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EDITED BY
Yuichi Naito,
Central Research Institute of Electric Power Industry (CRIEPI), Japan

REVIEWED BY
Ichiro Tayasu,
Research Institute for Humanity and Nature, Japan
Thomas Larsen,
Max Planck Institute for Geoanthropology, Germany

*CORRESPONDENCE
Linda M. Reynard
lindareynard@boisestate.edu
Thomas Tütken
tuetken@uni-mainz.de

†PRESENT ADDRESS
Jennifer N. Leichliter,
Emmy Noether Group for Hominin Meat Consumption (HoMeCo), Max Planck Institute for Chemistry,
Mainz, Germany

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Hydrogen and oxygen isotopes in vertebrate tissues vary by diet type

Linda M. Reynard[1]*, Jennifer N. Leichliter[2†], Daniela E. Winkler[3], Marcus Clauss[4] and Thomas Tütken[2]*

[1]Department of Geosciences, Boise State University, Boise, ID, United States, [2]Institute for Geosciences, Johannes Gutenberg University Mainz, Mainz, Germany, [3]Department of Biology, Zoology and Functional Morphology of Vertebrates, Kiel University, Kiel, Germany, [4]Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

Hydrogen and oxygen isotope ratios in proteinaceous tissues have been used for some time in migratory, ecological, and archaeological studies. While the result of isotopic variation in drinking water and diet has been investigated with controlled feeding experiments and studies in the wild, there are few controlled feeding studies that manipulate the diet components and diet type, and this across different taxa. In this experiment, the diet fed to rats, guinea pigs, and quail varied from plant-based to insect-based and meat-based pelleted diets. We report the diet to tissue offsets for δ^2H (denoted $\Delta\delta^2$H) and δ^{18}O ($\Delta\delta^{18}$O) of tissue-bound organic matter in two tissue types: muscle and dentine collagen. The diet to tissue offset varies by diet type in muscle of all three species, by up to 16 ‰ ($\Delta\delta^2$H) and 2 ‰ ($\Delta\delta^{18}$O). In dentine collagen, a range of ~20 ‰ in $\Delta\delta^2$H and ~1.5 ‰ in $\Delta\delta^{18}$O are observed across diets, though in a smaller number of samples. Additionally, we note large variation in $\Delta\delta^2$H and $\Delta\delta^{18}$O by tissue type (δ^2H = ~60 ‰, δ^{18}O = ~3–4 ‰) and more moderate differences by species (up to δ^2H = 7.4 ‰, δ^{18}O = 1.5 ‰). The difference in consumer tissue $\Delta\delta^2$H and $\Delta\delta^{18}$O by diet type is important to consider as a source of isotopic variability for some studies such as migratory research or diet or drinking water reconstructions and (palaeo-)climate inferences drawn from them, particularly in species that may vary their dietary habits.

KEYWORDS

feeding experiments, guinea pigs, rats, quail, diet changes, dentine, muscle

1 Introduction

Hydrogen isotopes in animal organic tissues have been exploited successfully to trace migration (a large literature; see the volume Hobson and Wassenaar, 2019) and are being investigated for ecological applications (reviewed by Vander Zanden et al., 2016) and palaeoenvironmental reconstruction (e.g., Gröcke et al., 2017; Reynard et al., 2020). Similarly, efforts are underway to include oxygen isotopes in organic matrices in these types of analyses (Kirsanow et al., 2008; Ehleringer et al., 2008; Koehler et al., 2019). The

isotopic relationship between inputs (diet, water, and inspired O_2; Longinelli, 1984; Kohn, 1996; Feng et al., 2022, 2024) and tissues is key to understanding tissue isotopic data and the limits of interpretation.

The literature on diet to tissue H and O isotope differences in organic tissues is modest, consisting of both controlled feeding experiments and observational studies. Most of the controlled studies vary the drinking water isotopic input to study its contribution to tissues; variations of the diet are fewer in number and manipulate the macronutrient proportions (Hobson et al., 1999) and/or the isotopic composition of diet components (Hobson et al., 1999; Wolf et al., 2012; Newsome et al., 2017; Topalov et al., 2019). It is not known how dietary habits (e.g., herbivory, omnivory) modulate the isotopic relationship from diet to tissue. Any isotopic variations between animals consuming different diet types are important to consider in interpretations based on underlying drinking water variation (e.g., migration studies) and to advance applications such as dietary reconstruction with tissue H and O isotopes.

In the controlled feeding study we report here, we hold the drinking water input isotopically constant and examine the H and O isotope relationship between diet and tissues on three different diet types: herbivorous, omnivorous, and insectivorous diets. Each of these diets was supplied to three different model animals (rats, guinea pigs, quail), and we analyzed muscle in all taxa and dentine collagen in rats and guinea pigs.

2 Materials and methods

The feeding experiments were performed at the Vetsuisse Faculty, University of Zurich and are the same as described thoroughly in Weber et al. (2020; Sr isotopes in tooth enamel) and Leichliter et al. (2021; nitrogen isotopes in tooth enamel). Tissues from three species were investigated: muscle from rats, guinea pigs, and quail; dentine collagen from incisors of rats and guinea pigs. We also used some quail feathers to supplement the other tissues as part of a complementary methodological study of δ^2H measurements in proteinaceous tissues and animal diets (Supplementary Information).

The animals were adult female WISTAR (RjHan : WI) rats (*Rattus norvegicus* forma domestica), adult female Dunkin Hartley (HsdDhl : DH) guinea pigs (*Cavia porcellus*), and quail (*Coturnix japonica*). After an acclimatization period of five days with still available supplier food, the animals were held on one of three experimental diets for 54 days, after which they were euthanized and tissues collected. Starting and final body weights, respectively, were 198 ± 17 g and 245 ± 17 g for rats, 253 ± 23 g and 290 ± 24 g for quail, and 401 ± 16 g and 577 ± 104 g for guinea pigs (Supplementary Table 1). After enzymatic maceration of the skulls at 55°C, the rootward portion (i.e., last mineralized section) of rat and guinea pig lower incisors were sampled, resulting in segments ~5 mm long. Rat and guinea pig incisors are ever-growing teeth and should be expected to reflect the experimental diet and approximately 17–25 days' growth (Law et al., 2003; Hillson, 2005; Müller et al., 2015). The Swiss Cantonal Animal Care and Use Committee, Zurich approved the experiment, licence N° ZH135/16.

