

RESEARCH ARTICLE

An experimental exploration of the incorporation of hydrogen isotopes from dietary sources into avian tissues

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Accepted 7 February 2012

SUMMARY

The analysis of hydrogen stable isotopes (δD) is a potentially powerful tool for studying animal ecology. Unlike other stable isotopes used in ecological research, however, we are less familiar with the physiological processes that influence the incorporation of hydrogen isotopes from dietary resources to animal tissues. Here we present the results of a controlled feeding experiment utilizing Japanese quail (*Cortunix japonica*) that was designed to: (1) estimate the relative contributions of diet to the δD signatures of blood plasma, red blood cells, intestine, liver, muscle and feathers; (2) investigate possible differences among these same tissues in diet to tissue discrimination; and (3) explore the differences in incorporation dynamics between deuterium (2H) and a well-studied isotope, ^{13}C , for blood plasma solids and red blood cells. Tissues differed in both the relative contribution of diet to tissue δD and diet to tissue discrimination. The average residence time of both hydrogen and carbon was significantly lower in plasma solids than in red blood cells. The average residence time of hydrogen was significantly lower than that of carbon in plasma solids, but not in red blood cells. Although the average residence times of hydrogen and carbon were positively correlated, the correlation was weak. Hence the incorporation of carbon seems to be a poor predictor of that of hydrogen.

Key words: δ^2H , $\delta^{13}C$, stable isotope, physiological ecology, bird.

INTRODUCTION

A thorough understanding of the processes that affect the incorporation of stable isotopes from resources to consumer tissues is fundamental to the reliable use of isotope analyses in animal ecology. This understanding is important for two reasons. First, because the rates at which consumer tissues incorporate the isotopic composition of resources differ (Phillips and Eldridge, 2005), knowledge of the dynamics of isotopic incorporation allows researchers to infer the temporal scope of resources used by an individual and to establish the contributions of intra- and inter-individual variation to the range of resources used by a population (Dalerum and Angerbjorn, 2005; Newsome et al., 2009). Second, knowledge of isotopic incorporation makes it possible to estimate the relative contributions of different resources to consumer tissue isotopic values, and to estimate discrimination factors between the isotopic composition of resources and consumer tissues (Wolf et al., 2011; Newsome et al., 2010) or between trophic levels (Post, 2002; Birchall et al., 2005). This need for a thorough understanding of the processes that influence the transfer of stable isotopes from the environment and resources to consumer tissues has motivated researchers to perform experiments and develop mathematical models to describe changes in the isotopic values of consumer tissues after a shift in dietary resources (Phillips and Gregg, 2001; Jardine et al., 2004; Carleton and Martínez del Río, 2005; Martínez del Río and Anderson-Sprecher, 2008). These investigations also examined the factors that determine variation in both incorporation rates, such as growth rate, temperature and protein turnover (Jardine et al., 2004; Carleton and Martínez del Río, 2005; Carleton et al., 2008), and

the discrimination between the isotopic values of resources and consumers (Caut et al., 2009).

When compared with carbon and nitrogen stable isotopes, the use of hydrogen stable isotopes in animal ecology is relatively nascent. Consequently, it is not surprising that our understanding of the processes that affect the incorporation of stable isotopes from resources to consumer tissues is not as complete for hydrogen as it is for carbon and nitrogen (reviewed by Martínez del Río et al., 2009). This is not, however, to say that we know nothing about these mechanisms. Several studies have examined the relative contributions of resources to the hydrogen stable isotopes (δ^2H) of consumer tissues (Estep and Dabrowski, 1980; Hobson et al., 1999; Ehleringer et al., 2008; Wang et al., 2009; Solomon et al., 2009; Nielson and Bowen, 2010) and the isotopic discrimination between resources and consumer tissues (Wolf et al., 2009). In addition, Podlesack et al. (Podlesack et al., 2008) conducted a study on the turnover of hydrogen isotopes in the body water, hair and tooth enamel of pack rats (*Neotoma* spp.). Although these studies have served to advance our knowledge on the relative contributions of resources to the δ^2H of consumer tissues and the incorporation rates and diet to tissue discrimination of 2H within individual tissues, we still lack information on how these processes may differ among tissues within an individual, among individuals within a species and between species. Without this information, we cannot yet confidently interpret the results of δ^2H analysis in a general comparative fashion. We performed a diet switch experiment using Japanese quail (*Cortunix japonica*) to examine the contributions of diet to the δ^2H values of quail body water, blood plasma, red blood cells, liver,

intestine, muscle and feathers and estimated discrimination values for these tissues. We chose these tissues based on their prevalence in the methodological and mechanistic literature for carbon and nitrogen stable isotopes (Carleton et al., 2008; Caut et al., 2009). Previous attempts to determine the relative contributions of diet and drinking water to tissue $\delta^2\text{H}$ were calculated using a variety of mixing models that have yielded a wide range of results (Hobson et al., 1999; Ehleringer et al., 2008; Wang et al., 2009; Solomon et al., 2009; Nielson and Bowen, 2010). In an attempt to combat this complexity, Wolf et al. (Wolf et al., 2011) adopted a simple phenomenological strategy that, rather than attempting to determine the exact contributions of drinking water to tissue $\delta^2\text{H}$, merely sought to characterize the relationship between drinking water $\delta^2\text{H}$ and the tissue $\delta^2\text{H}$ of house sparrows. Here we adopted this same strategy for Japanese quail.

