The Relationship between Drinking Water and the Hydrogen and Oxygen Stable Isotope Values of Tissues in Japanese Quail (*Cortunix japonica*)

Author(s): Nathan Wolf , Seth D. Newsome , Marilyn L. Fogel , and Carlos Martinez Del Rio

THE RELATIONSHIP BETWEEN DRINKING WATER AND THE HYDROGEN AND OXYGEN STABLE ISOTOPE VALUES OF TISSUES IN JAPANESE QUAIL (*CORTUNIX JAPONICA*)

Nathan Wolf,[1,3] Seth D. Newsome,[1] Marilyn L. Fogel,[2] and Carlos Martinez del Rio[1]

[1]*Department of Zoology and Physiology, University of Wyoming, 1000 University Avenue, Laramie, Wyoming 82071, USA; and*
[2]*Carnegie Institution of Washington, Geophysical Laboratory, 5251 Broad Branch Road NW, Washington, D.C. 20015, USA*

Abstract.—Developing an understanding of how differences in the dietary ecology and physiology of different species can influence the incorporation of hydrogen and oxygen from resources into consumer tissues is an important factor to consider when designing, or interpreting data from, ecological studies using stable isotope analyses of these elements. Here, we present the results of an experiment designed to examine the relationship between the δ^2H and δ^{18}O of drinking water and the δ^2H and δ^{18}O values of body water, blood plasma, red blood cells, intestine, liver, muscle, and feathers of Japanese Quail (*Cortunix japonica*). Because Japanese Quail have high drinking-water requirements compared with many other bird species, we hypothesized that the relative contribution of drinking water to tissue δ^2H and δ^{18}O values would be higher than that of bird species with lower drinking-water requirements. Our results demonstrate that the contribution of drinking water to the δ^2H and δ^{18}O values of tissues is generally higher in Japanese Quail than in other birds with lower drinking-water rates. However, we failed to find significant relationships between drinking-water isotope values and tissue isotope values in many of the tissues that we examined. We suspect that this lack of significant relationships is the result of variation in tissue isotope values caused by differences in drinking-water consumption rates among individual birds. Given these results, we recommend that researchers use caution when interpreting data from ecological investigations using δ^2H and δ^{18}O analyses. *Received 12 April 2012, accepted 27 January 2013.*

Key words: birds, deuterium, oxygen 18, physiological ecology, stable isotopes.

Relación entre el Agua Consumida y los Valores de Isótopos Estables de Hidrógeno y Oxígeno en Tejidos de *Cortunix japonica*

Resumen.—Desarrollar un entendimiento sobre cómo las diferencias en la ecología de la dieta y la fisiología de diferentes especies pueden influir en la incorporación de hidrógeno y oxígeno en los tejidos de los consumidores a partir de sus recursos es un factor importante a considerar cuando se diseñan estudios ecológicos que usan análisis de isótopos estables de estos elementos o cuando se interpretan datos producto de tales estudios. Presentamos los resultados de un experimento diseñado para examinar la relación entre los isótopos δ^2H y δ^{18}O del agua consumida y los valores de δ^2H y δ^{18}O en agua corporal, plasma sanguíneo, glóbulos rojos, intestino, hígado, músculo y plumas de *Cortunix japonica*. Dado que *C. japonica* tiene altos requerimientos de toma de agua en comparación con muchas otras especies de aves, planteamos la hipótesis de que la contribución relativa del agua consumida a los valores de δ^2H y δ^{18}O en los tejidos sería mayor que la de especies de aves con requerimientos más bajos de consumo de agua. Nuestros resultados demuestran que la contribución del agua consumida a los valores de δ^2H y δ^{18}O en los tejidos generalmente es mayor en *C. japonica* que en otras especies de aves con requerimientos más bajos de consumo de agua. Sin embargo, no encontramos relaciones significativas entre los valores de isótopos del agua consumida y de muchos de los tejidos que examinamos. Sospechamos que esta falta de relaciones significativas es el resultado de la variación en los valores de isótopos de los tejidos causada por diferencias en las tasas de consumo de agua entre los individuos. Dados estos resultados, recomendamos que los investigadores tengan cuidado al interpretar datos de investigaciones ecológicas que usan análisis de δ^2H y δ^{18}O.

[3]Present address: Environment and Natural Resources Institute, University of Alaska Anchorage, 3211 Providence Drive, Anchorage, Alaska 99508, USA. E-mail: nwolf4@uaa.alaska.edu

Using hydrogen and oxygen isotopes in animal ecology requires reliable estimates of the isotopic discrimination between animal tissues and local precipitation ($\Delta^2H_{tissue-precipitation}$ or $\Delta^{18}O_{tissue-precipitation}$; Hobson 2005, 2008; Hobson et al. 2009). In a recent review, Hobson (2008) showed that great variability exists in both the goodness-of-fit and the slopes of the relationship between the δ^2H of feathers and the δ^2H of precipitation reported by observational studies. Hobson (2008) reported coefficient of determination (r^2) values ranging from 0.36 to 0.91 (mean r^2 = 0.55, n = 24) and slopes ranging from 0.28 to 1.41 (mean ± 95%CI = 0.80 ± 0.11, n = 27). This variation in both the degree of association and slope of $\delta^2H_{feather}$ versus $\delta^2H_{precipitation}$ relationships has previously been attributed to variations in analytical techniques, including differences in laboratory protocols and inaccuracies in the modeling of the δ^2H values of precipitation (Lott and Smith 2006, Smith et al. 2009). However, the possibility that this variation may be attributable to differences in physiology or ecology among species has received less attention. One possible biological explanation for this variation that deserves further exploration is how differences in the dietary ecology of species may result in consequent variations in the relative contribution of the multiple sources from which the molecules (e.g., amino acids) that are used to synthesize proteinaceous animal tissues are derived (Wolf et al. 2011). These sources include food and preformed water (i.e., water in food and free drinking water) for hydrogen and food, preformed water, and atmospheric oxygen for oxygen (Hobson et al. 1999, 2009). Hobson et al. (1999) noted that variation in the isotopic composition of these sources could lead to variation in the relationship between the isotopic content of an animal's tissues and that of local precipitation. Another possibility is that individuals differ in the relative contribution of these sources to tissue hydrogen and oxygen. These differences can weaken the relationship between the isotopic content of an animal's tissues and that of local precipitation. This latter possibility has received relatively little attention.