The different pelleted diets were custom-made for the feeding experiments and comprise a) plant-based, with 56 wt% lucerne (hereafter *plant*); b) insect-based, with 26 wt% black soldier fly larvae insect protein meal (hereafter *insect*); c) meat-based, with 25 wt% lamb meat (hereafter *meat*). The balance of the diets was plant-based, including wheat- and oat-meal (Table 1). The diets were formulated to be isonitrogenous but not controlled for total hydrogen and oxygen content (Table 1; Leichliter et al., 2021); however, the experimental diets were nevertheless similar to each other in total hydrogen amount (5.9–6.2% H in whole diets, 5.5–5.6% H in non-lipid diets) and oxygen amount (37.7–38.4% O in whole diets, 36.9–40.2% O in non-lipid diets, Supplementary Table 3). Animals consumed food and water *ad libitum*. The diets from the animal suppliers were also analyzed (Supplementary

TABLE 1 Diet main ingredients, composition, and isotopic composition[a].

	plant-based	insect-based	meat-based
main ingredient	lucerne	insect protein meal[b]	lamb meat
weight % composition			
main ingredient	56	26	25
potato protein	13	–	–
wheat meal	10	18	18
oat meal	7	16	15
apple bits	5	14	15
soy husks	3	10	13
straw meal	–	10	9
molasses	3	3	3
vitamins & minerals	3	3	2
nutritional analysis %			
crude protein[c]	21.4	21.4	21.6
crude fat	4.5	5.8	5.1
neutral detergent fibre	26.7	33.0	28.4
acid detergent fibre	18.6	21.4	19.6
starch	10.0	18.9	17.4
sugar	3.2	2.2	2.2
isotopic composition			
δ^2H_n Cr (‰) non-lipid	-96 (2.0)	-77 (0.8)	-75 (0.6)
δ^2H_n Cr (‰) whole	-104 (2.6)	-95 (1.2)	-90 (2.3)
$\delta^{18}O$ (‰) non-lipid	21.8 (0.0)	23.8 (0.0)	23.1 (0.2)
$\delta^{18}O$ (‰) whole	22.0 (0.2)	23.7 (0.4)	23.2 (0.4)

[a]figures in parentheses are the measurement uncertainty based on duplicates.
[b]composed of black soldier fly larvae protein extract (Protix).
[c]total N x 6.25.

Table 6). Zurich tap water was supplied to all animals (δ^2H = -82 ‰, $\delta^{18}O$ = -11.6 ‰) using nipple drinkers (reducing evaporation), with the exception of a subset of quail who were supplied ^{18}O-enriched water ($\delta^{18}O$ = 5.9 ± 3.5 ‰, quail tissue results in Supplementary Table 2).

Tissue sample preparation and isotope ratio mass spectrometry were performed at Boise State University, in Boise, Idaho, USA. Muscle and diet samples were solvent-treated to remove lipids with 2:1 (v/v) chloroform:methanol for three sequential 24 hour treatments (Hobson et al., 1999; Soto et al., 2013; Newsome et al., 2017). After each soak the supernatant was pipetted off and refreshed. After the final 24 hour soak, the samples were given a brief final rinse in 2:1 chloroform:methanol and allowed to air dry. Tests showed minimal H and small and consistent O isotopic differences between petroleum ether (also used as a solvent for lipid removal, Wolf et al., 2012) and chloroform:methanol as a solvent on test rat and beef muscle samples (Supplementary Table 7). The diets were also analyzed without solvent-extraction (*whole diet*). Tooth segments (~ 5 mm long) were demineralized in 0.5 M EDTA over the course of several days, rinsed 8-10 times with deionized water in 2 mL tubes, and freeze dried, resulting in dentine collagen (Tuross, 2012). Feathers (for the methodological tests) were soaked overnight in 2:1 chloroform:methanol, rinsed in fresh 2:1 chloroform:methanol, and allowed to air dry.

Samples were prepared and analyzed by two methods: one, determination of δ^2H of nonexchangeable hydrogen with thermal conversion using a chromium-packed reactor (Reynard and Tuross, 2016; Reynard et al., 2019); two, determination of $\delta^{18}O$ with thermal conversion with a glassy carbon packed reactor. The latter technique also provided a second different δ^2H determination (unexchanged with a glassy carbon packed reactor). The chromium-powder reactor was modified to include 7 cm of chromium powder, rather than 3 cm as before, and correspondingly fewer glassy carbon chips to maintain the same height of total reactor filling (Reynard et al., 2019).

For δ^2H of nonexchangeable H (δ^2H_n), following Meier-Augenstein et al. (2011), ~300 µg sample aliquots of muscle, dentine collagen, or diet were packed into silver capsules and folded loosely. Each tray of samples was placed in a glass desiccator with a ground glass joint sealed with vacuum grease, along with a beaker of 50 ml of water of known H isotope composition (δ^2H_{waterA} = 155.2 ‰). Each sample was replicated in another tray and exchanged with a water of a second different known isotopic composition (δ^2H_{waterB} = -224.7 ‰). After 4 days' equilibration, the sample trays were quickly moved to a plastic vacuum desiccator and left under vacuum for 7 days, after which they were rapidly transferred to a zero-blank autosampler (Costech) and analyzed with the Cr-packed reactor configuration.

Samples were pyrolyzed at 1450°C (with glassy carbon reactor) or 1200°C (with Cr-packed reactor) in a Thermal Conversion Elemental Analyzer (TC/EA, Thermo Scientific). The resultant gases were separated with a 1.8 m long 5 Å molecular sieve gas chromatograph and then analyzed with a Delta V Plus mass spectrometer (Thermo Scientific). δ^2H and $\delta^{18}O$ values were normalized on the VSMOW-SLAP scale, using aliquots of VSMOW and SLAP in silver tubes (United States Geological Survey, Reston, VA) in each run. We estimate uncertainties of ±0.4 ‰ (1 SD) for $\delta^{18}O$ and ±3 ‰ (1 SD) for δ^2H_n (Cr-packed) based on long-term reproducibility data for the former and replicates of standard materials exchanged with water and analyzed in the same manner as the samples for the latter.

