}$, and Δ^2H_{Carbs}

The mean Δ^2H_{Water} was always negative and varied between -141 ± 28 and -70 ± 45 among tissues (Fig. 5). Likewise, Δ^2H_{Carbs} was also negative and varied between -74 ± 13 and -45 ± 21. In contrast to Δ^2H_{Water} and Δ^2H_{Carbs}, $\Delta^2H_{Protein}$ was positive for all tissues (Fig. 5), but statistically indistinguishable from zero for muscle ($2 \pm 10‰$) and RBC ($11 \pm 30‰$). $\Delta^2H_{Protein}$ for liver ($56 \pm 5‰$) and plasma ($50 \pm 2‰$) were higher than that for muscle and RBC. Mean tissue-specific discrimination factors for water, protein, and carbohydrates are represented in Fig. 5 (reported in SM Table 3). Mean $\Delta^2H_{Protein}$ was higher than Δ^2H_{Water} and Δ^2H_{Carbs} ($p < 0.001$). Unfortunately, we could not assess Δ^2H_{Water}, $\Delta^2H_{Protein}$, and Δ^2H_{Carbs} variations within each diet treatment, because the statistical method we used required the simultaneous analysis of data from all dietary treatments. We acknowledge that variation within treatments likely explains some of the variance observed in our Δ^2H estimates for specific macromolecules; however, we still observed significant differences in hydrogen isotope discrimination among macromolecules (Fig. 5).

Discussion

Using the combination of δ^2H and $\delta^{13}C$ data, we quantified (1) the proportion of hydrogen sourced from water versus individual dietary macromolecules and (2) hydrogen isotope discrimination between these potential sources of hydrogen and a variety of tissues commonly analyzed by ecologists. In the following sections, we discuss the mechanisms and metabolic pathways that are likely responsible for the patterns in hydrogen isotope discrimination within and among tissues across diet and water treatments in our experiment. Our results are a first step toward developing a mechanistic (biochemical) understanding of diet to tissue hydrogen isotope discrimination in an omnivorous mammal, which we anticipate will broaden the use of δ^2H as a tool to study foraging ecology and species interactions in terrestrial ecosystems.

Proportion of water (p_{Water})

The percent of hydrogen derived from drinking water (p_{Water}) in mice tissues (15–26%, Fig. 2) is within the range reported in previous experiments on both aquatic and terrestrial animals (Hobson et al. 1999; Ehleringer et al. 2008; Tuross et al. 2008; Solomon et al. 2009; Wolf et al. 2011; Soto et al. 2013a; Newsome et al. 2017). Although our experiment did not directly measure δ^2H of body water, we assumed that the large majority of body water in our laboratory mice was comprised of drinking water (Soto et al. 2013a and b) for several reasons. First, the previous work shows that ~ 70–85% of body water in mice is sourced from pre-formed (drinking or food) water (Podlesak et al. 2008), with most of the remaining portion consisting of metabolic water produced as a byproduct of respiration (Podlesak et al. 2008; Soto et al. 2013a, b). Second, house mice are obligate drinkers with high body water turnover rates (Podlesak et al.

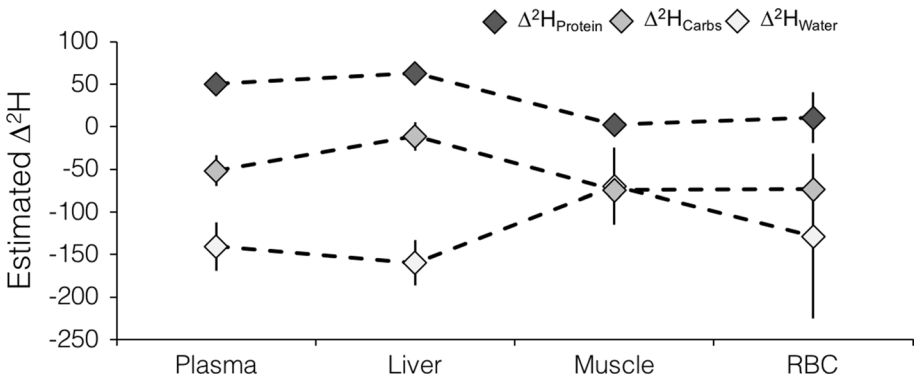

Fig. 5 Mean (±SE) of Δ^2H_{Water}, $\Delta^2H_{Protein}$, Δ^2H_{Carbs} for each tissue; error bars (±SD) not shown are smaller than symbol. Dashed lines are for graphical clarity and do not have statistical meaning

2008) and are offered drinking water ad libitum throughout our experiment. Finally, our diets were homogenized with water that had the same δ^2H value as the drinking water associated with each treatment.

