Stable isotopes of H, C and N in mice bone collagen as a reflection of isotopically controlled food and water intake*

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ABSTRACT

$^{2}$H/$^{1}$H ratios in animal biomass reflect isotopic input from food and water. A 10-week controlled laboratory study raised 48 mice divided in two generations (8 mothers Mus musculus and their offspring). The mice were divided into four groups based on the combination of $^{2}$H, $^{13}$C, $^{15}$N-enriched and non-enriched food and water. Glycine, the most common amino acid in bone collagen, carried the $^{2}$H, $^{13}$C, $^{15}$N-isotopic spike in food. ANOVA data analysis indicated that hydrogen in food accounted for $\sim$81% of the hydrogen isotope inventory in collagen whereas drinking water hydrogen contributed $\sim$17%. Air humidity contributed an unspecified amount. Additionally, we monitored $^{13}$C and $^{15}$N-enrichment in bone collagen and found strong linear correlations with the $^{2}$H-enrichment. The experiments with food and water indicate two biosynthetic pathways, namely (i) de novo creation of non-essential amino acids using hydrogen from water, and (ii) the integration of essential and non-essential amino acids from food. The lower rate of isotope uptake in mothers’ collagen relative to their offspring indicates incomplete bone collagen turnover after ten weeks. The variance of hydrogen stable isotope ratios within the same cohort may limit its usefulness as a single sample proxy for archaeological or palaeoenvironmental research.

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Carbon-13; diet; food; hydrogen-2; isotope ecology; mice; nitrogen-15; tracer technique

1. Introduction

Stable isotope ratios of the most common elements found in living organisms (carbon C, hydrogen H, oxygen O, and nitrogen N) vary in nature due to physically, chemically, and biologically driven abundances and fractionations. The traditional use of carbon and nitrogen stable isotope ratios in biology has been subsequently joined by $^{2}$H/$^{1}$H ratios as an additional independent isotopic dimension [1]. Hydrogen isotopes found in animal tissues provide important insight into metabolism [2–6], ecology [7], and environmental

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conditions [8] of both modern vertebrates and their well-preserved fossil remains. Fruitful applications have been found in archaeology [9–11] and forensic sciences [12–14].

The hydrogen isotope ratio of bone collagen and other tissues is ultimately a mixture of the isotopic signature of water taken from the local environment by drinking and the signature of ingested food. The raising of biota under isotopically controlled trophic conditions and subsequent mass-balances can constrain the isotopic contributions from various sources [e.g. 4–6]. Aqueous inorganic hydrogen can enter newly formed organic molecules either via metabolic biosynthesis or during isotope exchange of hydrogen in organic functional groups with body water. Hydrogen from food can be metabolised to $\text{H}_2\text{O}$ through respiration, or can become incorporated into structural tissues, such as when amino acids are biopolymerised to form proteins. The diverse hydrogen transfer routes across and within water and biomass yield a wide range of isotope ratios among different organic compound classes, taxa, environments, seasons, and geographic locations [15]. Most interestingly the range of hydrogen isotope ratios can be wide even within a single population. The intrapopulation variability seems to increase with the trophic level, being the narrowest in herbivores (bison, deer) and the largest among carnivores [16]. As the rapidly growing matrix of stable-isotope data on natural populations of plants and animals demonstrates complex relationships, the quantitative influence of individual factors on hydrogen stable isotope ratios in protein remains best constrained by carefully controlled laboratory experiments [17,18]. The interpretation of hydrogen stable isotope data from modern and palaeoenvironments should be based on insight from ground truthing experiments under lab-controlled conditions. Most commonly dietary-controlled isotopic studies utilised tissues whose sampling was relatively harmless to animals, such as hair and feather keratin (birds: [19]; rodents: [20]; humans: [21]). However, in comparison to soft tissues, bone collagen represents a longer-term record of environmental conditions and an animal’s ecology during its lifetime due to the relatively slow turnover rate of most bones in mature animals (versus a more momentary, weekly, or monthly record inferred from hair and nail keratin) [22,23]. Collagen can be well preserved inside the protective bone matrix, and established techniques can diagnose fossil bone collagen’s integrity for palaeoenvironmental reconstruction [24,25]. Due to the detrimental nature for their subjects, the studies involving mammal bone collagen have been few (e.g. [6,26–28]).

Our study tested the effects of diet and metabolism on hydrogen isotopes in animal tissues. The focus was on population diversity of hydrogen stable isotope ratio in mice bone collagen in controlled laboratory conditions. In addition to the isotopic influence of food and water, we evaluated whether age and sex differences contribute to the isotopic composition of bone collagen. The carbon and nitrogen stable isotope systems were used in parallel with stable hydrogen ratios to provide multidimensional isotopic control on experimental variables.

2. Materials and methods

2.1. Preparation of mouse food

As starting material for mouse diets, we used commercially available Purina® 5015 Mouse Diet that was optimised for long-term lab mouse experiments (caloric contributions in % of
This dietary formula was designed to minimize the variability of nutrient composition (amino acids, carbohydrates, lipids, etc.) in natural ingredients and thus aided in dietary mass-balance calculations of stable isotopes $^2$H, $^{13}$C, and $^{15}$N. We prepared two types of mouse food representing isotopically enriched and control food (Figure 1) by adding small amounts (approximately 0.01 weight %) of glycine with $^2$H, $^{13}$C and $^{15}$N-enrichments to the first, and pure non-enriched glycine to the latter. Glycine represents about every third amino acid in bone collagen [29]. The glycine enrichment was prepared as a common aqueous solution of (1) $[^{2}$H$_5$]glycine with 98 atom % $^2$H and (2) doubly-substituted $[^{13}$C$_2$$^{15}$N]glycine with $\geq$98 atom % $^{13}$C and 98 atom % $^{15}$N (Medical Isotopes, Inc., Pelham, New Hampshire). The use of a triply enriched ($^2$H, $^{13}$C, and $^{15}$N) glycine provided complementary, independent isotopic controls for recognition of contributions of $^2$H to collagen from food versus water. In order to avoid taste preference, the isotopically non-enriched regular kibble (i.e. small chunks) food had an equivalent addition of regular, non-enriched glycine with the same nutritional value as the isotopically enriched kibbles. The small amounts of added $^2$H-enriched glycine were not considered to have adverse effects on mice because the $^2$H-enrichment in kibbles amounted to only $\sim$30 % elevated $^2$H-abundance in bulk organic hydrogen. In absolute terms, the $^2$H abundances in regular and isotopically