Previous studies that have investigated the relative contributions of free drinking water and diet to the δ^2H and $\delta^{18}O$ values of avian tissues have reported variable results. Hobson et al. (1999) reported that 18–32% of the hydrogen in Japanese Quail (*Cortunix japonica*) tissues (blood, muscle, liver, lipids, feathers, and claws) was derived from preformed water. These results contrast with those of Wolf et al. (2011), who reported much lower contributions of preformed water (14%, 17%, and 18% of the hydrogen in red blood cells, plasma, and feathers, respectively; and 15% and 27% of the oxygen in red blood cells and feathers, respectively) to the tissues of House Sparrows (*Passer domesticus*). Wolf et al. (2011) speculated that the lower preformed water contributions might be the result of the low drinking-water requirements and small-drinking water uptake of granivorous birds, such as House Sparrows (MacMillen 1990), compared to those with higher water intake, such as Japanese Quail (Bartholomew and MacMillen 1961). Unfortunately, Hobson et al. (1999) and Wolf et al. (2011) used slightly different methods to determine the fractional contributions of drinking water to the tissue isotopic values of the birds in their studies. Given the difference between the two studies, we cannot reliably determine whether the difference in the fractional contribution of drinking water to consumer tissues reported by Hobson et al. (1999) and Wolf et al. (2011) is, indeed, the result of a physiological or ecological mechanism and not of differences in experimental protocol.

Here, we present the results of a study designed to reexamine the relationship between drinking-water δ^2H value and the δ^2H values of Japanese Quail tissues (body water, plasma, red blood cells, intestinal muscle, liver, skeletal muscle, and feathers) using the same methods used by Wolf et al. (2011) to examine this relationship in House Sparrows. Our experiment also allowed us to examine the relationship between the isotopic value of preformed drinking water and the $\delta^{18}O$ signatures of these same seven Japanese Quail tissues, which was not examined by Hobson et al. (1999). This examination will aid in our understanding of the causes of variation seen in the relationship between the isotopic signature of tissues and that of local precipitation in birds (Hobson 2008). Such an understanding will, in turn, allow us to design and interpret data from ecological studies that use δ^2H and $\delta^{18}O$ analysis more precisely and confidently. A secondary goal of our work was to determine the variation in the δ^2H and $\delta^{18}O$ values among the seven Japanese Quail tissues listed above. On the basis of previous observations that discrimination values for hydrogen and oxygen can vary greatly between tissues (Wolf et al. 2011), we expected the tissue-to-drinking-water discrimination factors to differ among the seven Japanese Quail tissues examined. These tissue-specific discriminations have significant implications for comparisons between ecological studies that analyze different tissues, and serve to emphasize the need for accurate tissue-specific discrimination values when attempting to use hydrogen or oxygen isotopes to study animal movements. By exploring the differences in these aspects of the relationship between consumer tissues and drinking water for two birds with different drinking-water needs, we can develop a more complete understanding of the mechanisms that shape the relationship between the δ^2H and $\delta^{18}O$ values of resources and those of consumer tissues.

Methods

Fifteen adult Japanese Quail (6 males and 9 females, body mass [M_b] = 176 g) were housed at 26.0°C in individual cages (40 × 30 × 25 cm) at the University of Wyoming Department of Zoology and Physiology Animal Care Facility (Laramie, Wyoming). The birds were kept on an initial diet of Common Wheat (*Triticum aestivum*, δ^2H = −85‰, $\delta^{18}O$ = 25‰) and provided with tap water (δ^2H = −132‰, $\delta^{18}O$ = −17‰) ad libitum until their red blood cells and blood plasma achieved an asymptotic value that reflected that of the food and water (90 days). The birds also received a vitamin and mineral supplement (Nekton S, Nekton GmbH, Pforzheim, Germany). Following this 90-day equilibration period, the birds were randomly assigned to one of three treatment groups of five birds each. The three treatment groups continued to receive the same Wheat diet (and vitamin supplement), but each group was provided with a drinking-water treatment with different δ^2H and $\delta^{18}O$ values [δ^2H ($\delta^{18}O$) = −111(−15)‰, −87(−13)‰, −58(−11)‰]. The δ^2H and $\delta^{18}O$ of the drinking water in each treatment was created by adding 99 atom percent deuterated water (D_2O) and 98 atom percent ^{18}O water (H_2O^{18}) to distilled local tap water. Both diet and drinking-water sources were purchased and mixed in large volumes to ensure isotopic consistency. The variation in the isotopic values of the three water treatments was designed to approximate the natural variation in environmental waters along the Global Meteoric Water Line (Craig 1961).