A second function of our experiment was to compare incorporation rates for ^2H and a well-studied isotope, ^{13}C , in blood plasma and red blood cells. A thorough understanding of the differences in the dynamics of incorporation between these two isotopes will determine whether reliable comparisons can be made between the two isotopes. There are distinct differences in several aspects of the processes that may affect incorporation rates between hydrogen and carbon stable isotopes. For example, the carbon in the organic molecules that comprise animal tissues is entirely derived from diet (DeNiro and Epstein, 1978) whereas the hydrogen in these same molecules can come from several sources (Estep and Dabrowski, 1980; Hobson et al., 1999; Wang et al., 2009; Solomon et al., 2009; Nielson and Bowen, 2010; Wolf et al., 2011). Although the rate of hydrogen incorporation is not likely to be directly caused by that of carbon, it is reasonable to hypothesize that the processes that determine the magnitude of incorporation of carbon (e.g. protein turnover) will also have an effect on the incorporation of hydrogen.

MATERIALS AND METHODS

Quail maintenance, experimental design and sample collection

Fifteen Japanese quail (*Coturnix japonica* Temminck and Schlegel 1849) [nine males and six females, initial mean body mass (M_b)=129 g, final mean M_b =121 g; paired t -test, t_{12} =0.69, P =0.25] were kept on an initial diet of wheat (*Triticum aestivum*, $\delta^2\text{H}$ =−85‰, $\delta^{13}\text{C}$ =−26‰) and tap water ($\delta^2\text{H}$ =−133‰) until their red blood cells and blood plasma achieved an asymptotic value that reflected that of the food and water (90 days). For the entire course of the experiment, the birds also received a vitamin and mineral supplement (Nekton S, Nekton GmbH, Pforzheim, Germany). Following this 90-day equilibration period, two female birds and three male birds were randomly assigned to each of three corn (*Zea mays*, $\delta^{13}\text{C}$ =−11‰) and distilled water ($\delta^2\text{H}$ =−133‰) diets. The deuterium isotopic composition of corn was varied in each treatment. The levels of $\delta^2\text{H}$ in the corn diets were determined by three collections of corn harvested in three different geographical locations of differing latitude (Florida, USA: $\delta^2\text{H}$ =−22.3‰, Missouri, USA: $\delta^2\text{H}$ =−44.9‰ and Ontario, Canada: $\delta^2\text{H}$ =−54.0‰). Following the diet switch, blood samples were taken from each bird on days 0, 2, 4, 8, 16, 32, 64 and 90. The blood samples were centrifuged to separate blood cells from plasma. After 90 days, we plucked several primary feathers from each bird and allowed them to regrow. The newly grown feathers were sampled to examine the relationship of diet $\delta^2\text{H}$ and tissue $\delta^2\text{H}$ in feathers, a popular tissue in isotope-based avian movement studies. Following the feather sampling, the quail were euthanized and intestine, liver and muscle samples were collected to determine the relationship between dietary isotopic

values and the isotopic values of a variety of tissues. We chose these tissues based on their prevalence in the isotope literature (Carleton et al., 2008; Caut et al., 2009).

Sample preparation and stable isotope analysis

All tissue samples except feathers were dried to a constant mass in an oven at 50°C and ground into a fine powder using a mortar and pestle. Feathers were rinsed with distilled water and air-dried. Using surgical scissors, we removed the feather barbs from the rachis and minced and homogenized the barbs. We did not include the rachis in the isotopic analysis. The powdered samples were then soaked in three 24-h treatments of petroleum ether to remove lipids. Following lipid removal, each powdered tissue sample was divided into two equal aliquots and a randomly selected sub-sample of each aliquot was loaded into a silver capsule (mean aliquot mass=0.14±0.03 mg, Costech Analytical Technologies Inc., Valencia, CA, USA) for deuterium analysis. To control for exchangeable hydrogen, encapsulated samples for deuterium analysis were equilibrated for at least 2 weeks prior to analysis in a 50°C oven under a constant stream of N_2 alongside protein reference materials (chicken and turkey feathers) with known non-exchangeable $\delta^2\text{H}$ values (chicken feather: $\delta^2\text{H}$ =−92±2.0‰; turkey feather: $\delta^2\text{H}$ =−49±2.0‰). Despite the varied natures and large potential range of $\delta^2\text{H}$ values of the tissues we analyzed, we used these reference materials for all analyses because of a lack of availability of specific reference materials for other tissues. $\delta^2\text{H}$ values were determined using a Finnigan thermal conversion/elemental analyzer (TC/EA) (Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled to a Thermo-Finnigan Delta Plus XL mass spectrometer (Thermo Fisher Scientific) at the Carnegie Institution of Washington (Washington, DC, USA). Samples were introduced into the TC/EA using a Costech Zero Blank autosampler (Costech Analytical Technologies). While in the autosampler, possible hydrogen exchange between ambient water vapor and samples was controlled for by flooding the sealed autosampler carousel with N_2 . In addition, all samples were equilibrated and analyzed using the same methods during the same time period. Consequently, we assume that the $\delta^2\text{H}$ composition of ambient water vapor was consistent throughout the analysis period and that any hydrogen exchange that occurred between ambient water vapor and tissues samples during the analysis process was also consistent. Analysis of two organic (keratin) and two inorganic (pump oil and mineral oil) reference materials showed that the precision of $\delta^2\text{H}$ analysis was ≤4‰. $\delta^{13}\text{C}$ values were determined using a Costech 4010 elemental analyzer (Costech Analytical Technologies) interfaced with a Thermo Finnigan Delta^{PLUS} XP Continuous Flow Stable Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific) at the University of Wyoming Stable Isotope Facility (Laramie, WY, USA). Analysis of beef blood and acetanilide reference materials showed that the precision of $\delta^{13}\text{C}$ analysis was ≤0.1‰.