Figure 1. (A) Drilled mice kibbles; (B) Individual kibble size was slightly variable; (C) Kibble with injected 15 µL of green food dye test solution to visually examine the distribution of injected aqueous solutions in a ‘spiked’ kibble; (D) Cross section of a split kibble after the green coloured test solution had been injected. The image shows how the solution permeated and dried in the drilled holes.
enriched kibbles were 0.0155 and 0.0199 atom % of organic hydrogen, respectively. The difference has no physiological effect [30]. The atom % enrichments in terms of $^{13}$C and $^{15}$N were below that of $^2$H.

The experimental design strictly followed practical and ethical norms for research on laboratory animals set by Indiana University’s Bloomington Institutional Animal Care and Use Committee (BIACUC). BIACUC regulations mandated that the structural integrity of each kibble was preserved even after the addition of the glycine spike. Accordingly, we spiked each kibble by drilling multiple holes with a diameter of approximately 1 mm in a domino pattern and injecting approximately 15 µL of aqueous glycine solutions (Figure 1). The holes did not compromise the mechanical integrity of kibbles and at the same time guaranteed quantitative absorption of glycine solutions in the kibbles’ interior. The injected kibbles were left to air-dry and averaged approximately 5 g dry weight. Spiking the interior of kibbles resulted in a non-homogenous isotopic distribution of glycine within each kibble. We randomised the glycine injection sites and tried to encourage mice to eat entire kibbles to produce an isotopically homogenous intake on average. The food was prepared freshly in $\sim$1 kg batches according to the need of the growing mice. The food was freely accessible to the mice (ad libitum), it was added to cages as needed, and the food levels were checked daily.

### 2.2. Preparation of drinking water

Two isotopically different types of Bloomington (Indiana) tap water were prepared. This study adheres to the current IUPAC definition of $\delta$-values expressing stable isotope ratios, for example for hydrogen (Equation 1)

$$\delta^2H = \left[ R\left(2^{\text{H}}/1^{\text{H}}\right)_{\text{sample}} - R\left(2^{\text{H}}/1^{\text{H}}\right)_{\text{standard}} \right] / R\left(2^{\text{H}}/1^{\text{H}}\right)_{\text{standard}},$$

where $R$ expresses a ratio of $^2$H versus $^1$H.

Regular, non-$^2$H-enriched Bloomington tap water averaged $\delta^2H_{\text{water}} = -36 \, \%$ for the duration of our experiment. The observed weekly tap water $\delta^2H_{\text{water}}$ variations of up to 10 % agree with previously reported seasonal $\delta^2H_{\text{water}}$ variations in tap water [31]. The $^2$H-enriched water with a $\delta^2H_{\text{water}}$ value of $\sim+952 \, \%$ was prepared by adding a calculated amount of $^2$H$_2$O to Bloomington tap water. In compliance with BIACUC regulations, the two waters were available to mice ad libitum, freshly prepared and replaced in cages weekly for the duration of the experiment.

We used standardised drip bottles for mice cages that limited evaporation. The bottles were hung upside-down with a metal tube protruding from a cap. The tube contained a metal ball acting as a valve that released water only by a mouse licking it. Occasional sampling and isotopic monitoring of water samples from bottles yielded no evidence for isotopic drift due to partial evaporation.

### 2.3. Raising of laboratory mice under controlled dietary conditions

Eight two-month old pregnant female mice (Mus musculus L.; inbred strain C57BL/6) raised by Harlan Laboratories, Inc. (Indianapolis, Indiana) were fed common lab mouse diet and local Indianapolis tap water (annual precipitation average $\delta^2H_{\text{water}} = -48 \, \%$, while it is $-47 \, \%$ for Bloomington, Indiana, based on The Online Isotopes in Precipitation Calculator
At the end of their second gestational week they arrived at Indiana University and delivered their offspring in the next 5–6 days.

The experiment was conducted for four months (i.e. 17 weeks and five days) in the lab animal facilities of the School of Optometry at Indiana University, Bloomington, Indiana, and the animals had unrestricted access to the specially prepared food of known H, C, N-isotopic compositions and water of known H-isotopic composition (Figure 2). The mice were divided into four groups A to D, which received different combinations of isotopically enriched and non-enriched (i.e. regular) Bloomington tap water and mice kibble food (Figure 2; Table 1). One water type was $^2$H-enriched and the other was not, and one food type was enriched with $^2$H, $^{13}$C, and $^{15}$N-enriched glycine and the other was not. Control group A received regular food and water. Group B received isotopically enriched food and regular Bloomington tap water. Group C received regular food and $^2$H-enriched tap water. Finally, group D received both isotopically enriched food and $^2$H-enriched water. These diets were introduced to pregnant mice from their first day at Indiana University, thus their offspring were first exposed to the enrichments in utero at 2 weeks gestational age or 1 week prior to birth. At this point of mouse foetal development the limb bone ossification had started but was not completed until one to three weeks after birth [33] thus exposing the bone collagen to enrichment for the entire duration of our experiment.

After weaning at about 3 weeks of age, the offspring were separated from mothers. Ten pups for each of the four dietary groups were separated by sex and continued to be raised on the same diet and water as had been given to their mothers. The mothers were continued on their pre-weaning diets and waters as well, yielding a total of 48 mice (i.e. 4 groups with 10 offspring + 2 mothers in each group). Throughout the experiment, all mice were cared for and ultimately euthanised by decapitation under deep anesthesia using sodium pentobarbital (50 mg/kg) administered intraperitonially in strict accordance to BIACUC guidelines at Indiana University.