Following the drinking-water switch, drinking-water consumption of individual Japanese Quail was measured (mL) every 2 days, and blood samples (~0.5 mL) were taken from each bird on days 0, 2, 4, 8, 16, 32, 64, and 90. Blood samples were taken from the brachial vein using a 0.5-mL syringe equipped with a 30-gauge needle, and centrifuged (2 min in a microhematocrit centrifuge)

to separate red blood cells and plasma. All blood and tissue samples were collected at the same time of day (~1300 hours Mountain Standard Time). Body water was cryodistilled from plasma using the method described by Ehleringer (1989). Red blood cells were sampled over time to determine whether the isotopic value of the birds' blood had reached a constant asymptotic value reflecting that of the drinking water. After 90 days, several primary feathers were plucked from each bird and allowed to regrow. The newly grown feathers were sampled to examine the relationship of drinking-water δ^2H and δ^{18}O and tissue δ^2H and δ^{18}O in feathers, a common tissue used in isotope-based studies of avian movement. Following feather sampling (day 104), birds were euthanized using CO_2 asphixiation, and intestine, liver, and muscle samples were collected. We chose these tissues on the basis of their prevalence in the isotope literature (Carleton et al. 2008, Caut et al. 2009). Tissue samples were stored frozen at −80°C until processing.

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. Paritte and Kelly (2009) have explored the implications of different feather-cleaning methods on δ^2H values. They concluded that the best method for cleaning feathers is a 2:1 chloroform methanol rinse, followed by a detergent rinse. Although we recognize that this may be the most effective method for feather cleaning prior to hydrogen stable isotope analysis, we chose to use a simple distilled-water rinse because this is the same method used by Wolf et al. (2011) to prepare House Sparrow feathers. Using surgical scissors, we removed the feather barbs from the rachis and minced and homogenized the barbs; thus, samples for isotope analysis did not include the rachis. All powdered or minced tissue samples were then soaked in three 24-h treatments of petroleum ether to remove lipids. Following lipid removal, two randomly selected subsamples of each tissue sample were loaded into silver capsules (mean aliquot mass = 0.14 ± 0.03 mg; Costech Analytical Technologies, Valencia, California) for hydrogen and oxygen stable isotope analyses. In order to control for exchangeable hydrogen, encapsulated samples were equilibrated for at least 2 weeks prior to analysis in a 50°C oven under a constant stream of N_2 alongside keratin reference materials (Domestic Chicken [*Gallus gallus*] and Domestic Turkey [*Meleagris gallopavo*] feathers) with known non-exchangeable δ^2H values (chicken feather: δ^2H = −92 ± 2.0‰ and turkey feather: δ^2H = −49 ± 2.0‰). These reference materials were calibrated to the non-exchangeable hydrogen of a whale baleen laboratory standard (δ^2H = −108‰) at Environment Canada. Measured δ^2H values of the chicken and turkey feather values were compared with their known non-exchangeable δ^2H values. The observed relationship between the measured and known non-exchangeable δ^2H values of these reference materials was used to adjust for the effect of exchangeable hydrogen (Britzke et al. 2009) in unknown samples. Despite the varied natures and large potential range of δ^2H 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. The δ^2H values were determined using a Finnigan TC/EA (Thermo Fisher Scientific, Waltham, Massachusetts) coupled to a Thermo-Finnigan Delta Plus XL mass spectrometer (Thermo Fisher Scientific) at the Carnegie Institution of Washington (Washington, D.C.). Samples were introduced into the TC/EA using a Costech Zero Blank autosampler. While in the autosampler,

possible hydrogen exchange between ambient water vapor and samples was controlled-for by flooding the sealed autosampler carousel with He. In addition, all samples were equilibrated and analyzed using the same methods during the same period. Analysis of the two keratin and two oil (pump oil and mineral oil) reference materials showed that the precision of δ^2H analysis was ≤4‰. Prior to δ^{18}O analysis, tissue materials were equilibrated alongside protein reference materials (beef blood, δ^{18}O = 6 ± 0.3‰; beef liver, δ^{18}O = 8 ± 0.5‰; and feather keratin, δ^{18}O = 8 ± 0.3‰) for at least 2 weeks. The δ^{18}O values were determined using a Thermo Finnigan TC/EA coupled to a Thermo-Finnigan Delta Plus XP continuous-flow mass spectrometer (Thermo Fisher Scientific) at the University of Wyoming Stable Isotope Facility. Analysis of reference materials (beef blood, beef liver, feather keratin, and benzoic acid) showed that the precision of δ^{18}O analysis was ≤0.5‰. The δ^2H and δ^{18}O values of liquid water and body water cryodistilled from soft tissues were determined using the Los Gatos Research (LGR) Laser Water Analyzer (Los Gatos Research, Mountain View, California) at the University of Wyoming Stable Isotope Facility.

To assess whether the isotope values of bird tissues had achieved constant asymptotic values, the delta values of red blood cells from repeated sampling events were compared using paired *t*-tests. Following Ehleringer et al. (2008), Bowen et al. (2009), and Wolf et al. (2011), we used standard least-squares linear regression to establish the relationship between the isotopic composition of individual tissues and that of drinking water. When the isotopic signatures of consumer tissues had a linear relationship to drinking-water isotopic signatures, we used the slope of the relationship as a first-order estimate of the fractional contribution of drinking water to the hydrogen and oxygen content of tissues (Ehleringer et al. 2008). Differences in the isotopic values among tissues were analyzed using multivariate analysis of variance (MANOVA) with treatment, tissue, and treatment * tissue as independent variables and isotopic value as a response variable. In cases where tissue values were missing, the entire bird was removed from the MANOVA. Post hoc comparisons among means for all MANOVAs were done using Tukey's HSD tests. Differences in tissue stable isotope values between sexes and drinking-water consumption volume levels were analyzed using repeated-measures analysis of variance (RM ANOVA). We used RM ANOVA because tissues sampled from the same bird are not statistically independent. By using RM ANOVA, we were able to compare the δ^2H or δ^{18}O values among the tissues without violating any test assumptions. For each RM ANOVA, we completed a Mauchly's test for sphericity to ensure that all possible pairs of groups met the RM ANOVA's assumption that variances of groups were equal. If this test was statistically significant, we used the Greenhouse-Geisser corrected values for RM ANOVA. The Greenhouse-Geisser correction is a conservative technique that prevents the Type I error associated with sphericity (Zar 1996). In cases where tissue values were missing, the entire bird was removed from the RM ANOVA analysis. All statistical analyses were performed in the program JMP, versions 9.0 and 10.0 (SAS Institute, Cary, North Carolina).