Statistical analysis

Ehleringer et al. (Ehleringer et al., 2008), Bowen et al. (Bowen et al., 2009) and Wolf et al. (Wolf et al., 2011) have used the slope of the linear relationship between the isotopic value of tissues and that of drinking water as a first-order approximation of the percent contribution of drinking water to tissue isotopic value. In this study, we used standard least-squares regression to characterize the relationship between the $\delta^2\text{H}$ of diet and that of individual tissues. Differences in the isotopic values among tissues were analyzed using repeated-measures (RM) ANOVA. RM ANOVAs were performed as univariate analyses using an RM MANOVA function. For each RM ANOVA, we completed a Mauchly's test for sphericity. If this

test was statistically significant, we used the Greenhouse–Geisser-corrected values for RM ANOVA. In cases where tissue values were missing, the entire bird was removed from the analysis. *Post hoc* means comparisons among means for all RM ANOVAs were carried out using Tukey’s honestly significant difference (HSD) tests. Differences in tissue $\delta^2\text{H}$ values between the sexes were determined using two tailed *t*-tests.

To determine the dynamics of incorporation for hydrogen and carbon isotopes, we used the models described by Martínez del Río and Anderson-Sprecher (Martínez del Río and Anderson-Sprecher, 2008) to characterize the change in the isotopic composition of a tissue as a function of time (*t*):

$$\delta X(t) = \delta X_\infty - (\delta X_\infty - \delta X_0) \sum_i p_i e^{-\frac{t}{\tau_i}}, \quad (1)$$

where *X* is the isotope in question (in our case, ^2H or ^{13}C), δX_∞ and δX_0 are the initial and asymptotic values of the tissue, respectively, p_i is the functional size of each pool in the model (in this case, $p_i=1$ and τ_i is the average residence time of the isotope in the tissue in question. Following Carleton et al. (Carleton et al., 2008), we used small-sample Akaike’s information theoretic criterion (AIC_c) to determine whether the data were best described by one-compartment or two-compartment models. We chose the model with the lowest AIC_c value (Burnham and Anderson, 2002). The chosen model permitted estimation of the isotopic incorporation rates (in days), described as average residence time (τ , the reciprocal of the fractional incorporation rate), for carbon and hydrogen in blood plasma and red blood cells. Martínez del Río and Anderson-Sprecher (Martínez del Río and Anderson-Sprecher, 2008) provide the statistical details of these estimates. Differences in average residence times among tissues and isotopes were determined using paired *t*-tests. Two tailed *t*-tests were used to examine differences in average residence times between the sexes. All statistical analyses were performed using JMP 8.0 (SAS Institute, Cary, NC, USA) and had assumed alpha values of 0.05.

RESULTS

The $\delta^2\text{H}$ values of blood plasma, red blood cells, intestine and muscle showed significant linear correlations with the $\delta^2\text{H}$ values of the corn diets (blood plasma: $t_{10}=5.37$, $P=0.0003$; red blood cells: $t_{11}=3.16$, $P=0.009$; intestine: $t_{11}=3.75$, $P=0.003$; muscle: $t_{11}=2.39$, $P=0.03$). However, the $\delta^2\text{H}$ values of liver and feathers did not show significant linear relationships with those of diet (liver: $t_{11}=1.55$, $P=0.15$; feathers: $t_6=0.34$, $P=0.74$; Fig. 1). Two birds (one from treatment group 1 and one from treatment group 3) died before the end of the experimental course. Consequently, we did not include any data from these birds in our analyses. In addition, only one bird from treatment group 1 and two birds from treatment group 3 re-grew their feathers after they had been plucked. As a result, we had very small sample sizes for feathers for these two groups. All birds from treatment group 2 re-grew their feathers after they had been plucked. For blood plasma and red blood cells, we used the asymptote of the incorporation curve (δX_∞ in Eqn 1) as the final tissue $\delta^2\text{H}$ value. For blood plasma, we were able to calculate these values for 12 of the 13 birds that finished the experimental course. For red blood cells, we were able to calculate these values for all 13 of the surviving birds. Using Ehleringer et al. (Ehleringer et al., 2008), Bowen et al. (Bowen et al., 2009) and Wolf et al.’s (Wolf et al., 2011) interpretation of the linear relationship between the isotopic values of tissues and those of drinking water, the estimated percent contributions (\pm s.e.m.) of diet to the $\delta^2\text{H}$ values of tissues were $74\pm 15\%$ for plasma, $48\pm 17\%$ for red blood cells, $79\pm 21\%$ for intestine and $30\pm 12\%$ for muscle (Fig. 1).

We observed significant differences in $\delta^2\text{H}$ values among blood plasma red blood cells, intestine, liver, muscle and feathers (Greenhouse–Geisser-corrected RM ANOVA, $F_{1,97,15,75}=44.76$, $P<0.0001$; Table 1, Fig. 2). In addition, there was a significant interaction between tissue type and drinking water treatment group (Greenhouse–Geisser-corrected RM ANOVA, $F_{3,94,15,75}=5.58$, $P=0.0055$). This interaction prevents comparisons across