At the end of the 4-month period we collected multiple tissues (bone, muscle, skin, and liver). The only animal tissue used in the present study is bone collagen, while the other

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**Figure 2.** Timeline of the mice experiment. (I) Prior to the start of our experiment, all mothers were raised on typical lab mouse diet and tap water in a breeding facility in central Indiana; (II) After relocation to our facility at Indiana University, pregnant mothers were separated into four dietary groups with contrasting isotopic characteristics; (III) During late gestation and after birth, offspring were isotopically imprinted via their mother and later directly via the food and water that were available in cages shared with their mothers; (IV) Weaned offspring were separated from mothers by sex and kept receiving the same food and water as their mothers. The experiment ended with euthanasia at the end of the fourth month at Indiana University.
tissues were frozen for future research. In order to prevent ambiguity from different growth and turnover rates in different bones, we only used front and rear leg bones for this study (femur, tibia, fibula, humerus, radius, and ulna).

2.4. Preparation of collagen

We used the same classical method for preparing collagen that was described in [16]. In brief, bones were mechanically cleaned, ultrasonicated to remove remnants of soft tissue, and demineralised in 1N hydrochloric acid (HCl) for one week at room temperature with occasional stirring. The demineralised material was rinsed to neutral pH, freeze-dried, lipid-extracted with dichloromethane, dried, and stored in glass vials until isotopic measurements were conducted. The C:N ratios of the resulting collagen indicated good preservation for stable isotope analysis. We note that after completion of our analytical work, Cersoy et al. [34] compared several published bone demineralisation protocols and found that a method’s length and complexity had little effect on the collagen isotopic composition.

2.5. Isotope analyses of mice food and water

Stable isotope analyses were used to determine the average isotopic values of the treated and untreated food and water. Water samples were analysed by David Finkelstein at the University of Massachusetts Amherst using a Picarro L2120-i Isotopic Water Analyzer. Samples were calibrated with a set of in-house water standards spanning a wide range of δ²H values that had been calibrated against VSMOW, SLAP, GISP, and δ²H-enriched water standards IAEA-302 A and B. For isotopic characterisation of the two kibble food types we created a small ‘master batch’ representing a combination of individual kibbles subsampled from all weekly food batches. The composite master batch was homogenised by grinding in a ball mill and dried in the vacuum chamber of a freeze-dryer. However, even extensive grinding could not sufficiently homogenise food at the 0.3-mg level for on-line analyses in silver and tin cups. Also, the lipid content of the food kibbles would have been partially steam-distilled out of any container when exposed to equilibration in hot steam, thus causing partial loss of the ¹H-enriched lipid fraction. Our choice to equilibrate the food in moist air at room temperature avoided partial mobilisation and loss of the lipid fraction, but probably entailed a slightly different average hydrogen fractionation factor between organic and steam hydrogen when compared with steam equilibration of collagen. While this may impart a small error in food δ²H versus

Table 1. Groups of mice receiving isotopically distinct combinations of kibble food and tap water.

<table>
<thead>
<tr>
<th>Group</th>
<th>Food*</th>
<th>Water**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (control)</td>
<td>regular</td>
<td>regular</td>
</tr>
<tr>
<td>B</td>
<td>regular</td>
<td>²H, ¹³C, ¹⁵N-enriched</td>
</tr>
<tr>
<td>C</td>
<td>²H, ¹⁵N-enriched</td>
<td>regular</td>
</tr>
<tr>
<td>D</td>
<td>²H, ¹³C, ¹⁵N-enriched</td>
<td>²H-enriched</td>
</tr>
</tbody>
</table>

*Regular kibble food contained additional regular, isotopically non-enriched glycine in the same concentration as the isotopically enriched kibble food containing ²H, ¹³C, and ¹⁵N-enriched glycine.

**δ²H values of Bloomington tap water were −36 ± 7 ‰. ²H-enriched water was +952 ± 134 ‰ and, in compliance with BIACUC regulations, was prepared fresh weekly by spiking regular tap water with ²H₂O.
collagen $\delta^{2}H$ of a few per mil (e.g. [35]), the resulting analytical uncertainty was expected to be small relative to the signal of the $^{2}H$-enriched food and water.

Multiple 4-mg aliquots of regular or isotopically enriched mouse food samples were placed into open silica (‘quartz glass’) combustion ampoules with additional cupric oxide, copper and silver. Samples were hydrogen-isotopically equilibrated at room temperature in closed chambers (‘desiccators’) in the presence of two isotopically distinct waters ($\delta^{2}H_{\text{water}} = -147$ and $1348 \permil$) at 100% humidity for one week, followed by drying of the samples under vacuum, sealing of evacuated quartz ampoules, and combustion at 800 °C overnight. Nitrogen and carbon dioxide were separated from water cryogenically in a vacuum line. Water was reduced to $H_2$ gas by passage over uranium metal at 800 °C [36]. The volumes of $N_2$, $CO_2$ and $H_2$ were measured manometrically in a vacuum line for the determination of elemental ratios. $H_2$ and $N_2$ gases were collected by a Toepler pump and temporarily sealed in pre-evacuated glass tubes, whereas $CO_2$ was frozen into evacuated glass tubes and sealed off. Isotope ratios were determined mass-spectrometrically by manual dual-inlet analysis with a Thermo Finnigan Delta Plus XP stable isotope mass spectrometer. Two-point normalisations relied on international standards VSMOW, SLAP, NBS 19, LSVEC, IAEA-N-1, and IAEA-N-2.

2.6. Isotope analyses of collagen

We measured non-exchangeable hydrogen ($H_n$) in collagen, most of which is tightly bonded to a carbon chain, does not exchange with the environment, and therefore preserves the isotopic information recorded during the life of an individual. Exchangeable hydrogen ($H_x$), bound to O, N, and S potentially exchanges with environmental water and humidity in air after collagen biosynthesis and does not preserve a primary isotopic signature [37], similar to some of the organic oxygen in collagen [38].

The isotope composition of the total pool of hydrogen ($\delta^{2}H_t$) can be represented by Equation (2):

$$\delta^{2}H_t = x_e \times \delta^{2}H_x + (1-x_e) \times \delta^{2}H_n$$

(2)

The $x_e$ represents the fraction of exchangeable hydrogen in the total pool of hydrogen [39,40].