We computed δ^2H_n as follows (Meier-Augenstein et al., 2011):

$$\delta^2 H_n = \frac{\delta^2 H_A - (f \times \delta^2 H_{waterA})}{1 - f}$$

$$f = \frac{\delta^2 H_A - \delta^2 H_B}{\delta^2 H_{waterA} - \delta^2 H_{waterB}}$$

where δ^2H_A and δ^2H_B are δ^2H values of sample exchanged with water A or B, respectively; δ^2H_{waterA} and δ^2H_{waterB} are the δ^2H values of water A or B, respectively; and f is the fraction of H that is exchangeable under these experimental conditions.

We compute the diet-tissue isotopic offsets as $\Delta\delta^2H = \delta^2H_{tissue} - \delta^2H_{diet}$ and $\Delta\delta^{18}O = \delta^{18}O_{tissue} - \delta^{18}O_{diet}$.

We also computed a 'Cr-equivalent' δ^2H_n value for four dentine collagen samples where only the glassy carbon reactor result was available, using the mean offset between δ^2H_n-Cr and δ^2H-glassyC of other dentine collagen samples in this data set, resulting in δ^2H_n-Cr = δ^2H-glassyC +10.4 ‰. This offset agrees with the result previously obtained from collagen δ^2H_n-Cr = δ^2H-glassyC +10.1 ‰ (Reynard et al., 2019). The δ^2H_n-Cr and δ^2H-glassyC relationship for all sample types analyzed here is given in the Supplementary Material.

Statistical analysis was performed with the program R and the 'stats' statistical package, using standard methods including analysis of variance (ANOVA) and Tukey's Honest Significant Differences (Tukey's HSD) for multiple comparisons (R Core Team, 2023). Tukey's HSD test can be used with groups of different sample size (Quinn and Keogh, 2002).

3 Results

3.1 Isotopic differences by diet: muscle

Hydrogen and oxygen isotope values in muscle are grouped by diet treatment (Figures 1, 2). Diet-tissue offsets ($\Delta\delta^2H$ and $\Delta\delta^{18}O$) in muscle vary by diet in a consistent manner in all three species (Figures 3, 4; Table 2). $\Delta\delta^2H$ is smallest for plant, mid-sized for insect, and largest for the meat diets. The difference between the diet groups is in the range of $\Delta\delta^2H$ = 4–16 ‰ (Table 3). Similarly, $\Delta\delta^{18}O$ is smallest for the plant diet and a greater and overlapping difference for the insect and meat diets, for all three animal groups (excluding ^{18}O-enriched treatment quail), with differences of ~1.2 –2.0 ‰ (Figure 4, Table 4). The eight quail given ^{18}O-enriched drinking water have higher $\delta^{18}O$ in muscle than the quail consuming regular drinking water (12.9–13.4 ‰ vs. 8.1–9.7 ‰, respectively, Supplementary Table 2), consistent with the quail incorporating the drinking water and experimental new diets into the muscle tissue.

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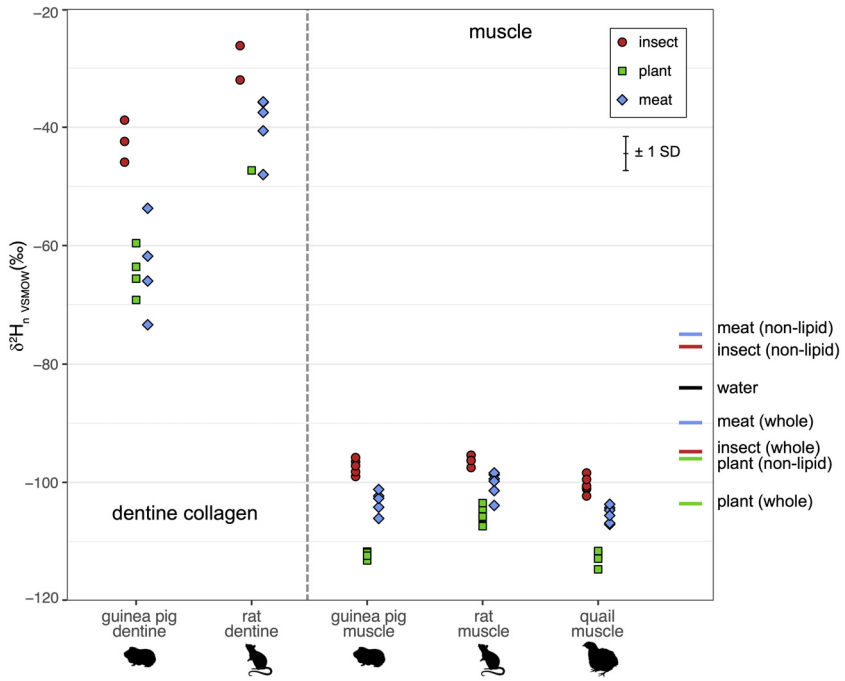

FIGURE 1
Tissue δ^2H_n in muscle and dentine collagen of guinea pigs, rats and quails (the latter muscle only) fed different pelleted plant-based (green squares), insect-based (red circles), and meat-based (blue diamonds) diets (see Table 1 for details). The error bar shows the estimated measurement uncertainty of ± 3 ‰ (1 SD). For comparison the diet (lipid and non-lipid extracted) and water δ^2H values are shown by the bars at the right.