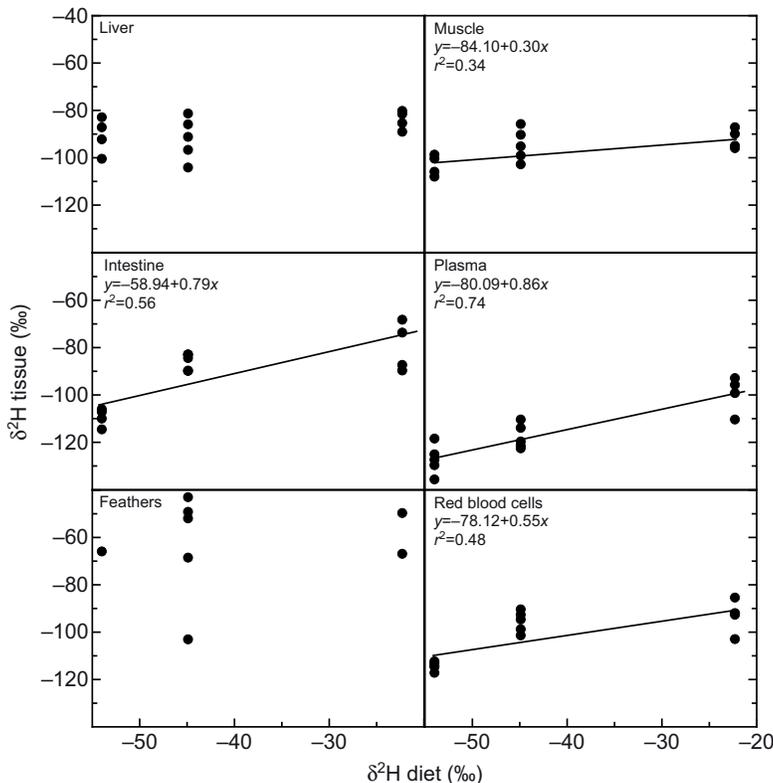


Fig. 1. Relationships between the $\delta^2\text{H}$ of diet and that of seven tissue types in Japanese quail: liver, muscle, intestine, blood plasma, red blood cells and feathers. Regression lines are provided for significant relationships. The $\delta^2\text{H}$ values of muscle, intestine, blood plasma and red blood cells were significantly correlated with those of diet.

Table 1. Mean (\pm s.d.) $\delta^2\text{H}$ values of Japanese quail tissues for the three diet treatments

	Plasma	Red blood cells	Muscle	Liver	Intestine	Feathers
Corn diet 1 ($\delta^2\text{H}=-54.0\text{‰}$)	-127.2 ± 7.3^d	-114.5 ± 1.9^c	-103.2 ± 4.4^c	-90.6 ± 7.5^b	-109.4 ± 3.8^c	-65.9^a
Corn diet 2 ($\delta^2\text{H}=-44.9\text{‰}$)	-117.6 ± 5.2^c	-95.6 ± 4.5^c	-94.6 ± 6.7^b	-91.8 ± 8.9^b	-85.9 ± 3.5^b	-63.1 ± 24.2^a
Corn diet 3 ($\delta^2\text{H}=-22.3\text{‰}$)	-99.7 ± 9.4^c	$-93.1\pm 7.3^{b,c}$	$-91.9\pm 4.1^{b,c}$	$-84.0\pm 3.9^{b,c}$	$-79.7\pm 10.4^{a,b}$	-58.2 ± 12.1^a

Tissues displayed significant variation in their mean $\delta^2\text{H}$ values. We were not able to calculate standard deviations for feathers in diet group 1 because of a very small sample sized caused by a lack of feather re-growth in several birds. Tissues with different letters are significantly different (Tukey's HSD, $P<0.05$).

independent treatments. There was no significant effect of sex (Greenhouse–Geisser-corrected RM ANOVA, $F_{1,97,15,75}=0.88$, $P=0.43$) on the tissue $\delta^2\text{H}$ values.

For carbon, the one-compartment model had lower AIC_c values for both plasma and red blood cells. ΔAIC_c ($\Delta\text{AIC}_c=\text{AIC}_{c,\text{one}}-\text{AIC}_{c,\text{two}}$) values ranged from -80.35 to -11.08 . For hydrogen, the one-compartment model was also better supported by data for both tissues. However, for hydrogen, the one-compartment model was favored because the two-compartment model was unable to converge in most cases. In those cases where the two-compartment model was able to generate results, the one-compartment model had lower AIC_c values for both blood plasma and red blood cells. For hydrogen, ΔAIC_c values ranged from -40.66 to -21.20 . Consequently, for the rest of our analyses, we used the one-compartment model. Using this one-compartment model, we were able to generate average residence times for carbon for all 13 birds that finished the experimental course for red blood cells and 12 birds for blood plasma. For hydrogen, we were able to generate average residence times for 12 birds for both red blood cells and blood plasma.

There were no significant differences in average residence times among corn diet treatments for either carbon or hydrogen isotopes in either tissue (ANOVA, carbon: red blood cells, $F_{2,10}=1.19$, $P=0.34$, plasma, $F_{2,9}=1.38$, $P=0.29$; hydrogen: red blood cells, $F_{2,9}=0.87$, $P=0.45$, plasma, $F_{2,9}=2.70$, $P=0.12$). In addition, there were no significant differences in average residence times between the sexes for either carbon or hydrogen isotopes in either tissue (carbon: red blood cells, $t_{11}=1.11$, $P=0.29$, plasma, $t_{10}=0.71$, $P=0.49$; hydrogen: red blood cells, $t_{10}=1.15$, $P=0.28$, plasma, $t_{10}=1.59$, $P=0.14$). Isotopic average residence times for hydrogen were lower than those for carbon in red blood cells (mean \pm s.d. difference= 7.72 ± 12.69 days, paired t -test, $t_{11}=2.10$, $P=0.05$; Fig. 3). Average residence times of hydrogen isotopes were also lower than those of carbon isotopes in blood plasma, but this difference was not statistically significant (mean \pm s.d. difference= 3.30 ± 8.90 days, paired t -test, $t_{11}=1.30$, $P=0.22$; Fig. 3). Average residence times for both carbon and hydrogen isotopes were significantly lower in blood plasma than in red blood cells (carbon:

mean \pm s.d. difference= 17.18 ± 4.88 days, paired t -test, $t_9=11.11$, $P<0.001$; hydrogen: mean \pm s.d. difference= 10.16 ± 10.37 days, paired t -test, $t_8=2.94$, $P=0.02$; Fig. 4). The average residence times of carbon and hydrogen isotopes were weakly but significantly correlated ($r^2=0.19$, $N=24$, $P=0.03$; Fig. 4).