We accounted for the contribution of $H_x$ to $H_t$ by equilibrating samples with steams of known isotope composition. This is done through Equation (3):

$$\varepsilon_{x-w} = [(1 + \delta^{2}H_x)/(1 + \delta^{2}H_w)] - 1,$$

(3)

where $\varepsilon_{x-w}$ represents the equilibrium isotope enrichment factor unique for each compound and dependent on ambient temperature; $\delta^{2}H_x$ represents exchangeable hydrogen in the sample, and $\delta^{2}H_w$ represents hydrogen in ambient water [37]. To constrain $x_e$ and $\varepsilon_{x-w}$ we equilibrated aliquots of our samples together with a set of laboratory standards of known stoichiometry in two water vapours with $\delta^{2}H_{\text{water}} = -136 \permil$ or 1246 %o [39].

Collagen samples were cut into aliquots weighing approximately 0.3 mg, and two samples per individual were loaded into silver capsules (Costech Analytical Technologies Inc., Valencia, CA, USA). Sequences of capsules containing samples and stable isotope standards were loaded into 50-position carousels, which were placed into aluminum chambers. Inside each tightly sealed chamber the samples were equilibrated for 6–8 h.
at 115 °C with water vapour of a known hydrogen isotope composition as described in [39]. At the end of equilibration, the chamber was dried in a flow of gaseous N₂, while simultaneously being cooled to room temperature, and then rapidly loaded into a Costech ZeroBlank autosampler, followed by immediate flushing of the loaded autosampler with helium. For measurement of hydrogen isotope ratios in collagen we used a TC/EA (i.e. thermal conversion elemental analyzer) coupled with a Delta Plus XP mass spectrometer yielding δ²H values. The isotope data are reported in standard δ²H notation relative to VSMOW (0 ‰) and SLAP (−428 ‰) according to Coplen’s [41] guidelines. Our analyses had been performed prior to the realisation that nitrogen-containing organic samples generate an HCN byproduct during TC/EA conversion, therefore resulting in incomplete hydrogen yields and systematically lowering of δ²H values by up to 30 ‰ depending on TC/EA conditions and stoichiometry of analytes [42,43]. In fact, Reynard and Tuross [44] found that these differences in collagen samples are 10–11 ‰. Fortunately, data analysis for this study is restricted to samples within this study only, which were all treated equally based on constant TC/EA operating conditions and equal treatment of all samples (internal lab standards, control group, and experimental group). The goal in this study is to examine differences between values, not necessarily actual values. Any systematic offset due to using a glassy carbon reactor as opposed to a chromium reactor will be inherent in all samples and will not affect calculated differences between treatment groups. The low variance of δ²H values in the control group (group A) makes the presence of HCN unlikely to have caused noise, since the noise from varying doses of spike was larger than that caused by incomplete hydrogen yields from TC/EA reactors. And finally, the TC/EA techniques we used match those of all pre-2016 hydrogen isotope work on collagen that used TC/EA as an inlet. The overall pattern of δ²H values therefore remains meaningful, even though the δ-scale underlying δ²H values is likely compressed. The same considerations affect published δ²H values of proteins that had been measured with conventional TC/EA (e.g. [21,45–47]).

For carbon and nitrogen isotope analyses of collagen we used a Costech Elemental analyzer coupled with on-line connection to a stable isotope mass-spectrometer Delta Plus XP. Stable isotope ratios are expressed as conventional δ-values relative to isotopic scales that are anchored by VPDB and Air. We used primary reference materials NBS 19, LSVEC, IAEA-N-1, and IAEA-N-2 for normalisation, and all our collagen runs utilised additional in-house organic isotopic reference materials C₃₆ n-alkane, coumarin, and acetanilides that had been developed at Indiana University [48,49]. The analytical precision (1σ) is generally better than ± 3 ‰ for δ²H, ±0.1 ‰ for δ¹³C, and ± 0.2 ‰ for δ¹⁵N.

We tested the effects of enriched diet on the pups using a two-way ANOVA Mathematica® (Ver. 11.3, Wolfram Research, 2018) between four groups (control, enriched water, enriched foot, and enriched water and food). The factors were δ²H of water, δ²H of food, and an interaction factor. This test determines whether the isotope values recorded from the collagen samples differ significantly between treatments. The test also determines whether there is a significant interaction effect between isotope enrichment of water and food (in other words, whether receiving both enriched food and enriched water influenced collagen that is different from what one would expect based on the effects when only one source is enriched).
3. Results

The isotopic characterisation of pairs of bulk kibble food and drinking waters yielded strong differences between isotopically enriched and non-enriched diets. The average \(^{2}H\)-enrichment (i.e. isotopic difference) among pairs of tap water and mice kibble food was +988 ‰ and +298 ‰, respectively (Table 2). The net \(^{15}N\)-enrichment in kibbles was +21.5 ‰. In comparison, the seemingly low \(^{13}C\)-difference of 3.0 ‰ between enriched and non-enriched bulk food is due to the large amount of organic carbon relative to the limited amount of added, \(^{13}C\)-enriched glycine.

Mothers had been raised at a breeding facility in Indianapolis, central Indiana, whereas the offspring were born and raised at Indiana University in south-central Indiana (74 km flight distance from Indianapolis). The limited isotopic differences between mothers and their offspring in control group A (i.e. receiving isotopically non-enriched food and water; Table 3) indicate that the food and water mothers had received during their upbringing in central Indiana were isotopically almost identical to non-enriched food and water used during our experiment at Indiana University (Table 2).