Results

In the 90 days following the drinking-water switch, the birds did not experience a significant change in body mass (initial mean M_b = 176 g, final mean M_b = 174 g, t = 1.71, df =14, P = 0.06). Mean daily

drinking-water volumes for individual birds were relatively high and displayed high variability (total daily average = 36.78 ± 15.38 mL [approximately 21% ± 8% of Japanese Quail M_b]). One bird from treatment group 3 died before the end of the experiment. We did not include any tissues from this bird in our analyses. We were also unable to collect feathers from one bird in treatment group 3 because of a lack of feather regrowth. In addition, we were unable to collect day-64 red blood cells, plasma, and body water samples from one bird in treatment group 2, and we lost day-64 plasma samples for δ^2H for one bird from treatment group 3 and for $\delta^{18}O$

for one bird from treatment group 1 because of autosampler error. Within each treatment, the delta values of red blood cells reached constant values by day 32 for both hydrogen (t = 1.4, df = 12, P = 0.18) and oxygen (t = 0.8, df = 12, P = 0.38).

The δ^2H values of body water and feathers were linearly related to the δD values of drinking water (Fig. 1). However, the δ^2H values of plasma, red blood cells, intestine, liver, and skeletal muscle, did not show a statistically significant linear relationship with those of drinking water (Fig. 1). The estimated percent contributions (± SE) of drinking water to the hydrogen isotopic signatures
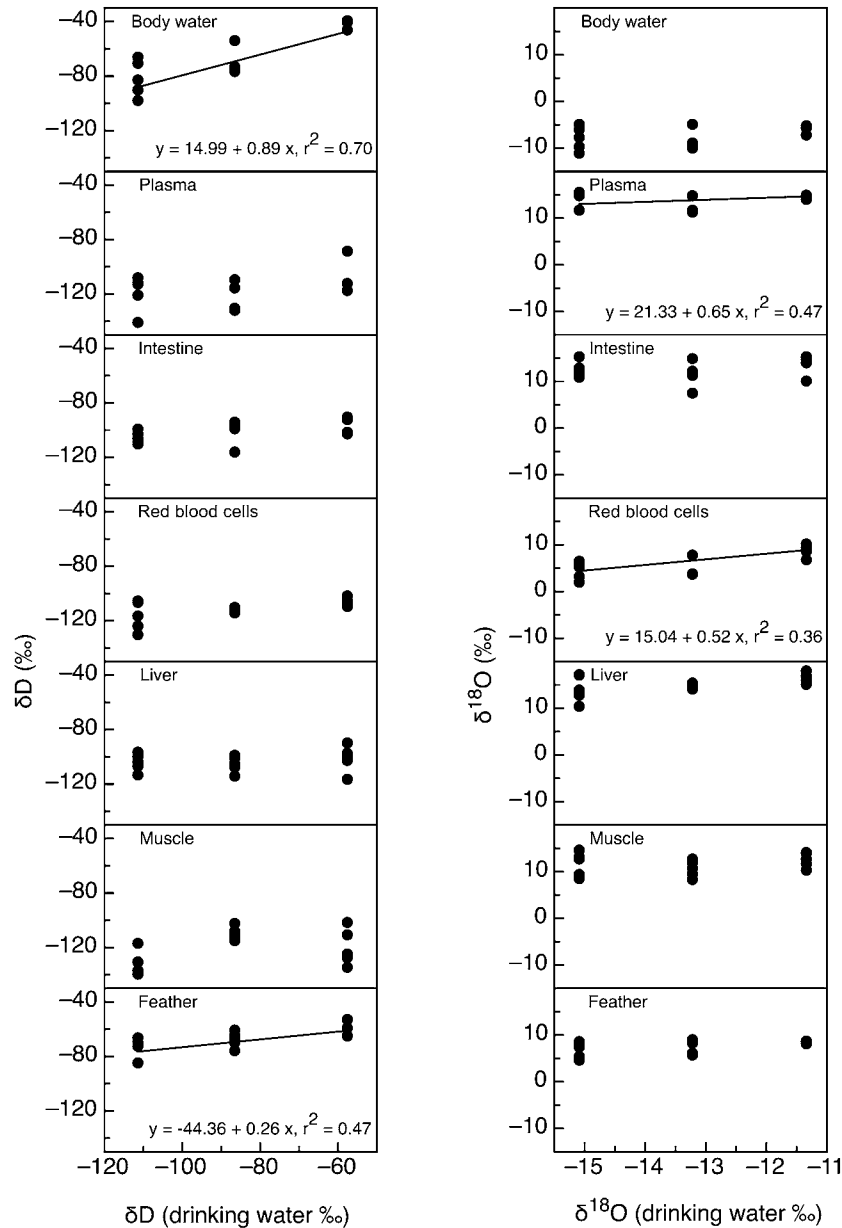

FIG. 1. Relationships between the δ^2H (left) and $\delta^{18}O$ (right) of drinking water and that of seven tissue types in Japanese Quail: body water, blood plasma, intestine, red blood cells, liver, muscle, and feathers. Regression lines are provided for significant relationships. The δ^2H values of body water and feathers were significantly correlated with those of drinking water. The $\delta^{18}O$ values of red blood cells and plasma were significantly correlated with those of drinking water.

Table 1. Mean (± SD) isotopic values of Japanese Quail tissues in three drinking water treatments. Within treatments, these means varied significantly. For each treatment, tissues are arranged from the lowest isotopic value to the highest. Tissues with different letters are significantly different (Tukey's HSD, $P < 0.05$).