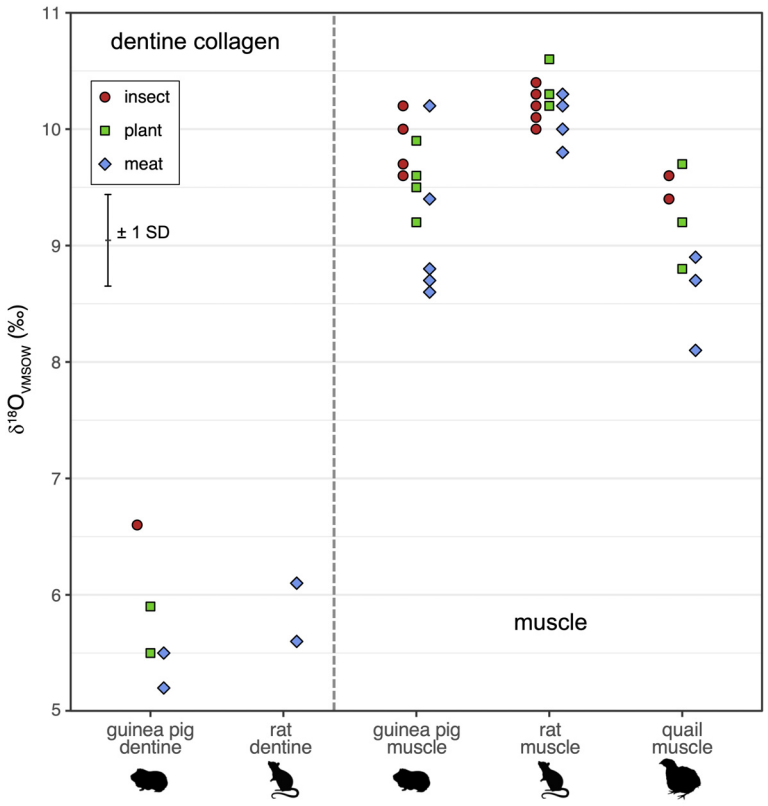

FIGURE 2
Tissue $\delta^{18}O$ in muscle and dentine collagen of animals fed on plant-based (green squares), insect-based (red circles), and meat-based (blue diamonds) diets, in rats, guinea pigs, and quail (the latter muscle only). The error bar shows the estimated measurement uncertainty of ± 0.4 ‰ (1 SD). The diet and water $\delta^{18}O$ values are outside the range of the figure (diets ~22–24 ‰, water -11.6 ‰, see Table 1).

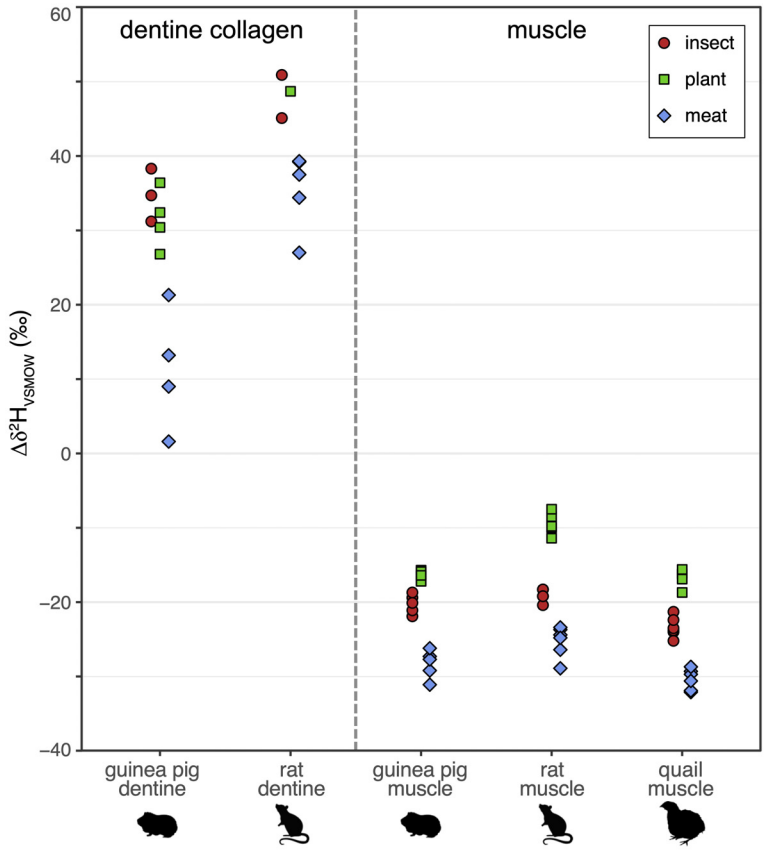

FIGURE 3
Tissue $\Delta\delta^2H_n$ (non-lipid diet) in muscle and dentine collagen of animals fed with plant-based (green squares), insect-based (red circles), and meat-based (blue diamonds) diets, in rats, guinea pigs, and quail (the latter muscle only).

3.2 Isotopic differences by diet: dentine

In dentine collagen, there is a large variation in the $\Delta\delta^2H$ and $\Delta\delta^{18}O$ values, but some isotopic patterning by diet broadly similar to the muscle results. In dentine collagen, $\Delta\delta^2H$ is smaller in the meat-containing diet than the plant- or insect-based diets for the guinea pigs by ~20 ‰ (Tukey HSD $p \leq 0.0031$) and ~12 ‰ for rats (non-significant differences); there is a larger and equal $\Delta\delta^2H$ for the insect and plant diets (Figure 3; Table 5). While the small number of samples precludes a strong comparison, $\Delta\delta^{18}O$ is smallest on the plant diet and larger on the meat and insect diets for the guinea pig, in agreement with the pattern observed for $\Delta\delta^{18}O$ in muscle (Figure 4).

3.3 Isotopic differences by tissue

There are large and systematic isotopic differences by tissue between muscle and dentine collagen for both H and O in both rats and guinea pigs (Table 2): higher δ^2H and $\delta^{18}O$ values in dentine collagen than muscle (Figures 1, 2); a big positive diet-tissue $\Delta\delta^2H$ in dentine collagen (~ +10–40 ‰) vs. ~ -20 ‰ (opposite direction) $\Delta\delta^2H$ in muscle (Figure 3); and ~4 ‰ greater magnitude (negative) $\Delta\delta^{18}O$ in dentine collagen than muscle (Figure 4).

3.4 Isotopic differences by species

Inter-species isotopic differences are generally smaller than the differences between diets. However, there are δ^2H differences between rat and guinea pig dentine collagen (by ~20 ‰ on average, though with a large range and SD of the mean of 7–11 ‰), and in muscle for plant-fed rats compared to plant-fed guinea pig and quail (7–8 ‰). Rat muscle $\delta^{18}O$ is somewhat higher than in guinea pigs and quail on all three diets (by 0.7–1.1 ‰, Table 2).