Our results of liver and blood plasma also showed that when dietary protein is limited, p_{Water} was significantly higher (24–26%) in comparison with the intermediate or high protein treatments (15–19%); no difference was observed in p_{Water} for muscle or RBC among diet treatments (Fig. 3, SM Table 1). It is not surprising that patterns observed in liver are mirrored by blood plasma, which carries molecules to/from the liver, and thus, these two tissues are expected to be in isotopic steady state (Karasov and Martinez del Rio 2008). A recent experiment on *Escherichia coli* fed a protein-free, glucose medium found that 52% (±7) of the hydrogen in non-essential amino acids synthesized de novo was sourced from environmental water; p_{Water} decreased to only 23% (±5) when *E. coli* was cultured in a protein-rich (tryptone) medium (Fogel et al. 2016). House mice and *E. coli* share the same basic metabolic machinery in which body water is incorporated into amino acids via the formation of pyruvate in glycolysis and through a series of hydration reactions in the TCA cycle. The degree to which body water hydrogen is used to synthesize non-essential amino acids for tissue growth and maintenance depend on organ-specific requirements. For example, the liver is a more metabolically active tissue than muscle or RBC, and is a critical organ in the metabolism of all macromolecules (Karasov and Martinez del Rio 2008). Due to the livers' high metabolic activity and isotopic incorporation rate (Martinez del Rio et al. 2009), there is greater likelihood for hydrogen from body water to be incorporated into the synthesis of compounds (e.g., non-essential amino acids) required to maintain liver tissue.

Proportion of protein ($p_{Protein}$) and carbohydrates (p_{Carbs})

As expected, the large majority of hydrogen used to synthesize plasma (81.0±4.0%), liver (78.0±4.0%), red blood cells (79.0±1.0%), and muscle (82.0±1.0%) was derived from organic hydrogen in food (Fig. 3). Our experimental design allowed us to quantify the amount of hydrogen sourced from dietary protein versus carbohydrates utilizing associated $\delta^{13}C$ data, because the dietary protein in our experiments had a much lower, C_3-based $\delta^{13}C$ value (−24.8±0.2‰) than dietary, and C_4-based carbohydrates (−11.3±0.8‰) or lipids (−15.5±0.2‰). We contend that this carbon-based approach provides a robust first-order approximation of protein routing of hydrogen for both theoretical and empirical reasons. First, most hydrogen (~60%) in the amino acids found in the tissues we analyzed is tightly bound to carbon (SM Table 11), which does not exchange with other pools of hydrogen (e.g., body water) during assimilation, metabolism, and tissue synthesis. Second, empirical data from hydrogen exchange experiments (Wassenaar and Hobson 2000, 2003; Bowen et al. 2005) show that the majority of hydrogen in proteinaceous tissues (80–90%) does not exchange with ambient water vapor. Furthermore, research on the exchangeability of specific amino acids in *E. coli* found that when bacteria had access to protein (tryptone) in their medium, most amino acids had an exchangeability of ≤20% during cellular synthesis (Fogel et al. 2016).

The mouse tissues we analyzed in this study are largely constructed of non-essential amino acids (~53–71%; Wolf et al. 2015), which can be directly routed from dietary protein or synthesized de novo from non-protein macromolecules such as carbohydrates, lipids, and/or body water (Berg et al. 2002). In contrast, the essential amino acids in these tissues (29–47%) must be routed directly from those in dietary protein (Hare et al. 1991; O'Brien et al. 2002; Newsome et al. 2014; Fogel et al. 2016), or may be supplied by gut microbiota, especially when dietary protein is in limited supply (Metges 2000; Newsome et al. 2011). The strong positive but non-linear relationship between dietary protein content and $p_{Protein}$ in each tissue (SM Fig. 1, SM Table 5) suggests that higher degrees of amino acid routing occur when dietary protein is limited. However, as dietary protein content approaches ~20%, protein supply is greater than that required for tissue synthesis and excess protein is likely metabolized for energy (Hayes 2000) by being converted to glucose (via gluconeogenesis), glycerol (via gluconeogenesis then glyceroneogenesis), fatty acids (lipogenesis), or deaminated to ketone bodies. This altered path of proteins, when they are in high abundances in the diet of animals, has been shown to influence the results of mixing models by overestimating the contribution of dietary protein to tissue synthesis (Kelly and Martinez del Rio (2010).