	Muscle	Plasma	Red blood cells	Intestine	Liver	Body water	Feathers
Drinking water 1 δD = −111.4‰	−130.9 ± 8.7 C Plasma	−118.9 ± 13.1 B C Red blood cells	−116.5 ± 10.7 B C Muscle	−105.4 ± 4.5 B Liver	−104.0 ± 6.4 B Intestine	−81.5 ± 13.2 A Body water	−72.9 ± 7.0 A Feathers
Drinking water 2 δD = −86.5‰	−121.9 ± 11.1 C Muscle	−112.3 ± 1.9 B C Plasma	−110.0 ± 5.0 B C Red blood cells	−105.3 ± 5.9 B Liver	−100.8 ± 8.6 B Intestine	−69.5 ± 10.5 A Feathers	−67.5 ± 5.6 A Body water
Drinking water 3 δD = −57.6‰ δ^{18}O	−119.8 ± 13.3 B Body water	−119.1 ± 28.9 B Feathers	−106.2 ± 3.0 B Red blood cells	−101.2 ± 9.7 B Plasma	−95.6 ± 6.0 B Muscle	−59.0 ± 6.0 A Intestine	−33.9 ± 16.2 A Liver
Drinking water 1 δ^{18}O = −15.0‰	−7.8 ± 2.5 D Body water	6.7 ± 1.7 C Feathers	7.3 ± 1.5 B C Red blood cells	11.5 ± 1.2 A B Muscle	11.6 ± 2.5 A B Intestine	12.7 ± 1.6 A Plasma	14.2 ± 2.9 A Liver
Drinking water 2 δ^{18}O = −13.2‰	−8.2 ± 2.2 D Body water	7.0 ± 1.5 C Feathers	7.9 ± 1.3 C Red blood cells	10.6 ± 1.7 B C Muscle	12.1 ± 3.0 A B Intestine	12.8 ± 1.5 A B Plasma	14.7 ± 0.5 A Liver
Drinking water 3 δ^{18}O = −11.3‰	−2.1 ± 7.9 C	8.2 ± 0.5 B	9.4 ± 0.4 B	12.5 ± 1.5 A B	13.8 ± 2.1 A B	14.1 ± 1.0 A B	16.5 ± 1.0 A

of tissues were 89 ± 17% for body water and 26 ± 8% for feathers. For oxygen, red blood cells and plasma were the only tissues for which the δ^{18}O values were significantly linearly related to the δ^{18}O values of drinking water (Fig. 1). No other tissue had δ^{18}O values that displayed significant relationships with those of drinking water (Fig. 1). The estimated percent contribution (± SE) of drinking water to the oxygen isotopic signatures of tissues was 52 ± 20% for red blood cells and 65 ± 21% for plasma.

We found significant differences in the isotopic values among tissues for hydrogen (MANOVA, $F = 4.088$, df = 7 and 88, $P < 0.0001$; Table 1) but no significant interaction between tissue type and drinking-water treatment group for hydrogen (MANOVA, $F = 0.28$, df = 14 and 88, $P = 0.051$). The δ^2H values of feathers and body water were more enriched in deuterium than other tissues, whereas muscle and blood plasma tended to be more depleted than other tissues (Table 1 and Fig. 2). There was no significant effect of sex (uncorrected RM ANOVA, $F = 0.30$, df = 6 and 42, $P = 0.93$) or daily drinking-water volume (uncorrected RM ANOVA, $F = 0.24$, df = 6 and 42, $P = 0.24$) on tissue δ^2H values. We also found significant differences in the isotopic values among tissues for oxygen (MANOVA, $F = 14.96$, df = 7 and 82, $P < 0.0001$; Table 1 and Fig. 2), but no significant interaction between tissue type and drinking-water treatment group for oxygen (MANOVA, $F = 0.12$, df = 14 and 82, $P = 0.74$). Body water was significantly more depleted in ^{18}O than other tissues, and liver was significantly more enriched in ^{18}O than other tissues (Table 1 and Fig. 2). There was no effect of sex (Greenhouse-Geisser corrected RM ANOVA, $F = 0.57$, df = 1.50 and 9.03, $P = 0.53$) or daily drinking-water volume (Greenhouse-Geisser corrected RM ANOVA, $F = 0.21$, df = 1.50 and 9.03, $P = 0.74$) on tissue δ^{18}O values.

The δ^2H and δ^{18}O values were significantly correlated in body water ($r = 0.837$, $P < 0.05$) and feathers ($r = 0.700$, $P < 0.05$; Fig. 3).

There were no significant correlations between δ^2H and δ^{18}O values in any other tissue (Fig. 3).

Discussion

Estimating the contribution of drinking water to tissue δ^2H and δ^{18}O signatures using slopes.—Given Hobson et al.'s (1999) results, we expected the slopes of the relationships between drinking-water δ^2H values and Japanese Quail tissue δ^2H values to be greater than those found in House Sparrows (Wolf et al. 2011). For hydrogen, this expectation proved correct for body water and feathers. Both of these tissues displayed significant linear relationships between tissue and drinking-water δ^2H that indicated drinking-water contributions of 26% and 89% for feathers and body water, respectively. These estimates are similar to those found by Hobson et al. (1999). By contrast, for House Sparrows, drinking-water contributions to tissue δ^2H values ranged between 14% and 18% (Wolf et al. 2011). For the majority of the Japanese Quail tissues in our experiment, however, no significant relationships between the hydrogen isotopic signatures of tissues and those of drinking water were observed.