4 Discussion

4.1 Isotopic differences by diet

The muscle δ^2H and $\delta^{18}O$ isotope values are tightly grouped by diet and species, and diet type affects the diet-tissue H and O isotope offset in muscle in all three species in the same way. In this and other controlled feeding experiments, tissue H and O isotope values are often tightly grouped within each diet or water treatment; the typical intra-group uncertainties are 2.4 ‰ for δ^2H and 0.44 ‰ for $\delta^{18}O$ (median values of 2 x the standard error of the mean, compiled in Figures 5, 6; Hobson et al., 1999; Tuross et al., 2008; Podlesak et al., 2008; Kirsanow and Tuross, 2011; Wolf et al., 2012; Wolf et al., 2013; Newsome et al.,

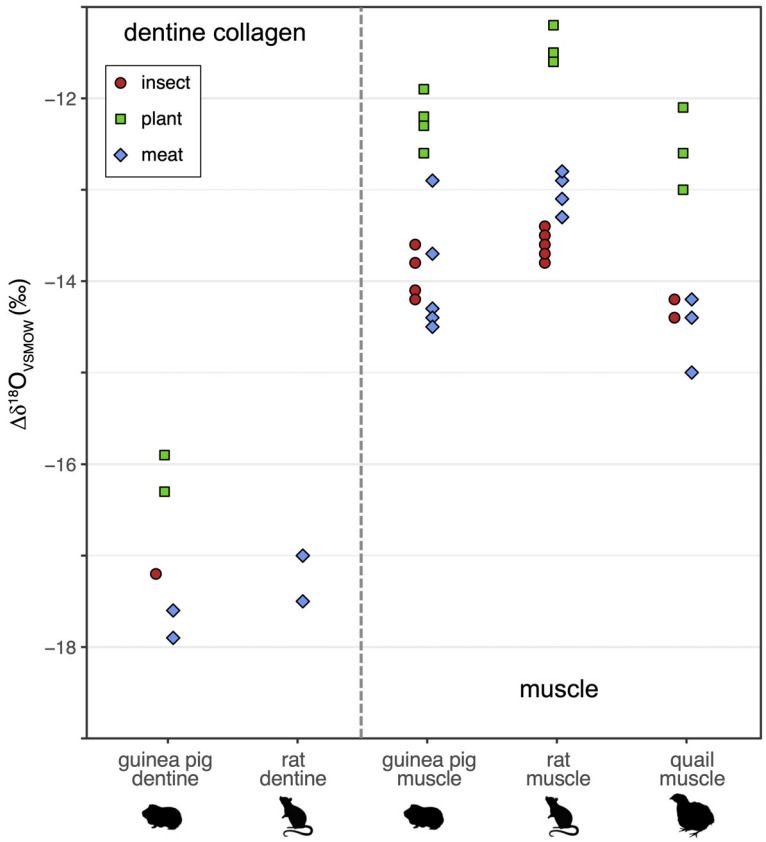

FIGURE 4
Tissue $\Delta\delta^{18}O$ (non-lipid diet) in muscle and dentine collagen of animals fed with plant-based (green squares), insect-based (red circles), and meat-based (blue diamonds) diets, in rats, guinea pigs, and quail (the latter muscle only).

2017; Rodriguez Curras et al., 2018; Topalov et al., 2019). Our isotopic differences by diet greatly exceed this intra-group variability. The magnitude of the difference for $\Delta\delta^2H$ of ~ 4–16 ‰ and $\Delta\delta^{18}O$ up to ~2 ‰ is relatively small but not negligible (Figure 4; Tables 3, 4); greater than typical intra-group uncertainties, but also smaller than the variance found in some studies (discussed further in sections 4.4 and 4.5, e.g. δ^2H can range up to 20–80 ‰ in bird keratin, Hobson et al., 2014). In dentine collagen in the guinea pig $\Delta\delta^2H$ varies by ~20 ‰ and $\Delta\delta^{18}O$ by ~1.5 ‰ between the diets (diet group mean, Figures 3, 4).

In the same individual animal, dentine collagen and muscle $\Delta\delta^2H$ and $\Delta\delta^{18}O$ values are generally offset in parallel (Figure 7), indicating that both tissues are reflecting the same isotopic dietary input, and further, that conclusions drawn from the muscle results are probably generalizable to collagen (dentine and bone) and other proteinaceous tissues such as feathers.

This study does not directly elucidate the mechanisms responsible for these observed diet-tissue H and O isotope differences, and further work would be required to address this. Digestion, absorption, biochemical transformation, and differential incorporation of diet macronutrients and amino acids into tissues impart isotopic change (Macko et al., 1986; Bowen et al., 2009; Vander Zanden et al., 2016; Newsome et al., 2017; Magozzi et al., 2019; Newsome et al., 2020). The diet-tissue offset is plausibly affected by the protein composition and/or lipid and carbohydrate amounts (e.g. for nitrogen isotopes, protein quality influences diet-tissue offsets, e.g. Robbins et al., 2005).

The diets in this present study are isonitrogenous (Leichliter et al., 2021), but they differ in their major component of lucerne, lamb, or soldier fly larval insect meal (Table 1). We first consider the meat- and insect-based diets. These two diets are nearly the same in the non-meat/non-insect components (Table 1), so that the only difference is between the main component, the larval meal or lamb meat. The δ^2H values of the lipid-removed diets are the same (*non-lipid*, -77 ‰ vs. -75 ‰, Table 1), which means that the main ingredient in these diets must have the same δ^2H values as each other. There is no carbohydrate in lamb (U.S. Department of Agriculture and Agricultural Research Service, 2019), so the dried and defatted lamb meat is principally protein. The larval meal contains chitin, a glucosamine-based polymeric carbohydrate. In black soldier fly meal chitin levels can be in the range of 5–9% (dry matter basis, variable defatting; Finke, 2013; Schiavone et al., 2017; Caligiani et al., 2018). Recent work has shown that some mammals and birds express chitinases and can therefore hydrolyze chitin to the glucosamine monomer, but the expression and activity of Chia (one of the chitinases) varies between species (Tabata et al., 2018). Mice, chickens, and pigs have higher Chia expression than dogs or cattle; and guinea pigs lack the Chia gene (Tabata et al., 2018). One might predict rats and quail to have some ability to hydrolyze chitin. Glucosamines are absorbed by the gastrointestinal tract and can be further metabolized (Setnikar and Rovati, 2001; Anderson et al., 2005).