As expected, the proportion of hydrogen from carbohydrates (p_{Carbs}) used to synthesize tissues varied across diet treatments (Fig. 3; O'Brien et al. 2002, Howland et al. 2003, Jim et al. 2006). In the low-protein diets, p_{Carbs} was 28–35%, but declined to ~10–15% in the high protein diet treatments. These patterns clearly show that carbohydrates are not only used as an energy source, but the hydrogen atoms from simple (sucrose) and complex (corn meal) carbohydrates are also used to synthesize structural proteinaceous tissues, even when mice were fed diets with adequate (20%) and even excess (35%) amounts of protein. Again, because the majority of amino acids in tissues are non-essential forms (~53–71%; Wolf et al. 2015), it is not surprising that p_{Carbs} exceeded 30% when dietary protein was limiting.

Hydrogen discrimination: water (Δ^2H_{Water}), protein ($\Delta^2H_{Protein}$), and carbohydrates (Δ^2H_{Carbs})

Our study provides the first estimates hydrogen isotope discrimination factors (Δ^2H) for different molecular (Δ^2H_{Water}) and macromolecular ($\Delta^2H_{Protein}$ and Δ^2H_{Carbs}) sources of hydrogen available for tissue synthesis (Eq. 4, Fig. 4). Interestingly, the range of values for Δ^2H_{Water} (− 150 to − 80‰) for mice tissues (Fig. 5) are comparable to the discrimination between plant leaves and leaf water (Estep and Hoering 1980, 1981; Dawson et al. 2002). Hydrogen from body water is assimilated via hydration reactions into intermediaries in the TCA cycle that are precursors for non-essential amino acid synthesis (Hayes 2000; Berg et al. 2002). Most hydrogen is metabolized via a pool of ^{2}H-depleted NADPH or NADH (Bizouarn et al. 1995; Leavitt et al. 2016), which plays an important role in the transformation of intermediaries in the TCA cycle, precursors to the de novo synthesis of (most) non-essential amino acids (Estep and Hoering 1981; Zhang et al. 2009). The very negative Δ^2H_{Water} values observed in our experiment suggest that the ^{2}H-depleted NADPH or NADH pool is a very important source of hydrogen for de novo synthesis of non-essential amino acids used to build tissues. The relative influence of the NADPH or NADH pool on tissue synthesis likely varies with dietary macromolecular content; however, our experimental design did not allow us to examine the influence of dietary protein content on Δ^2H_{Water}.

Macromolecule-specific Δ^2H patterns in blood plasma and liver suggest that these tissues are synthesized from a hydrogen pool that is ^{2}H-enriched relative to dietary protein. Interestingly, Δ^2H_{Carbs} for liver is much closer to zero (Fig. 5) than other tissues, suggesting more direct routing of carbohydrates toward the synthesis of non-essential amino acids used to maintain liver tissue. While direct routing of amino acids avoids the cost of de novo synthesis (Berg et al. 2002; Horton et al. 2002), our results suggest that hydrogen from the most abundant and easily assimilated dietary macromolecule (i.e., sucrose) is used to maintain liver, which is the most metabolically active and important tissue that we measured.

Negligible $\Delta^2H_{Protein}$ for muscle and RBC suggests direct routing of dietary protein with much less de novo synthesis of non-essential amino acids in comparison with liver and plasma. Muscle and RBC are the major reservoirs of endogenous protein, and the former tissue is especially important for maintaining locomotion. Thus, routing of dietary protein to maintain muscle tissue might be higher priority than routing to liver, especially if other sources of hydrogen (e.g., water or dietary carbohydrates) can be readily used to build non-essential amino acids needed to maintain liver tissue. Muscle also has lower metabolic demands (Altman and Dittmer 1968) and by extension slower isotopic incorporation rates than liver and plasma (Tieszen et al. 1983; Martinez del Rio et al. 2009), thus decreased demand in metabolic amino acids may result in decreased de novo synthesis of amino acids and higher direct isotopic routing of non-essential and essential amino acids from dietary protein.