Enrichments in \(^{2}H\), \(^{13}C\), and \(^{15}N\) were detected in bone collagen from groups B, C and D of mice that had consumed isotopically enriched diets and/or waters. In contrast, consistently lowest \(\delta\)-values were found for collagen from control group A that had received isotopically non-enriched food and water (Figures 3 and 4). \(^{2}H\), \(^{13}C\), and \(^{15}N\)-abundances in bone collagen were consistently larger in offspring relative to bone collagen of their respective mothers and reflect faster biochemical use and incorporation of dietary glycine into the collagen of a growing bone (Table 3). The ingestion of \(^{2}H\)-enriched food and \(^{2}H\)-enriched drinking water by group D resulted in even higher bone collagen \(\delta^{2}H\) values than from ingestion of either \(^{2}H\)-enriched water (group B) or \(^{2}H\)-enriched food alone (group C) in both mothers and offspring, indicating a cumulative effect from ingestion of \(^{2}H\) derived from food and water.

The \(\delta^{2}H\)-values of bone collagen from our individual mice ranged by about \(\sim\)900 ‰ (Figure 3; Supplementary material 1). Because of the large range of \(\delta^{2}H\) values we report the data in \(^{2}H\) atom % as well (Tables 2 and 3; Supplementary material 1).

Both mothers and offspring in control group A have collagen that is \(^{2}H\)-depleted by \(\sim\)100 ‰ relative to food and water. Collagen from offspring in group A averages \(\delta^{2}H = -123 \pm 100 \) ‰ and is slightly less \(^{2}H\)-depleted than collagen from respective mothers averaging \(\delta^{2}H = -138 \pm 100 \) ‰. Group B, having received \(^{2}H\)-enriched water and non-enriched food, features \(\delta^{2}H\) values which are higher than that of their food and continue the trend of greater enrichment in offspring compared to their mothers. The mothers receiving enriched food and regular tap water (group C) show values in-between their food and

### Table 2. Mean \(\delta^{2}H\), \(\delta^{13}C\), and \(\delta^{15}N\) values with standard deviations of isotopically enriched and non-enriched water and food (bulk enrichment in kibble).

<table>
<thead>
<tr>
<th></th>
<th>Water (\delta^{2}H) (‰)</th>
<th>Food (\delta^{2}H) (‰)</th>
<th>(\delta^{13}C) (‰)</th>
<th>(\delta^{15}N) (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-enriched</td>
<td>-36 ± 7 (10)</td>
<td>-52 ± 13 (2)</td>
<td>-22.2 ± 0.1 (3)</td>
<td>+1.0 (1)</td>
</tr>
<tr>
<td>Enriched</td>
<td>+952 ± 134 (9)</td>
<td>+246 ± 38 (2)</td>
<td>-19.2 ± 0.3 (4)</td>
<td>+22.5 (1)</td>
</tr>
<tr>
<td>Net enrichment</td>
<td>+988</td>
<td>+298</td>
<td>+3</td>
<td>+21.5</td>
</tr>
</tbody>
</table>

Note: The numbers of samples tested are given in parentheses.
Table 3. Mean δ²H, δ¹³C, and δ¹⁵N values (in ‰) with standard deviations and ²H atom % of bone collagen for the four groups and two generations of mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>δ²H</th>
<th>²H atom %</th>
<th>δ¹³C</th>
<th>δ¹⁵N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (no enrichment)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>−138 ± 5</td>
<td>0.0134</td>
<td>−18.5 ± 0.6</td>
<td>+5.3 ± 0.2</td>
</tr>
<tr>
<td>Offspring</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B (enriched water)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>−8 ± 6</td>
<td>0.0154</td>
<td>−19.0 ± 0.2</td>
<td>+5.2 ± 0.2</td>
</tr>
<tr>
<td>Offspring</td>
<td>+119 ± 16</td>
<td>0.0174</td>
<td>−19.4 ± 0.3</td>
<td>+5.0 ± 0.5</td>
</tr>
<tr>
<td>Group C (enriched food)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>+197 ± 0.5</td>
<td>0.0186</td>
<td>−8.7 ± 0.5</td>
<td>+22.0 ± 1.5</td>
</tr>
<tr>
<td>Offspring</td>
<td>+409 ± 48</td>
<td>0.0219</td>
<td>+6.8 ± 3.7</td>
<td>+49.1 ± 5.7</td>
</tr>
<tr>
<td>Group D (enriched food and water)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>+254 ± 26</td>
<td>0.0195</td>
<td>−8.3 ± 1.7</td>
<td>+22.9 ± 1.8</td>
</tr>
<tr>
<td>Offspring</td>
<td>+666 ± 49</td>
<td>0.0259</td>
<td>+6.2 ± 3.5</td>
<td>+49.1 ± 5.6</td>
</tr>
</tbody>
</table>

Note: Each group consists of two mothers and 10 offspring.

Figure 3. Diagram showing individual bone collagen δ²H values divided into 4 test groups of mice consisting of 2 generations (mothers and offspring). Dashed horizontal lines indicate δ²H long-term average values of food and water types used to raise mice (data given in Table 2).

However, their offspring demonstrate collagen δ²H higher than both their water and kibble. Mothers receiving fully enriched diet (group D) have achieved an ²H-enrichment of nearly 400 ‰ by the end of the experiment which makes them seemingly...
identical to their enriched baseline, while data from the offspring plot midway between the respective water and food values.

The addition of $^{13}$C and $^{15}$N-enriched glycine to food for groups C and D tested for dietary $^{13}$C and $^{15}$N-enrichment in biosynthesised bone collagen, whereas mice in groups A and B received food with the equivalent addition of glycine with natural $^{13}$C and $^{15}$N-abundances. For simplicity, we combined the mice into pairs of groups (A+B) and (C+D) since all of the carbon and nitrogen is received through food so the differences in water treatment do not have an effect. The collagen values from mothers and offspring of the control group (A+B) cluster tightly with averages $\delta^{13}$C = −19 ‰ and $\delta^{15}$N = 5 ‰ (Table 3). Offspring from group (C+D) have larger values for both $\delta^{13}$C and $\delta^{15}$N and greater ranges (0–12 and 40–58 ‰, respectively) than offspring in group (A+B) with a strong linear correlation ($r^2 > 0.99$; Figure 4). The average isotopic differences between offspring of group (A+B) versus group (C+D) are approximately 25 ‰ for carbon and 45 ‰ for nitrogen. The respective isotopic differences for treated mothers are smaller than their offspring (~10 and ~17 ‰, respectively), which corresponds to the results observed in hydrogen and is most likely due to an unknown portion of the mother’s bone collagen having been synthesised before the dietary and water treatments began.