Despite the main component having the same δ^2H value, $\Delta\delta^2H$ in muscle varies significantly between the meat and insect diets (6–8

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TABLE 2 Mean δ^2H, $\delta^{18}O$, $\Delta\delta^2H$, and $\Delta\delta^{18}O$ by diet group[a].

animal	diet	δ^2H_n mean (‰)	sd	$\Delta\delta^2H_n$ non-lipid mean (‰)	sd	$\Delta\delta^2H_n$ whole diet mean (‰)	sd	n δ^2H and $\Delta\delta^2H$	$\delta^{18}O$ mean (‰)	sd	$\Delta\delta^{18}O$ non-lipid mean (‰)	sd	n $\delta^{18}O$ and $\Delta\delta^{18}O$	note
muscle														
guinea pig	plant	-112	0.7	-16	0.7	-8	0.7	5	9.5	0.3	-12.3	0.3	5	
guinea pig	insect	-97	1.3	-20	1.3	-3	1.3	5	9.9	0.2	-13.9	0.2	5	
guinea pig	meat	-103	1.9	-28	1.9	-13	1.9	5	9.1	0.7	-14.0	0.7	5	
rat	plant	-105	1.5	-9	1.5	-1	1.5	5	10.3	0.2	-11.5	0.2	5	
rat	insect	-96	0.9	-19	0.9	-1	0.9	5	10.2	0.2	-13.6	0.2	5	
rat	meat	-100	2.1	-25	2.1	-10	2.1	6	10.4	0.9	-12.7	0.9	6	
quail	plant	-113	1.2	-17	1.2	-9	1.2	5	9.2	0.5	-12.6	0.5	3	b
quail	insect	-100	1.4	-23	1.4	-6	1.4	6	9.5	0.1	-14.3	0.1	3	b
quail	meat	-105	1.4	-30	1.4	-15	1.4	6	8.6	0.4	-14.5	0.4	3	b
dentine collagen														
guinea pig	plant	-65	4.0	32	4.0	40	4.0	4	5.7	0.3	-16.1	0.3	2	c
guinea pig	insect	-42	3.6	35	3.6	52	3.6	3	6.6		-17.2		1	
guinea pig	meat	-64	8.2	11	8.2	26	8.2	4	5.4	0.2	-17.8	0.2	2	
rat	plant	-47		49		57		1						
rat	insect	-29	4.1	48	4.1	66	4.1	2						
rat	meat	-40	5.1	35	5.1	50	5.1	5	5.9	0.3	-17.2	0.3	3	c

[a]δ^2H and $\Delta\delta^2H$ are data obtained with the Cr-packed TC/EA reactor configuration.
[b]excluding $\delta^{18}O$ data for quail in ^{18}O-enriched drinking water treatment.
[c]including samples measured on glassy carbon for δ^2H and adjusted to Cr-equivalent δ^2H using δ^2H_n-Cr = δ^2H-glassyC + 10.4 ‰.

‰, Tukey HSD p ≤0.002, Table 3). This suggests a few possibilities: first, if the protein δ^2H value is the same in the meat- and insect-main components, then the protein quality or type affects the protein H utilization and diet-tissue fractionation ($\Delta\delta^2H$). Second, if the chitin has a different δ^2H value than the insect-meal protein, then the inferred different protein δ^2H between meat- and insect-diets could be the cause of the $\Delta\delta^2H$ difference. Third, any incorporation of chitin H into tissue could also affect δ^2H on the insect-based diet. Given the chitin component is likely small (<10% w/w of the main component), it is perhaps more likely that differences in protein quality and amino acid composition are important in the resulting $\Delta\delta^2H$ offsets and the differences between the meat-based and insect-based diets.

In contrast, $\Delta\delta^{18}O$ presents a more mixed picture in comparing the insect and meat diets. The insect and meat diet muscle $\delta^{18}O$ values are fairly close at ~0.6 ‰ apart, and the resulting $\Delta\delta^{18}O$ is significantly different between those diets only for the rat (0.6 ‰, Tukey HSD p < 0.001, Table 4).

The plant diet results in very different and smaller $\Delta\delta^2H$ and $\Delta\delta^{18}O$ in muscle than the other two diets (Figures 3, 4). The

TABLE 3 ANOVA and Tukey's honest significant differences for muscle $\Delta\delta^2H$ by diet.

	guinea pig[a]			rat[b]			quail[c]		
	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p
plant-insect	3.8	± 2.3	0.0026	9.6	± 2.7	< 0.001	5.7	± 4.1	0.013
meat-insect	-8.1	± 2.3	< 0.001	-6.2	± 2.6	< 0.001	-8.3	± 4.1	0.0020
meat-plant	-11.8	± 2.3	< 0.001	-15.8	± 2.6	< 0.001	-14.1	± 4.1	< 0.001

[a]ANOVA: F-statistic: 95.98 on 2 and 12 DF, p-value: < 0.001.
[b]ANOVA: F-statistic: 134 on 2 and 13 DF, p-value: < 0.001.
[c]ANOVA: F-statistic: 55 on 2 and 6 DF, p-value: < 0.001.

TABLE 4 ANOVA and Tukey's honest significant differences for muscle $\Delta\delta^{18}O$ by diet.

	guinea pig[a]			rat[b]			quail[c]		
	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p
plant-insect	1.6	± 0.8	< 0.001	2.1	± 0.3	< 0.001	1.8	± 0.9	0.0023
meat-insect	-0.1	± 0.8	0.98	0.6	± 0.3	< 0.001	-0.2	± 0.9	0.78
meat-plant	-1.6	± 0.8	< 0.001	-1.6	± 0.3	< 0.001	-2.0	± 0.9	0.0013

[a]ANOVA: F-statistic: 21.81 on 2 and 12 DF, p-value: < 0.001.
[b]ANOVA: F-statistic: 164.1 on 2 and 13 DF, p-value: < 0.001.
[c]ANOVA: F-statistic: 27.03 on 2 and 6 DF, p-value: < 0.001.

difference of the plant diet $\Delta\delta^{2}H$ to that of other diets is significant ($\Delta\delta^{2}H$ = 12–16 ‰ plant-meat, 4–10 ‰ plant-insect, Tukey HSD p ≤0.013, Table 3). Similarly, $\Delta\delta^{18}O$ differences between plant and other diets are up to 2.1 ‰ (Table 4). The plant diet has a higher proportion of the main ingredient (lucerne), additional potato protein, and a lower proportion of the remainder of the diet components (to maintain an iso-nitrogenous condition, Table 1). The nutritional composition of the diets is similar, except the plant-based diet is somewhat lower than the other two diets in neutral detergent fibre and starch (Table 1). The variable dietary macronutrients (proportion of each and their isotope values) in the plant diet and/or the protein composition itself are possible reasons for the observed diet-tissue offset differences from the other two diets in both H and O.