DISCUSSION

Our results demonstrated a significant correlation between $\delta^2\text{H}_{\text{tissue}}$ and $\delta^2\text{H}_{\text{diet}}$ in four of six tissues. We also found differences in the $\delta^2\text{H}$ composition of different tissues. Finally, our results also showed a weak, but statistically significant, correlation between the mean residence times of hydrogen and carbon isotopes. The mean residence time of both carbon and hydrogen isotopes differed between plasma and red blood cells. In subsequent sections, we will examine possible explanations for these results, and the implications of these results for the use of $\delta^2\text{H}$ analysis in ecological studies.

The relationship between the $\delta^2\text{H}$ of diet and the $\delta^2\text{H}$ of quail tissues

Previous estimates of the contribution of diet to the $\delta^2\text{H}$ of tissues have yielded values ranging from 68 to 86% in a variety of avian tissues (Hobson et al., 1999; Wolf et al., 2011), 51–80% in aquatic invertebrates (Wang et al., 2009) and 88% in salmonid fish muscle (Solomon et al., 2009; Nielson and Bowen, 2010). We observed significant relationships between the $\delta^2\text{H}$ value of tissues and diet for four of six tissues: blood plasma, red blood cells, intestine and muscle (Fig. 3). The estimated percent contributions of diet to the hydrogen isotopic compositions of these tissues ranged from 30 to 86%. In addition, the correlation between the hydrogen isotopic value of these tissues and those of food varied. The coefficient of determination of these relationships ranged from 0.34 to 0.79 (Fig. 3). We observed these variations among tissues and individuals in the estimated contributions of diet to tissue $\delta^2\text{H}$ values despite the controlled nature of our experiment. This demonstrates that much of the variation observed by previous studies is the result of differences in the biochemical composition of tissues and the

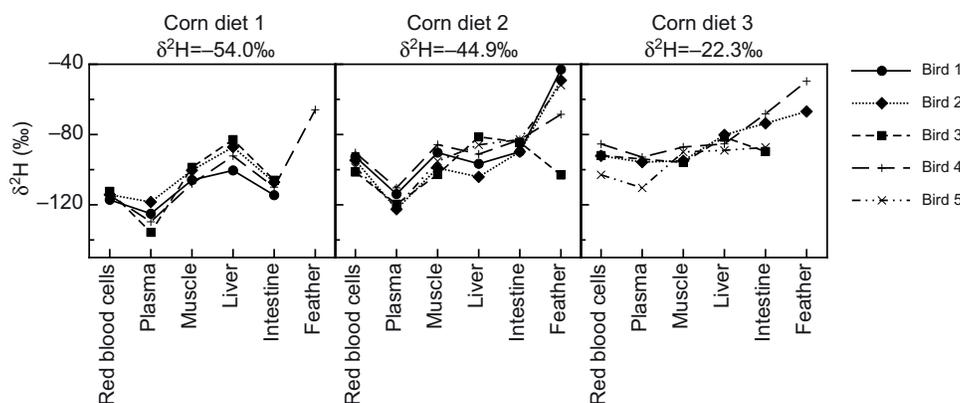


Fig. 2. $\delta^2\text{H}$ values of different tissues for individual Japanese quail. Lines connect individual tissues from the same bird and are provided to obviate the overall pattern in the data. Feathers were more enriched in ^2H than other tissues. This pattern was consistent among individual birds and diet treatments.

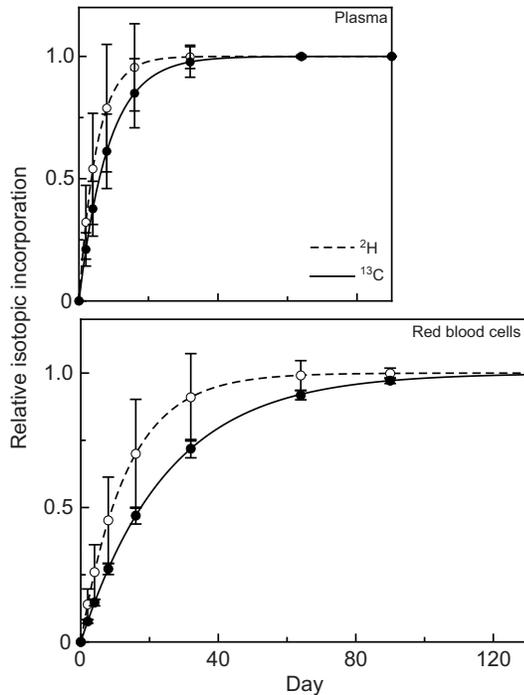


Fig. 3. Isotopic average residence times (τ) (\pm s.d.) for hydrogen and carbon in blood plasma and red blood cells of Japanese quail. Average residence times for hydrogen were lower than those for carbon in red blood cells. Average residence times of hydrogen isotopes were also lower than those of carbon isotopes in blood plasma, but this difference was not statistically significant. For comparison, we standardized the isotopic incorporation curves by setting $\delta^2H_{\infty}=1$ and $\delta^2H_0=0$.

physiology and/or ecology of individuals. These results demonstrate the need for further exploration of the physiological mechanisms that influence the incorporation of hydrogen isotopes from resource to consumer tissues. Of particular interest is the influence of the δ^2H of diet on the δ^2H of individual amino acids using compound-specific analysis (Howland et al., 2003). Such an approach would provide insight into the relative contributions of dietary resources to the δ^2H values of individual amino acids and the influence of a tissue's amino acid composition on its bulk δ^2H value.