The bone collagen of mice should exhibit commensurate $^2$H, $^{13}$C and $^{15}$N-enrichments in the various dietary groups when food is the only factor responsible for isotopic variance. To test this, we only looked at the mice in control groups A (non-enriched food) and C (isotopically enriched food) who were never exposed to $^2$H-enriched drinking water so the only $^2$H-enrichment would come from food. Cross-plots of $\delta^2$H values versus either $\delta^{13}$C or $\delta^{15}$N exhibit strong linear correlations ($r^2 = 0.96$; Figure 5). Offspring in group C exhibit far wider ranges of isotopic values than offspring in group A for all three elements.
We made an estimate of metabolic tissue replacement rate ($m$) for collagen based on MacAvoy’s [50] model for soft tissues in adult mice (Equation (4))

$$m = -\ln\left(\frac{C - C_E}{C_O - C_E}\right)$$

where $C$ is the isotopic value of mothers at the end of the experiment, $C_O$ is the isotopic value for the control group (assumed initial value), $C_E$ is the isotopic value in equilibrium with the enriched diet, and $t$ is time with $t = 0$ representing the arrival of the pregnant mice at Indiana University and the instantaneous introduction of isotopically enriched food and water. The following four a priori assumptions were made: (i) the baseline for mothers was the bone collagen isotopic signal reflected in control group mothers (group A); (ii) the offspring grew up in a steady state and their bone collagen at 18 weeks reflected their food and/or water intake (i.e. there is a negligible amount of protein from the earliest gestation prior to arriving in Bloomington at the end of the experiment); (iii) the mothers who received enriched food and/or water (groups B, C, and D) all experienced a step-change in their food, water, or both; (iv) the bone collagen of mothers who received enriched food and/or water started asymptotically approaching the value that was observed by the offspring in their treatment. $^{2}\text{H}$, $^{13}\text{C}$, and $^{15}\text{N}$ were observed as separate signals to indicate the final isotopic shifts (Table 4).

With the two time points ($t = 0$ and $t = 18$ weeks) and the assumptions above, we were also able to calculate the collagen half-life (Equation (5)).

$$t_{1/2} = \frac{\ln 2}{m}$$

Based on our calculations, 3 %–5 % of adult mouse leg bone collagen turns over every week. After 18 weeks (the end of the experiment) of consuming enriched food and/or water, the mothers had 40 %–60 % of the original bone collagen remaining.
4. Discussion

Our results demonstrate that hydrogen from both water and food contribute to the organic hydrogen in bone collagen. We observed strong effects on the bone collagen δ²H of groups of mice according to ²H-abundances in their diets and drinking waters (Figure 3), and the strongest ²H-enrichment was observed in mice that received both ²H-enriched food and water. The large differences in isotope enrichment signals resulting from different treatments, coupled with the relatively small variations within individual groups, allow us to separate the ²H transfer rates of hydrogen from water and food into collagen δ²H. For all enriched treatments, the offspring collagen values were higher than the maternal values, which is consistent with the offspring’s high metabolic rates and ossification since the early stages of skeletal development in utero, whereas the mothers had new isotopic signal incorporated via turnover only. Our complementary findings for δ¹³C and δ¹⁵N support the conclusions on hydrogen biochemical incorporation into bone collagen.

4.1. Age effects on δ²H of collagen

Mothers and offspring in control group A had similar δ²H collagen values indicating no difference in their metabolic processes (though a statistical comparison is impossible because only two mothers were measured). Hydrogen isotope results for group A also show that the food and water the mothers received prior to the start of our experiment for the first two months of their lives were similar to the treatment’s non-enriched food and water. While having had constant isotopic intakes during their entire lives, both the mothers and offspring of control group A had collagen δ²H values that were 100 ‰ lower than the values of their respective food and water. This finding agrees with previous data on bone collagen from herbivores and omnivores in natural environments [16,51]. While all mice in groups B, C, and D produced collagen that reflected the enriched food and/or water, the amount of ²H-enrichment in mothers was 40 %–50 % lower than that observed in their offspring. This points to faster turnover rates for offspring, which can be due to their faster metabolism, greater integration of essential and nonessential amino acids created de novo, and juvenile ossification process.

While there is a strong correlation between the turnover rates from carbon and nitrogen data (Figure 4), the hydrogen shows a faster turnover (Table 4), which may be due to additional ways ²H-enriched hydrogen is being integrated into the protein. For example, during the formation of peptide bonds, one N-bound H is removed, and the observed ²H-enrichment is consistent with preferential removal of ¹H over ²H. Moreover, the isotope data for carbon and nitrogen, which are not lost during peptide bond formation, show

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>m (daily %)</th>
<th>δ²H</th>
<th>δ¹³C</th>
<th>δ¹⁵N</th>
<th>t₁/₂ (days)</th>
<th>δ²H</th>
<th>δ¹³C</th>
<th>δ¹⁵N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched water (group B)</td>
<td>0.57</td>
<td>0.57</td>
<td>122.5</td>
<td></td>
<td></td>
<td>0.57</td>
<td>122.5</td>
<td></td>
</tr>
<tr>
<td>Enriched food (group C)</td>
<td>0.77</td>
<td>0.40</td>
<td>0.39</td>
<td>90.5</td>
<td>174.0</td>
<td>179.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched food &amp; water (group D)</td>
<td>0.54</td>
<td>0.43</td>
<td>0.42</td>
<td>128.3</td>
<td>161.9</td>
<td>166.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
almost identical turnover rates, as expected assuming that the $^{15}$N- and $^{13}$C-enriched glycine is incorporated into the bone collagen along the same pathway. While the observed shift in $^{15}$N results from the dietary protein (the only source of N), the shift in $^{13}$C may be diluted by the incorporation of C from lipids and carbohydrates in the diet. However, the close match in turnover time estimates using N and C isotope data implies that the fraction of C derived from non-protein sources is consistent among all mice groups.