For oxygen, significant linear relationships between tissue and drinking-water δ^{18}O were observed for red blood cells and plasma. Previous studies (O'Brien and Wooller 2007, Ehleringer et al. 2008, Wang et al. 2009, Nielson and Bowen 2010, Wolf et al. 2011) using a variety of tissues have reported larger contributions of drinking water to the oxygen in tissues than those observed for the hydrogen in tissues. These contributions for oxygen range from 15% in House Sparrow feathers (Wolf et al. 2011) to 70% in the chitin produced by larval chironomids and brine shrimp (Wang et al. 2009, Nielson and Bowen 2010). Our estimates of a 50% contribution of drinking water for red blood cells and a 65%
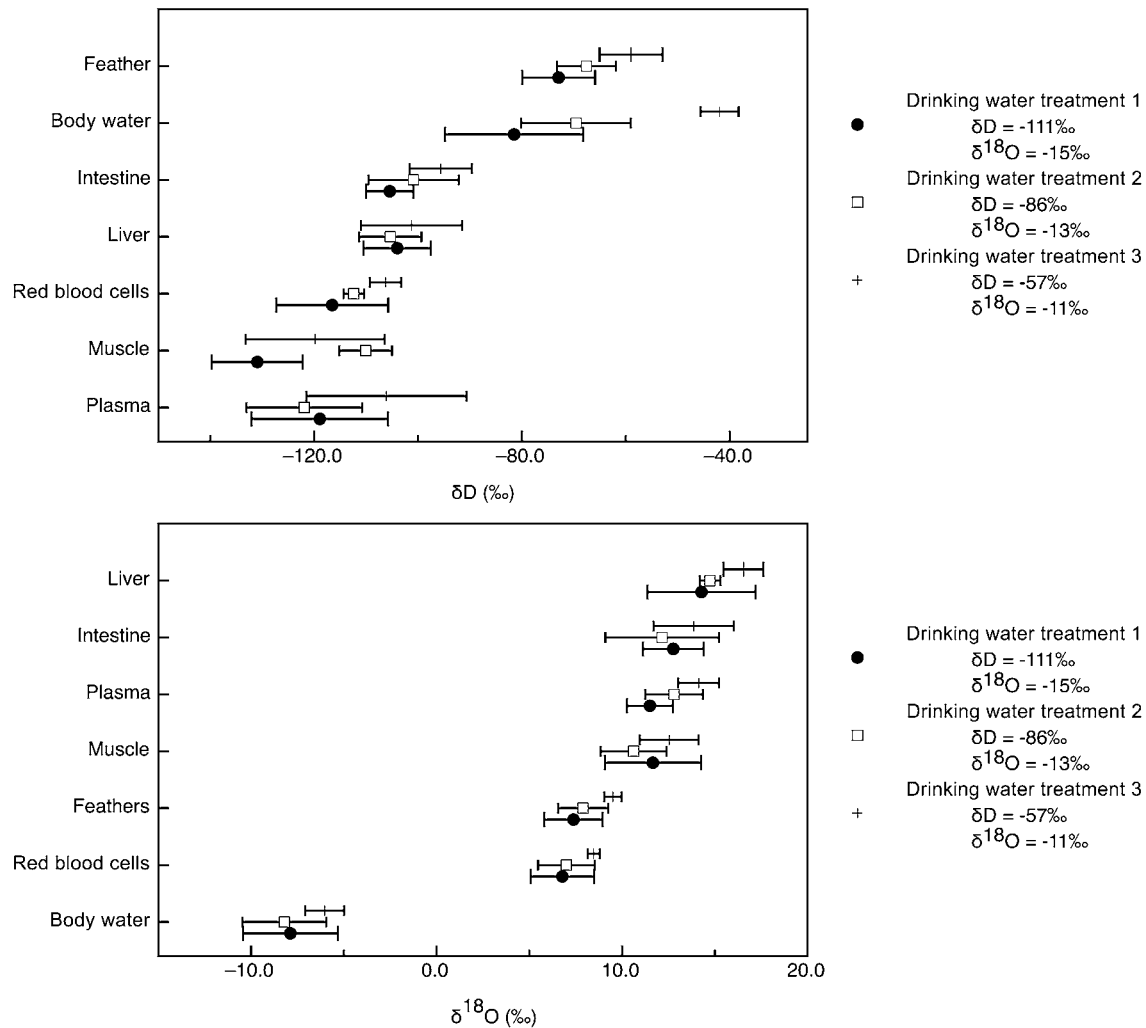
FIG. 2. Mean (± SE) of δ^2H and $\delta^{18}O$ values for each tissue type from Japanese Quail in three drinking-water treatments. We found significant differences in the isotopic values between tissues for both hydrogen and oxygen. There was no significant interaction between tissue type and drinking-water treatment group for either isotope. There was no significant effect of treatment or sex.

contribution for plasma are both well within this general range, and higher than the estimated contributions of drinking water to the $\delta^{18}O$ values of House Sparrow tissues reported by Wolf et al. (2011).

Our results demonstrate that the mechanism behind the difference in the fractional contributions of drinking water to tissue isotopic signature between Japanese Quail (Hobson et al. 1999) and House Sparrows (Wolf et al. 2011) remains unknown. Ehleringer et al. (2008) and Wolf et al. (2011) have demonstrated that the slopes of the relationships between drinking-water δ^2H or $\delta^{18}O$ and tissue δ^2H or $\delta^{18}O$ are likely dependent on the proportion of body-water hydrogen or oxygen derived from drinking water. Thus, the relatively low contribution of drinking water to tissue δ^2H and $\delta^{18}O$ values in House Sparrows as compared with Japanese Quail likely results from the differences in water requirements of the two species (Bartholomew and MacMillen 1961, MacMillen 1990). The influence of drinking-water intake on the fractional contribution of drinking water to tissue isotopic values

would also explain the larger contribution of drinking water to tissue $\delta^{18}O$ than to tissue δ^2H values. Because the oxygen atoms in the carboxylic acid group of amino acids are exchanged with oxygen atoms from the body-water pool during digestion and proteolysis (Bernlohr 1972), a large proportion of the oxygen atoms in the amino acids comprising the animal's tissues will be derived from drinking water for animals in which the proportion of drinking water in the body-water pool is greater than that of metabolically produced water.