An unexpected result of our experiment was the lack of a positive linear relationship between the δ^2H values of diet and tissue for liver and feathers. For feathers, this could have been the result of our relatively small sample size. However for liver, we suspect that our study's failure to detect this relationship (Fig. 1) might be due to the relatively narrow range of δ^2H values employed on our diets and the lack of covariation between the δ^2H values of our diets and that of drinking water. The δ^2H values of the corn diets employed in our experiment only represent one-third of the variation seen in the δ^2H values of surface water (Waterisotopes.org, <http://wateriso.eas.purdue.edu/waterisotopes/>), and presumably producers, over the North American continent ($<33\%$, from -54 to -22%). Although biologically relevant, this range may not be sufficient for establishing the relationship between the δ^2H value of diet and that of tissues, especially because the δ^2H value of drinking water was held constant across diet treatments in our experiments.

To assess the effect of both increasing the range of δ^2H values of diet treatments and the covariation between the δ^2H value of diet

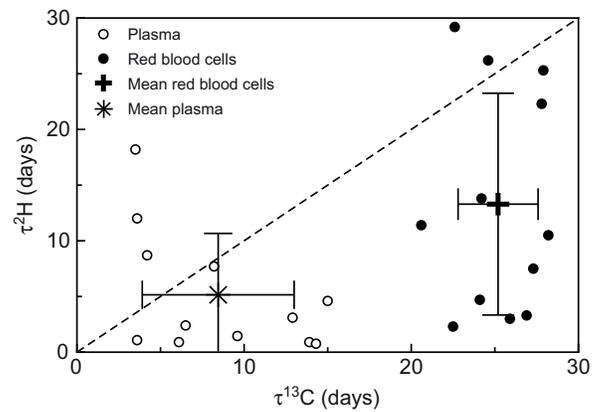


Fig. 4. Relationship between average residence times (τ) (\pm s.d.) of carbon and hydrogen isotopes in blood plasma and red blood cells of Japanese quail. Average residence times for both carbon and hydrogen isotopes were significantly lower in blood plasma than in red blood cells. The average residence times of carbon and hydrogen isotopes were weakly, but significantly, correlated. The dashed line represents a 1:1 line.

and drinking water, we performed a set of simulations. We varied the range of δ^2H values of diet from 30% (-52 to -22%) to 60% (-82 to -22%). We assumed that either the δ^2H values of drinking water were identical to those of diet, or that they were constant and independent of the value of diet ($\delta D=-135\%$). Based on the estimated percent contribution of diet to intestinal tissue δ^2H observed in our quail, we assumed that food contributed 79% of the hydrogen in tissues. We chose to use the value for the estimated percent contribution of diet to intestinal tissue δ^2H because of its similarity to values found by previous studies (Hobson et al., 1999; Wang et al., 2009; Solomon et al., 2009; Nielson and Bowen, 2010; Wolf et al., 2011). To generate error, we assumed that the incorporation fractions of both diet and drinking water to the hydrogen content in tissues had a normally distributed error with a mean of 21% and a standard deviation of 29% . This mean value was based on the standard error of the slope of the relationship between the δ^2H values of diet and intestinal tissue found in our experiments. The standard deviation was calculated from the contributions of diet δ^2H to bird tissue δ^2H observed in our data and two additional sources (Wolf et al., 2011) (N.W., S.D.N., M.L.F. and C.M.d.R., submitted). Based on our data for intestinal tissue, we assumed that the discrimination factor from diet to tissues was normally distributed with a mean of -92% and a standard deviation of -16% . The discrimination factor from drinking water to tissues was also normally distributed with a mean of -19% and a standard deviation of -17% (value for Japanese quail intestinal tissue from N.W., S.D.N., M.L.F. and C.M.d.R., submitted). To reflect the conditions of our experiment, we generated 15 points at regular intervals along the simulated range of δ^2H_{diet} values. Accordingly, the model relating the δ^2H value of diet with that of tissues was:

$$\delta^2H_{\text{tissue}} = [0.79 + \epsilon_p(0.21, 0.29)] [\delta^2H_{\text{diet}} + \epsilon(-92, -16)] + [0.21 - \epsilon_p(0.21, 0.29)] [\delta^2H_{\text{water}} + \epsilon(-19, -17)], \quad (2)$$

where ϵ_p is the error associated with the fractional contributions of diet and drinking water and ϵ is the error associated with diet to tissue discrimination. In these simulations, there was no significant relationship between the range of δ^2H values in diet treatments and the r^2 value of the relationship between the δ^2H values of diet and tissue ($r^2=0.02$, $N=40$, $P=0.36$, $y=0.037+0.001x$ (Fig. 5). In addition,

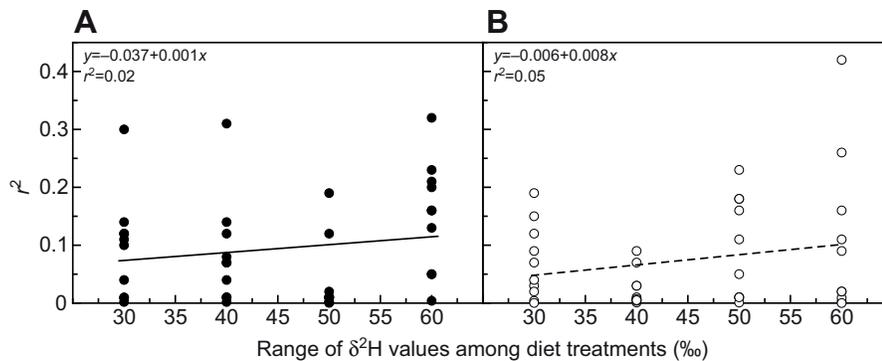


Fig. 5. Simulation to assess the effect of both increasing the range of $\delta^2\text{H}$ values of diet treatments and the covariation between the $\delta^2\text{H}$ value of diet and drinking water. (A) Drinking water $\delta^2\text{H}$ values covary with diet $\delta^2\text{H}$ values. (B) Drinking water $\delta^2\text{H}$ values are constant (-135%). There was no significant relationship between the range of $\delta^2\text{H}$ values among diet treatments and the r^2 value of the relationship between the $\delta^2\text{H}$ values of diet and tissue. In addition, the r^2 values for the simulations when $\delta^2\text{H}$ values were held constant were not significantly different from those for the simulations when $\delta^2\text{H}$ values of water covaried with those of diet.