The model elaborated by Magozzi et al. (2019) includes routing from dietary protein to tissue protein (keratin), meaning the protein component plays a strong role in setting the tissue isotope value (e.g., 60% of dietary protein H to keratin). Routing from the dietary protein with a different isotopic composition is plausibly why the plant diet in particular has different $\Delta\delta^{2}H$ and $\Delta\delta^{18}O$ than the insect and meat diets; a second effect may be variation in the fraction of dietary protein H and O routed to tissue between these diet types. In controlled feeding experiments where the water input is changed, the estimated total food contribution to tissues ranges from 70–85 % H in different proteinaceous tissues such as collagen/hair/feather/nail (Kirsanow and Tuross, 2011; Hobson et al., 1999; Topalov et al., 2019) and 30–80 % H in muscle (Hobson et al., 1999; Wolf et al., 2012); this variability may be a real reflection of different routing of atoms from diet component to tissue in different diet conditions as well as differences in how the experiments were conducted (see Vander Zanden et al., 2016 for more data and discussion).

4.2 Isotopic differences between tissues

The results here agree with previous studies showing that isotopic values vary by tissue (e.g., Tuross et al., 2008; Kirsanow and Tuross, 2011; Wolf et al., 2012, for H and O). Collagen H and O isotope values are reported in only a few other controlled feeding studies (rats: Kirsanow and Tuross, 2011; pigs: Tuross et al., 2008; mice: Topalov et al., 2019), and only one reports both muscle and collagen (Tuross et al., 2008). The isotopic pattern seen here agrees with bone collagen results found in pigs (Tuross et al., 2008): there are higher $\delta^{2}H$ values and lower $\delta^{18}O$ values in collagen (here, in dentine collagen) than in muscle. We find a large isotopic difference between dentine collagen and muscle of $\delta^{2}H$ = ~55 ‰ (range 40–67 ‰) and $\delta^{18}O$ = -3–4 ‰ with the same diet, species, and water combinations (Figures 1, 2). These inter-tissue differences are important to bear in mind in ecological or migration studies.

4.3 Isotopic differences between species

There is slight $\delta^{18}O$ patterning in muscle by species for all three diets, with $\delta^{18}O$ values following quail < guinea pigs < rats (0.2–1.5 ‰, Figure 2; Table 6). Hydrogen isotopes also vary between species on some diets in muscle, and more strongly in dentine collagen for all three diets (13–24 ‰, Figure 1; Table 7). Inter-species variation is not unexpected given the effect of metabolic rate and water flux (particularly from drinking water) on resultant tissue oxygen and hydrogen isotopes (O: Bryant and Froehlich, 1995; Kohn, 1996; H and O: Magozzi et al., 2019). Many of the relevant H and O fluxes scale with body mass with an allometric coefficient less than one (i.e., flux $\propto$ mass^{x}, where x < 1); some of these include the metabolic rate (and thus

TABLE 5 ANOVA, Tukey's honest significant differences, and t test results for dentine collagen $\Delta\delta^{2}H$ by diet.

	guinea pig[a]			rat[b]		
	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p
plant-insect	-3.2	12.8	0.76	0.7		
meat-insect	-23.5	12.8	0.0020	-12.5	13.6	0.06
meat-plant	-20.2	11.9	0.0031	-13.2		

[a]ANOVA: F-statistic: 17.47 on 2 and 8 DF, p-value: 0.0012.
[b]t test between meat and insect diet only, due to n=1 in the plant group for the rat.

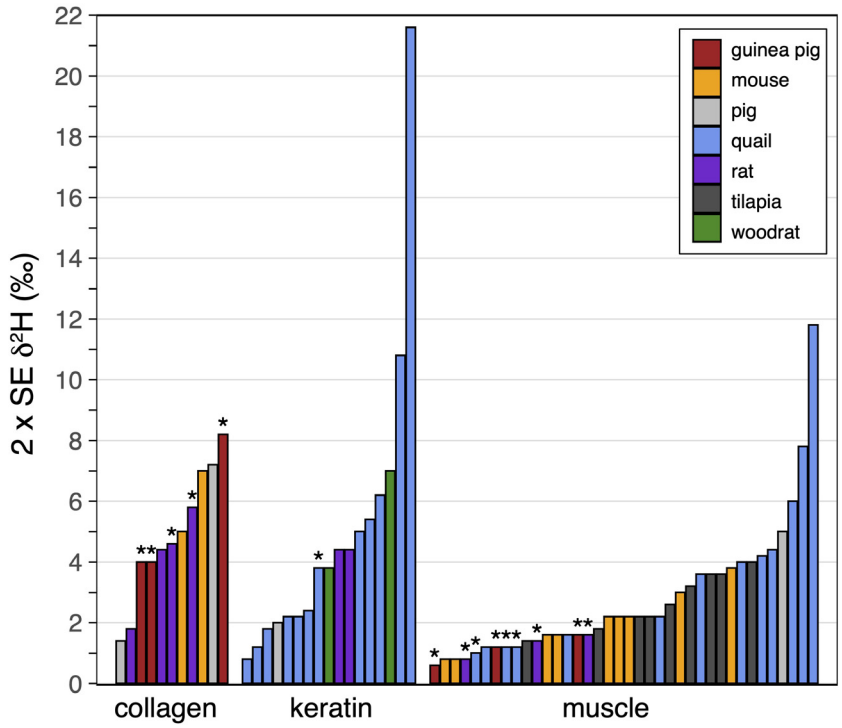

FIGURE 5
Variances (2 x Standard Error of the mean) in δ^2H by substrate and species for controlled feeding studies. Each bar represents one experimental group per study (same diet and water treatment). Results from the present work are denoted by asterisks (*). Data are from Hobson et al. (1999): quail keratin and muscle; Tuross et al. (2008): pig collagen and muscle; Podlesak et al. (2008): woodrat keratin; Kirsanow and Tuross (2011): rat collagen and keratin; Wolf et al. (2012): quail keratin and muscle; Wolf et al. (2013): quail keratin and muscle; Newsome et al. (2017): tilapia muscle; Rodriguez Curras et al. (2018): mouse muscle; Topalov et al. (2019): mouse collagen. Analytical uncertainties are ±1–4 ‰ (1 SD, or not specified) in these studies.

O_2 and food consumption), total water flux, and water vapour loss. These fluxes have slightly different scaling relationships with body mass, so that the balance between them is important in determining the resultant body water and tissue isotopic compositions. Total water flux (and often thus the liquid water drinking rate) increases with body mass (Nagy and Peterson, 1988; Bryant and Froehlich, 1995; Kohn, 1996); albeit with different scaling relationships for captive and wild animals as well as between wild herbivorous mammals, wild omnivorous mammals, and wild birds (Nagy and Peterson, 1988).