Earlier we had reported a shift towards less negative $\delta^{2}$H values related to weaning in carnivores [16]. A similar trend was not observed in this study of laboratory mice, likely due to the fact that the offspring generation of mice reached adulthood at the end of our experiment. In small mammals such as mice the faster biomass turnover rate and an accelerated pace of reaching maturity (within a few weeks) can obscure the weaning effect (at 3 weeks of age), so any temporary juvenile effects would have been erased by the subsequent turnover.

4.2. Influence of water on $\delta^{2}$H of collagen

Mice who drank $^{2}$H-enriched water and ate regular food (group B) showed elevated $\delta^{2}$H collagen values. This indicates that water has an effect on collagen isotope values through amino acids, which are synthesised de novo, rather than being ingested as food. We surmise that ingested $^{2}$H-enriched water isotopically exchanges with intermediate organic compounds during the biosynthesis of amino acids and generated non-exchangeable polypeptide hydrogen. Approximately every third amino acid building block in the bone collagen biopolymeric chain is the non-essential amino acid glycine [29] that can be biosynthesised by many organisms [52]. Non-essential amino acids proline and hydroxyproline each represent every sixth amino acid in collagen, and the remaining 50 % are other amino acids, some of which are essential and must be supplied by food. Even though animals can synthesise non-essential amino acids like glycine, they readily incorporate them from protein-rich food and use them as building blocks in fibrous proteins such as collagen [26,53]. The isotope enrichments in food kibbles given to mice were exclusively in the form of isotopically enriched glycine, while all other dietary amino acids had natural isotope compositions. The pattern of bone collagen $\delta^{2}$H differences between the offspring in control group (A) and the experimental groups (B, C, and D) is similar to the $\delta^{2}$H differences observed in their mothers (Figure 3). This indicates approximately equivalent water hydrogen integration in protein biosynthesis for (1) the mothers who only received $^{2}$H-enriched water during adulthood after reaching an age of two months, and (2) for the offspring generation that received $^{2}$H-enriched water throughout their growing (juvenile) period, excluding early time in utero. In other words, bone collagen biosynthesis in both groups partially relies on similarly balanced fluxes of de novo biosynthesised non-essential amino acids that carry an H-isotope signal from body water.

4.3. The influence of food on $\delta^{2}$H of collagen

The similarity of hydrogen isotope values between food and bone collagen (Figure 3) mainly comes from the artificial $^{2}$H-spike in food produced by a small addition of glycine rather than being randomly distributed among all biochemical components of
food. Essentially all the carbon incorporated into collagen during overturn derives from protein in the diet of mice, as indicated by almost identical turnover rates of carbon and nitrogen (Figure 4), which suggests that much hydrogen in newly incorporated bone collagen also derives from dietary protein rather than from carbohydrates or lipids. Proteins made up 19% of the kibble by weight (Purina® 5015 Mouse Diet), and this portion of the food, plus the glycine that was added to it, should therefore be the primary component contributing to the isotopic composition of collagen in the mice. In other words, two factors affect the collagen values measured in this experiment: the proportion of protein in the pre-treated kibble and the enrichment value of the glycine that was added to it. This complicates the possibility of directly equating the isotope values of bone collagen and food.

4.4. Extent of food and water effects on $\delta^{2}H$ of collagen

To determine the effects of food and water on bone collagen hydrogen isotope values we performed a two-way ANOVA statistical evaluation with water and food as the factors (Table 5). We also included an interaction effect in case the combination of water and food enrichment has an effect that is more or less than the sum of water and food independently. The ANOVA was performed on the offspring generation of mice only since these mice were exposed to experimental conditions from before their birth until the end of the experiment. We found that water and food both have significant effects on collagen ($p < 0.001$ in both cases), with food explaining 81% of the variance, and water 17%, but there is no interaction effect of food and water ($p = 0.5$). Collectively the values in the water and food explain more than 98% of the variance in the collagen values (adjusted $r^2$ value).

4.5. Comparisons of $\delta^{2}H$ with $\delta^{13}C$ and $\delta^{15}N$

In contrast to hydrogen, carbon and nitrogen in animal tissues come from food only and there is no exchange with body water. Nitrogen is almost exclusively incorporated via food protein [26,54]. Therefore, for the two isotope systems the organismal tissues reflect bulk food values with an additional enrichment in $^{13}C$ and $^{15}N$ (e.g. [54,55]).

All three elements were similarly enriched in the collagen of mice that received enrichment through food only (group C; Figures 4 and 5). The mothers from groups A (regular food) and C (isotopically enriched food) exclusively utilised regular water, which identifies the H-isotopic difference between their two bulk food sources of 298 ‰ as the primary reason for the mean H-isotopic difference between their bone collagen of 335 ‰ (Figure 5; Tables 2 and 3). The offspring in groups A and C follow the same pattern

Table 5. Two-way ANOVA with $\delta^{2}H$ values of water and food as the factors.

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F-statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1</td>
<td>622503</td>
<td>622503</td>
<td>492.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>food</td>
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<td>2911682</td>
<td>2911682</td>
<td>2305.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>food &amp; water</td>
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<td>577.6</td>
<td>577.6</td>
<td>0.4573</td>
<td>0.5</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>45471.4</td>
<td>1263.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>3580233</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: DF = degrees of freedom, SS = sum of squares, and MS = mean sum of squares.
with a collagen mean H-isotopic difference of 532 ‰, which is larger than for mothers because the latter retained some of their bone collagen from their earlier upbringing in central Indiana. The very strong signal transfer of $^2$H-enrichment into bone collagen is due to the $^2$H-spiking of glycine in isotopically enriched kibbles and the efficient incorporation of dietary glycine into bone collagen.