the r^2 value for the simulations when the $\delta^2\text{H}$ value of water covaried with that of diet was not significantly different from when the $\delta^2\text{H}$ value of water was held constant (ANCOVA, $F_{3,76}=1.18$, $P>0.05$; Fig. 5). Finally, most of the r^2 values of the simulated relationships ($r^2=0.27$) were lower than the P -values of the linear regression. These results demonstrate that the small number of tissues for which we found positive linear relationships between the $\delta^2\text{H}$ values of diet and tissue was not likely caused by the relatively narrow range of $\delta^2\text{H}$ values employed on our diets, nor was it caused by the lack of covariation between the $\delta^2\text{H}$ values of our diets and that of drinking water. Instead, the lack of significant relationships for several tissues was caused by the large intra-individual variation in both incorporation rates and discrimination factors exhibited by hydrogen.

This result has implications for the use $\delta^2\text{H}$ analysis for studying animal movements and migratory strategies. The most common use of hydrogen isotopes in the ecological literature compares the $\delta^2\text{H}$ value of an animal tissue (typically a bird feather) with the $\delta^2\text{H}$ value of precipitation in an 'isoscape' (West et al., 2006) to determine where the tissue was grown (Hobson, 2008; Wunder, 2010). Hobson et al. (Hobson et al., 1999) noted that large isotopic differences between drinking water and food at a single locality could lead to variation in the relationship between the isotopic content of an animal's tissues and that of the precipitation of the area in which this animal lives. The results of our simulations showed that the variation in tissue $\delta^2\text{H}$ values was similar for scenarios when drinking water δD values covaried with diet $\delta^2\text{H}$ values and for scenarios when drinking water δD values were held constant. Our simulation demonstrates that for birds (e.g. Japanese quail) with high and variable drinking water rates, the reliability of $\delta^2\text{H}$ analysis for studying animal movements might not decrease when the $\delta^2\text{H}$ values of diet and drinking water differ.

Variation in $\delta^2\text{H}$ among tissues

In our experiment, feathers were more enriched in ^2H than other tissues. In addition, the mean $\delta^2\text{H}$ values of all other tissues were significantly different (Table 1). This pattern of variation was consistent among individual birds from the same treatment (Fig. 2). In their drinking water switch experiment using house sparrows, Wolf et al. (Wolf et al., 2011) also found that feathers were significantly more enriched in ^2H than other tissues. Although we see similar patterns in the discrimination values of hydrogen isotopes between house sparrows and Japanese quail, the variations in tissue-specific discrimination values seen among tissues in both species demonstrates the need to develop a mechanistic understanding of the processes that influence variations in hydrogen isotopic discrimination among tissues. Although we suspect that

these processes might be similar to those that influence carbon and nitrogen [e.g. differences in nutrient routing, protein turnover and the macromolecular compositions of tissues (Martínez del Río et al., 2009)], future $\delta^2\text{H}$ analysis of individual amino acids is necessary to determine both the general patterns in hydrogen isotopic discrimination among tissues and the processes that determine these patterns.

Similarities in the residence times of ^{13}C and ^2H

Similarities in the average residence times of carbon and hydrogen isotopes would allow for valuable comparisons between the two isotopes. Our results demonstrate a weak, yet statistically significant, correlation in the average residence times of carbon and hydrogen. However, the regression of the relationship of $\tau^{13}\text{C}$ and τD only explained approximately 20% of the variation in deuterium retention (Fig. 4). In addition, the slope of the relationship of the residence times of the two isotopes was significantly lower than 1 (slope \pm s.e.m. = 0.51 ± 0.23 , $t=2.08$, $P=0.04$), and the intercept was not significantly different from 0 (intercept \pm s.e.m. = 2.27 ± 4.36 , $t=0.62$, $P=0.53$).

Differences in the average residence times of ^{13}C and ^2H

For red blood cells, the average residence times of hydrogen isotopes were significantly shorter than those of carbon isotopes. Average blood plasma residence times of hydrogen isotopes were also shorter than those of carbon isotopes, but this difference was not statistically significant (Fig. 3). The lack of a significant difference in $\tau^2\text{H}$ and $\tau^{13}\text{C}$ in blood plasma was likely the result of the large amount of variation in the retention time of hydrogen in plasma observed among individuals (Fig. 3). The coefficient of variation (CV) of residence time of hydrogen (CV=1.07) was approximately twice as high as that for carbon (CV=0.54) in blood plasma. This variation, and the shorter mean residence times observed for hydrogen isotopes, might be attributable to the greater number of potential sources for hydrogen in consumer tissues than for carbon in consumer tissues. The carbon in the molecules that comprise animal tissues is entirely derived from diet (DeNiro and Epstein, 1978), but hydrogen in animal tissues can come from either diet or body water (Hobson et al., 1999). In turn, the hydrogen in body water can come from two potential sources: pre-formed water (from food or drinking water) or metabolically produced water (Hobson et al., 1999; Wolf et al., 2011). Because the hydrogen in consumer tissues can be derived from multiple source pools, and each of these pools can have different dynamics (Ehleringer et al., 2008), the interaction of these pools can result in variations in the incorporation rates of hydrogen from each source. For example, in our experiment, birds were provided with water *ad libitum*. Variations in drinking water

rates among individual birds could lead to variations in the incorporation of hydrogen derived from the diet into the body water pool (*via* metabolic water production). This would cause resultant variation in the incorporation of diet-derived hydrogen into consumer tissues. Similarly, in animals with low water intake requirements, the incorporation of diet-derived hydrogen into body water will be faster and, consequently, mean residence times will be shorter than those found in animals with higher drinking water requirements. In addition to the rate at which hydrogen from diet is incorporated into tissues, differences in drinking water intake will influence the fraction of the hydrogen found in consumer tissues that is derived from diet sources (Wolf et al., 2011).