Δ^2H_{Net}

Our mathematically derived estimates of net hydrogen isotope discrimination (Δ^2H_{Net}) are the algebraic sum of isotope discrimination factors for each source of hydrogen (water, protein, and carbohydrates) multiplied by their associated source contributions (p) (Eq. 3). Therefore, Δ^2H_{Net} is sensitive to the proportion of hydrogen in consumer tissues that is sourced from dietary macromolecules ($p_{Protein}$ and p_{Carbs}) versus drinking or environmental water (p_{Water}). Our previous work reported Δ^2H_{Net} values for muscle (-47 ± 5‰) and liver (-41 ± 5‰) in captive tilapia (*Oreochromis niloticus*) fed a controlled diet containing 10% protein (Newsome et al. 2017). Here, we found that Δ^2H_{Net} varies with dietary protein content, but estimates of Δ^2H_{Net} for muscle (-37 ± 3‰) and liver (-20 ± 4‰) in mice fed a slightly smaller amount (5%) of the same type of protein (casein) were lower in magnitude than those previously reported for tilapia.

We hypothesize that several eco-physiological factors, and potentially their combined effects, could be responsible for the observed differences in Δ^2H_{Net} between mice and tilapia. First, as terrestrial organisms, mice can lose water to the environment via breath and urine, which results in evaporative ^{2}H-enrichment of the body water pool. Similar Δ^2H_{Net} for mice fed the same diet but consumed water that varied in δ^2H (Fig. 4) suggests that evaporative ^{2}H-enrichment of body water does not result in different Δ^2H_{Water}. Second, to maintain a higher body temperature, mice have higher metabolic rates with increased rates of energy production than tilapia. A higher metabolism may require the use of non-protein dietary macromolecules (e.g., carbohydrates or lipids) for the synthesis of non-essential amino acids, resulting in mice having more positive Δ^2H_{Carbs}, and by extension Δ^2H_{Net}, in comparison with tilapia. Finally, the degree of protein routing, which influences $\Delta^2H_{Protein}$, may be higher in mammals than in fish (e.g., Ambrose and Norr 1993, Kelly and Martinez del Rio 2010), namely, because of the speculated costs of high body temperatures and more efficient protein utilization in endotherms versus ectotherms (MacAvoy et al. 2006, Kelly and Martinez del Rio 2010).

Implications for using δ^2H to study animal resource use

As with other stable isotope systems, the use of δ^2H to study animal foraging and movement ecology requires

knowledge of hydrogen isotope discrimination during assimilation and tissue synthesis, which is mediated by biochemistry and physiology. Unsurprisingly, we found that Δ^2H_{Net} varies systematically among tissues and with diet quality (i.e., protein content), similar to patterns observed in $\Delta^{13}C$ and $\Delta^{15}N$ (Vanderklift and Ponsard 2003; Caut et al. 2009). A few controlled-feeding experiments and empirical observations suggest that Δ^2H_{Net} varies with trophic level (Birchall et al. 2005; Solomon et al. 2009), but the ecological and physiological factors described above—respiratory water loss, thermoregulatory strategy, and diet quality (e.g., protein content)—may also contribute to systematic differences across species. Such physiological mechanisms should apply to a variety of organisms, because eukaryotes and some prokaryotes share similar metabolic machinery (e.g., glycolysis and TCA cycle) used to synthesize tissues or cells and thus maintain protein homeostasis (Karasov and Martinez del Rio 2008). Eventually, improved estimates of Δ^2H_{Net} will allow for the use of mixing models that utilize a combination of $\delta^{13}C$, $\delta^{15}N$, and δ^2H data to better quantify diet composition (Estep and Dabrowski 1980; Solomon et al. 2009), species interactions (Birchall et al. 2005), and energy flow (e.g., Doucett et al. 2007) within and among ecosystems (Vander Zanden et al. 2016).

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Author contribution statement SDN and MLF formulated the idea; MRC, SDN, and MLF designed the experiments. MRC completed the experiments, analyzed the data, and wrote the manuscript; SDN and MLF provided editorial advice.

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