In contrast to carbon which is abundantly present in many compound classes in food, a nitrogen-isotopic comparison between diets and bone collagen is more straightforward because most N in food is present in the form of amino acids that are either directly integrated into collagen chains or are used as precursors in collagen biosynthesis [56]. Mothers and offspring that received non-enriched food (the combined group A + B) demonstrate a $\delta^{15}N$ increase of approximately 4 ‰ from their diet to bone collagen (Tables 2 and 3), which is in accordance with previously reported $\delta^{15}N$ dietary enrichment [54,57]. The mothers which received enriched food (group C + D) exhibit $\delta^{15}N$ collagen values that are similar to $\delta^{15}N$ values of enriched food, while the offspring’s collagen from the combined group (C + D) express strong $^{15}N$-enrichment ($\delta^{15}N \sim +50$ ‰) that is about 25 ‰ more positive than the respective bulk food. This maximum $^{15}N$-enrichment indicates enhanced utilisation of $^{15}N$-enriched glycine from food for biosynthesis of growing offspring’s bone collagen relative to their mothers. This increased integration of nonessential glycine in pre-adult versus adult animals can be explained by ongoing osteogenesis in juveniles and reached turnover equilibrium in adults. $^2$H-enrichment in group C derives exclusively from food and therefore follows the trend of $^{15}N$-enrichment. Although intensive utilisation of glycine is most noticeable in experimental groups that received isotopically enriched food (C + D), the same processes must have occurred in mice that ingested regular food without the additional benefit of carrying a strong isotopic signal. Our results are consistent with those from an earlier carbon isotope study on bone collagen from rats that had been raised in the laboratory on purified C$_3$ and/or C$_4$ macronutrients [58].

4.6. Sources of hydrogen isotope heterogeneity

Maternal mice in groups B, C and D were exposed to isotope enrichments during pregnancy at Indiana University after their bones were fully formed. Any isotope enrichment of their bone collagen over the control values must therefore have occurred via bone turnover. On the other hand, their offspring began receiving isotopically enriched nutrients and water during their bone growth period, first prenatally in utero beginning one week prior to birth, then for two weeks after birth via lactation, and finally, through water and/or food intake for the remaining 15 weeks and five days. The full exposure to artificial isotope enrichments for offspring at Indiana University phased in a few days after birth. Around the same time, the slower process of bone collagen turnover in adult mice began to shift their mothers’ bone collagen in the same isotopic directions as the rapidly growing collagen of offspring.

The large isotopic variances among collagen of offspring fed the enriched food (but not the offspring that received the enriched water only) can be explained by individual feeding habits that were possibly guided by the taste of glycine concentrated unevenly in the centres of kibbles. Some of the mice in the offspring generation may have preferred or avoided ingestion of the parts of kibbles containing most of the glycine infusions. Some offspring may have eaten more food than their siblings (glutton versus runt). The latter
seems to be less important because 1.5 months into the experiment one of the offspring was marked as underweight by a lab animal veterinarian and diagnosed with overgrowth of its incisors. From then on, the incisors had been filed down biweekly and the mouse regained its weight. Ultimately the mouse showed no extreme isotopic characteristic (Supplementary material 1, sample C7). Underfeeding stress may be insufficient to redirect metabolic pathways with significant long-term isotopic consequences. In the study of a wild mammal fauna around Bloomington, Indiana [16] a group of Eastern short-tailed shrews (6 individuals) showed a wide range of bone collagen $\delta^{2}H$ values ($-60$ to $+100$ ‰). Shrews have body mass similar to mice and like them, have a fast metabolism. Both of these traits may be responsible for dramatic variations in these two small mammal species’ collagen $\delta^{2}H$. In case of wild shrews, it is the heterogeneity of food sources available to individuals and in case of laboratory mice the heterogeneity comes from randomised points of glycine injections into food kibbles and individual preference to more or less sweet spots. In contrast, larger mammals have slower metabolism, more body mass, and in case of herbivores more homogenous food sources available (plants are an isotopically more uniform food source).

Individual isotopic variability in experimental groups of mice may indicate diversity in food and water uptakes, metabolic rates, and maybe even differences in evapotranspiration rates between mice of the same group and generation [20,28,50,59]. Similar differences within populations could therefore be expected in ecosystem isotope studies of wild fauna [60–62]. Stable isotopes in nature do not necessarily reach an isotopic diet–tissue steady state due to the seasonally changing environmental and food isotopic signals [50]. Moreover, on a day-to-day basis the type of food that animals find available varies, especially for carnivores and omnivores who have a broader range of potential food sources, thus making hydrogen stable isotope values harder to predict. Finally, metabolic pathways within the body change with age, growth rate, and potentially with stress, which further complicates the resulting stable hydrogen isotope characteristics. Animals on steady homogenous diets and possibly slower metabolism such as herbivores do better in reflecting similar bone collagen isotope values and can therefore be used as proxies for the populations. However, animals with more diverse and irregular diets ( omnivores and carnivores) and faster metabolism (small mammals) show too wide $\delta^{2}H$ collagen range and using one or few samples may not give an average for the entire group.

**Conclusions**

- Collagen $\delta^{2}H$, $\delta^{13}C$, and $\delta^{15}N$ values increased with addition of enriched food and in cases of $^2H$-enriched water.
- Enriched glycine in food was predominately responsible for collagen enrichment for all three elements.
- $^{2}H$-enrichment that came from water indicates (non-essential) amino acids were created *de novo* and integrated into collagen.
- The incorporation of a heavy isotope spike into mothers’ collagen is 40 %–60 % that of offspring and most likely due to mothers having slower turnover rates than offspring. The collagen turnover rate in adult mice was 3 %–5 % per week. This was observed in all three elements.
• Both mothers and offspring seemed to utilise the same collagen biosynthesis pathways (de novo creation of amino acids from water and integration of food-available essential and non-essential amino acids) at the same rate. This is indicated by the proportional increase of δ-values for all three H, C, N isotope systems.
• We found that food as a source of ²H-enrichment contributed ∼ 80 % and water ∼17 % to collagen (p < 0.001 in both cases), with no interaction effect.
• Variability within the same cohort indicates unique food preferences and uptakes, and possibly individual differences in metabolic rates and evapotranspiration. This effect should be expected in equal or even greater measure in nature.
• Individual bone collagen samples of mammals with fast metabolism and heterogenous diets may not accurately represent their populations and be used as proxies in isotopic ecological and archaeological studies of wildlife.

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