Differences in average residence times of ^{13}C and ^2H between red blood cells and blood plasma

The average residence times of both carbon and hydrogen isotopes were significantly shorter in blood plasma than in red blood cells. This pattern agrees with the results of previous studies on the incorporation rates of carbon isotopes among tissues (Carleton et al., 2008). Isotopic ecologists have capitalized on the significant differences in ^{13}C average residence times among tissues to study dietary ecology (Dalerum and Angerbjörn, 2005; Martínez del Rio et al., 2009). By conducting $\delta^{13}\text{C}$ analysis on multiple tissues from the same individual, ecologists can infer the timing of changes in resource use by an individual animal (Klassen et al., 2010). Although we observed significant differences in the average residence times of deuterium in blood plasma and red blood cells, our results prompt us to urge against using deuterium in a similar fashion. Estimating the timing of shifts in dietary resources using this method requires accurate estimates of the differences in isotopic incorporation rates between tissues (Klassen et al., 2010). In one-compartment incorporation models, such as those we used, average retention times are the reciprocal of fractional incorporation rates (Martínez del Rio and Anderson-Sprecher, 2008). The coefficient of variation of the difference between the fractional incorporation rate of blood plasma and red blood cells of hydrogen ($\text{CV}=1.25$) is almost double that of carbon ($\text{CV}=0.71$). Large variations in the difference between the fractional rates of incorporation of two tissues can lead to high error propagation and consequent large uncertainty in estimates of diet shift (Klassen et al., 2010). However, despite the variation we observed, we do see a significant difference in the average residence times of the two tissues. Consequently, we recommend further exploration of the average residence times of hydrogen isotopes in a variety of tissues before concluding that the average residence times of hydrogen isotopes in red blood cells and blood plasma are sufficiently variable so as to negate the reliable use of these tissues as independent indicators of hydrogen isotope incorporation in ecological studies.

Conclusions

Our results demonstrate the complexity of using $\delta^2\text{H}$ analysis for studying animal ecology. With our current state of knowledge on the physiological processes that influence the incorporation rates and diet to tissue discrimination of $\delta^2\text{H}$, we face great difficulty in achieving accurate results using hydrogen isotope analysis. The results of our study show that variations in the $\delta^2\text{H}$ values among animal tissues, both among individuals and individual tissues in the same animal, is a natural phenomenon that is likely driven by physiological and ecological causes. This variation is high even in controlled experimental situations in which individuals are provided with food and water with identical and constant $\delta^2\text{H}$ values. In addition, our results demonstrate the potential differences in the

incorporation of carbon and hydrogen isotopes. Given that hydrogen can be derived from two sources (diet and pre-formed water), it is likely that the hydrogen isotope composition of wild, free-ranging animals will be inherently more variable and unpredictable than for carbon isotopes. Hydrogen stable isotope analysis is a potentially powerful tool in animal ecology. Given this potential, we think that it is important that the limiting aspects of this tool are explored thoroughly. However, until more information is available on the reliability of the method, we recommend that future studies that aim to use $\delta^2\text{H}$ analysis in the field consider the variation demonstrated by our results in the design of their studies and the interpretation of their results.

LIST OF SYMBOLS AND ABBREVIATIONS

^{13}C	carbon isotope (6 protons, 7 neutrons) (moles)
^2H	hydrogen isotope (1 proton, 1 neutron) (moles)
AIC_c	Akaike's information theoretic criterion for small samples
CV	coefficient of variation
f_A	relative fractional contribution of source A
f_B	relative fractional contribution of source B
p_i	functional size of each pool in the incorporation model
$\delta^{13}\text{C}$	$[(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}] / (^{13}\text{C}/^{12}\text{C})_{\text{standard}} \times 10^3$ (‰, Vienna Pee Dee belemnite)
$\delta^2\text{H}$	$[(^2\text{H}/^1\text{H})_{\text{sample}} - (^2\text{H}/^1\text{H})_{\text{standard}}] / (^2\text{H}/^1\text{H})_{\text{standard}} \times 10^3$ (‰, standard mean ocean water)
δ_A	isotopic composition of source A
δ_B	isotopic composition of source B
δ_T	isotopic composition of a consumer tissue
δX_∞	asymptotic value of an isotope (X) in a tissue
δX_0	initial value of an isotope (X) in a tissue
ϵ	error associated with diet to tissue discrimination
ϵ_p	error associated with the fractional contributions of diet and drinking water
τ	average residence time of the isotope for a tissue

ACKNOWLEDGEMENTS

We thank Robert Carroll for his assistance with bird care and Roxanne Bowden for her assistance with $\delta^2\text{H}$ analysis.

FUNDING

N.W. was supported by the University of Wyoming Dr George E. Menken's Memorial Scholarship. N.W., C.M.d.R. and S.D.N. were supported by the National Science Foundation (DIOS 0848028). M.L.F. was supported by the Carnegie Institution of Washington.

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