The small number of tissues for which a significant linear relationship existed between tissue isotopic signature and drinking-water isotopic signature (Fig. 1) is surprising given the success of this technique in previous studies (Ehleringer et al. 2008, Bowen et al. 2009, Wolf et al. 2011). This unexpected result may be a consequence of the multiple sources from which the hydrogen and oxygen in animal tissues can be derived: food and preformed water (i.e., water in food and free drinking water) for hydrogen; and food, preformed water, and atmospheric oxygen
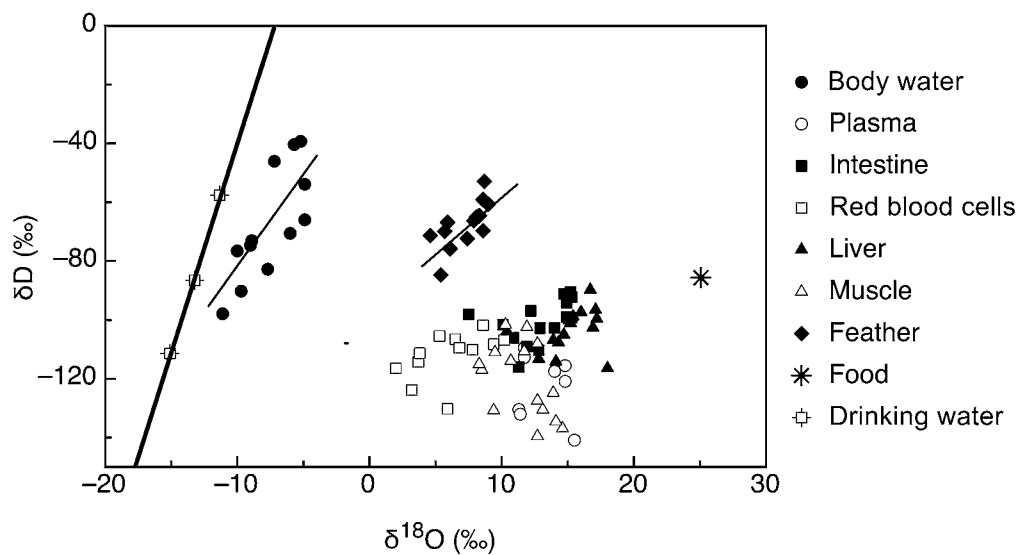
Fig. 3. The δ^2H and δ^{18}O values of body water and feathers were positively and linearly related ($y = -16.43 + 6.80x$, $r^2 = 0.60$ and $y = -94.79 + 3.74x$, $r^2 = 0.49$ for body water and feather, respectively; $P < 0.01$). The δ^2H and δ^{18}O values of no other tissues were statistically related. For reference, we included the meteoric water line and the isotopic composition of food.

for oxygen (Hobson et al. 1999, 2009). The interaction and relative contributions of these sources influence the final isotopic value of consumer tissues. Consequently, variations in the input of one of these potential sources can significantly influence the final isotopic value of the consumer tissue.

Variation in the isotopic signatures among tissues.—The large amount of variation we observed in the isotopic composition of tissues was similar to that found in House Sparrows (Wolf et al. 2011). The processes that contribute to variation in isotopic discrimination among tissues are well studied for carbon and nitrogen isotopes (Martínez del Rio et al. 2009). These include nutrient routing, differences in protein turnover, and differences in the amino acid compositions of tissues (Martínez del Rio et al. 2009). We suspect that the differences in the δ^{18}O and δ^2H values of tissues are also the result of these processes. Amino acid composition can vary greatly among tissue types within an individual. For example, bird feathers have ~1,400 µmoles g^{-1} dry mass of glycine and 540 µmoles g^{-1} dry mass of glutamic acid (Murphy et al. 1990). Bird muscle, on the other hand, has only ~798 µmoles g^{-1} dry mass of glycine but has ~1,115 µmoles g^{-1} dry mass of glutamic acid (Murphy 1994). Because the δD value of different amino acids can differ by >300‰ in a single tissue (M. L. Fogel et al. unpubl. data), differences in the amino acid composition of tissues are likely the primary cause for observed variation in the discrimination values among tissues (Fig. 2).

Implications for the use of δ^2H and δ^{18}O analysis in the laboratory and field.—Our results have several implications for the use of δ^2H and δ^{18}O analysis in ecological studies and for the interpretation of δ^2H and δ^{18}O data. First, it appears that the differences in the fractional contribution of drinking water to tissue δ^2H and δ^{18}O signatures observed by Hobson et al. (1999) and Wolf et al. (2011) are the result of interspecific differences in physiology and/or ecology and are not due to differences in experimental or laboratory protocol (Lott and Smith 2006, Smith et al. 2009). In Japanese Quail, the coefficient of determination of

the relationship between the δ^2H of feathers and the δ^2H of precipitation was 0.47, and the slope of this relationship was 0.25. For comparison, using the same experimental protocol, Wolf et al. (2011) found a coefficient of determination of 0.73 and a slope of 0.18 for this same relationship for House Sparrow feathers. These differences in the strength and slope of the relationships between the δ^2H values of drinking water and the δ^2H values of Japanese Quail and House Sparrow feathers suggest that interspecific differences in the mechanisms that influence the transfer of hydrogen from resources to consumer tissues may play an influential role in creating the variation reported by Hobson (2008). Although differences in water intake seem a likely candidate for this mechanism, more specific research is needed to confirm this suspicion.

Our research also showed that despite an isotopically uniform diet, significant differences in δ^2H and δ^{18}O values can occur among tissues from the same individual. Because of these differences, we urge caution when considering comparisons between different tissue types. Analyses using δ^2H and δ^{18}O are a powerful technique for studying animal ecology. Unlike carbon and nitrogen, however, the pathway(s) from resources to consumer tissues is not necessarily straightforward for hydrogen and oxygen. Consequently, researchers who wish to use δ^2H or δ^{18}O analysis to study animal ecology should recognize this complexity and interpret the results of such investigations conservatively.

Literature Cited

Bartholomew, G. A., and R. E. MacMillen. 1961. Water economy of the California Quail and its use of sea water. Auk 78: 505–514.

Bernlohr, R. W. 1972. ^{18}Oxygen probes of protein turnover, amino acid transport, and protein synthesis in *Bacillus licheniformis*. Journal of Biological Chemistry 247:4893–4899.

Bowen, G. J., J. R. Ehleringer, L. A. Chesson, A. H. Thompson, D. W. Podlesak, and T. E. Cerling. 2009. Dietary and physiological controls on the hydrogen and oxygen isotope ratios of hair from mid-20th century indigenous populations. American Journal of Physical Anthropology 139:494–504.

Britzke, E. R., S. C. Loeb, K. A. Hobson, C. S. Romanek, and M. J. Vonhof. 2009. Using hydrogen isotopes to assign origins of bats in the eastern United States. Journal of Mammalogy 90: 743–751.

Carleton, S. A., L. Kelly, R. Anderson-Sprecher, and C. Martinez del Rio. 2008. Should we use one-, or multi-compartment models to describe ^{13}C incorporation into animal tissues? Rapid Communications in Mass Spectrometry 22: 3008–3014.

Caut, S., E. Angulo, and F. Courchamp. 2009. Variation in discrimination factors (Δ^{15}N and Δ^{13}C): The effect of diet isotopic values and applications for diet reconstruction. Journal of Applied Ecology 46:443–453.

Craig, H. 1961. Isotopic variations in meteoric waters. Science 133:1702–1703.

Ehleringer, J. R. 1989. Carbon isotope ratios and physiological processes in arid land plants. Pages 41–54 *in* Stable Isotopes in Ecological Research (P. W. Rundel, J. R. Ehleringer, and K. A. Nagy, Eds.). Springer, New York.

Ehleringer, J. R., G. J. Bowen, L. A. Chesson, A. G. West, D. W. Podlesak, and T. E. Cerling. 2008. Hydrogen and oxygen isotope ratios in human hair are related to geography. Proceedings of the National Academy of Sciences USA 105:2788–2793.

Hobson, K. A. 2005. Using stable isotopes to trace long-distance dispersal in birds and other taxa. Diversity and Distributions 11:157–164.

Hobson, K. A. 2008. Applying isotopic methods to tracking animal movements. Pages 45–78 *in* Tracking Animal Migration with Stable Isotopes (K. A. Hobson and L. I. Wassenaar, Eds.). Academic Press, London.

Hobson, K. A., L. Atwell, and L. I. Wassenaar. 1999. Influence of drinking water and diet on stable-hydrogen isotope ratios of animal tissues. Proceedings of the National Academy of Sciences USA 96:8003–8006.

Hobson, K. A., S. H. DeMent, S. L. Van Wilgenburg, and L. I. Wassenaar. 2009. Origins of American Kestrels wintering at two southern U.S. sites: An investigation using stable-isotope (δD, δ^{18}O) methods. Journal of Raptor Research 43:325–337.

Lott, C. A., and J. P. Smith. 2006. A geographic-information-system approach to estimating the origin of migratory raptors in North America using stable hydrogen isotope ratios in feathers. Auk 123:822–835.

MacMillen, R. E. 1990. Water economy of granivorous birds: A predictive model. Condor 92:379–392.

Martínez del Rio, C., N. Wolf, C. Carleton, and L. Z. Gannes. 2009. Isotopic ecology ten years after a call for more laboratory experiments. Biological Reviews 84:91–111.

Murphy, M. E. 1994. Amino acid compositions of avian eggs and tissues: Nutritional implications. Journal of Avian Biology 25: 27–38.

Murphy, M. E., J. R. King, T. G. Taruscio, and G. R. Geupel. 1990. Amino acid composition of feather barbs and rachises in three species of pygoscelid penguins: Nutritional implications. Condor 92:913–921.

Nielson, K. E., and G. J. Bowen. 2010. Hydrogen and oxygen in brine shrimp chitin reflect environmental water and dietary isotopic composition. Geochimica et Cosmochimica Acta 74:1812–1822.

O'Brien, D. M., and M. J. Wooller. 2007. Tracking human travel using stable oxygen and hydrogen isotope analyses of hair and urine. Rapid Communications in Mass Spectrometry 21: 2422–2430.

Paritte, J. M., and J. F. Kelly. 2009. Effect of cleaning regime on stable-isotope ratios of feathers in Japanese Quail (*Cortunix japonica*). Auk 126:165–174.

Smith, A. D., C. A. Lott, J. P. Smith, K. C. Donohue, S. Wittenberg, K. G. Smith, and L. Goodrich. 2009. Deuterium measurements of raptor feathers: Does a lack of reproducibility compromise geographic assignment? Auk 126:41–46.

Wang, Y. V., D. M. O'Brien, J. Jenson, D. Francis, and M. J. Wooller. 2009. The influence of diet and water on stable oxygen and hydrogen isotope composition of Chironomidae (Diptera) with paleoecological implications. Oecologia 160:225–233.

Wolf, N., G. J. Bowen, and C. Martínez del Rio. 2011. The influence of drinking water on the δD and δ^{18}O values of House Sparrow plasma, blood, and feathers. Journal of Experimental Biology 214:98–103.

Zar, J. H. 1996. Biostatistical Analysis, 3rd ed. Prentice Hall, New Jersey.

Associate Editor: M. Klaassen