In terms of isotopic effects, body water and tissue δ^2H and $\delta^{18}O$ values are predicted to decrease (body water in the direction of the drinking water isotopic value), with increasing liquid drinking water inputs and increasing total water flux. More isotopically fractionated water loss (as vapour), results in higher body water and thus higher tissue δ^2H and $\delta^{18}O$ values (Kohn, 1996). Given the complexity of the varying influences on H and O fluxes, of which only a few were mentioned above, it is difficult to predict simply the expected H and O isotope behaviour for these three species. In any case, these results highlight that it is important to bear this inter-specific isotopic variation in mind.

4.4 Implications for migration and ecology

Typically, isotope-based animal migration studies use an accessible and inert tissue, such as hair, feathers or claws. Controlled feeding experiments have shown that feathers or hair and muscle are isotopically related to each other; i.e. broadly speaking δ^2H in tissues all shift systematically in concert with changes in water and/or dietary inputs (Hobson et al., 1999; Wolf et al., 2012; Newsome et al., 2017). As such the variations in muscle $\Delta\delta^2H$ and $\Delta\delta^{18}O$ by diet we observe here are likely relevant to migration and ecological studies using other tissues such as feathers.

Long-distance animal migration studies often have measured tissues with large variances in δ^2H values between individuals in a given group. This present experiment's $\Delta\delta^2H$ differences with diet in muscle of ~4–16 ‰ and in dentine collagen of ~13–23 ‰ are small/moderate relative to the long-range geospatial variation in precipitation δ^2H values and resultant tissue δ^2H values; e.g., feathers can range up to ~80 ‰ in one species from across North America captured in one over-wintering location, and can typically be ~20–30 ‰ (Hobson et al., 2014). Idaho-resident and non-resident American kestrel claws differ in δ^2H by up to ~40 ‰ (Ranck et al., 2023). The isotopic variation with diet type is small enough relative to the large geospatially-related variation in precipitation δ^2H but might be important in cases with less of a δ^2H gradient in environmental water and thus potentially blur the geospatial assignment of individuals.

Furthermore, an observational study also agrees with our experimental result that diet can affect tissue δ^2H values. In non-migrant wild birds from the same locale (caught by mist nets, mostly

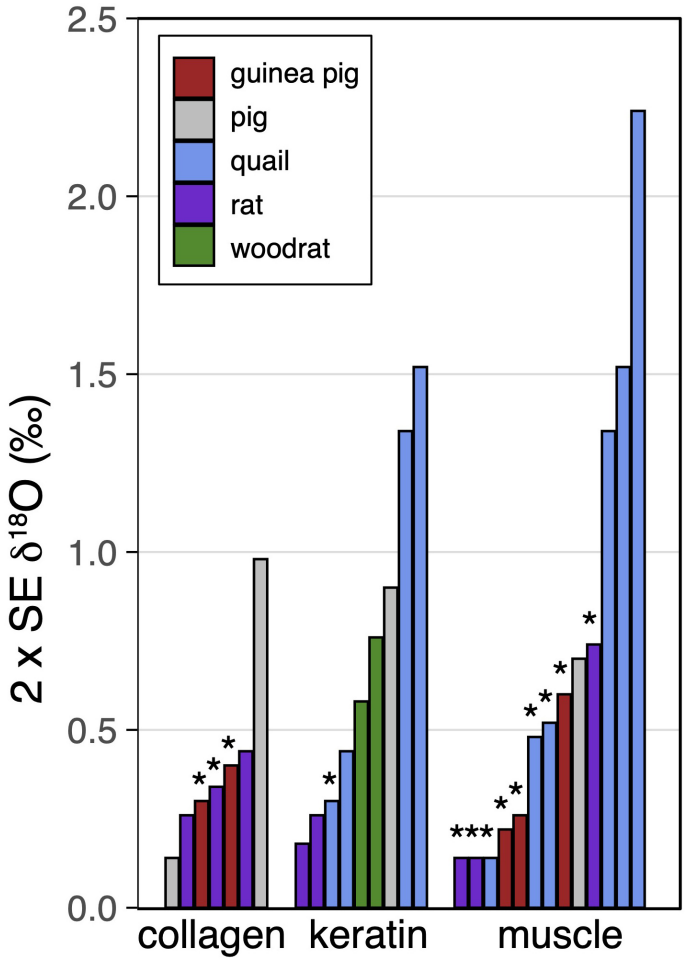

FIGURE 6
Variances (2 x Standard Error of the mean) in $\delta^{18}O$ by substrate and species for controlled feeding studies. Each bar represents one experimental group per study (same diet and water treatment). Results from the present work are denoted by asterisks (*). Data are from Podlesak et al. (2008): woodrat keratin; Kirsanow and Tuross (2011): rat collagen and keratin; Wolf et al. (2013): quail keratin and muscle; Tuross et al. (2008): pig collagen and muscle. Analytical uncertainties are ±0.2–1 ‰ (1 SD, or not specified) in these studies.

smaller birds, van Wijk et al., 2021), feather δ^2H values across species show differences between five dietary guilds (nectarivores, frugivores, granivores, omnivores, insectivores) of 2–30 ‰ – all much larger than the differences in muscle in this present controlled feeding experiment but somewhat closer to the differences we observe in dentine collagen. This variation in δ^2H in the wild birds may result from a large variation in the δ^2H values of the diet components, as well as any inter-species physiological differences.

The $\Delta\delta^{18}O$ variations by diet in muscle of up to ~1.2–2.0 ‰ (Figure 4; Table 4) and ~1.5 ‰ in dentine collagen are relatively large (Figure 4; Tables 2, 4). While studies of oxygen isotopes in organic tissues are few thus far, feather $\delta^{18}O$ reflects 60–80% of the variation in environmental water $\delta^{18}O$ and thus is dampened in its response to changing water $\delta^{18}O$ values, yielding a reduced range of tissue $\delta^{18}O$ values (Hobson and Koehler, 2015; Magozzi et al., 2019). The greater $\delta^{18}O$ variation in tissue (muscle, dentine collagen) relative to the reduced geospatially-related variation in $\delta^{18}O$ (for feathers) means that in oxygen isotope studies for migration- and ecology-related questions, greater attention is needed to consider differences in diet type as a modulating factor.

However, this varied diet-tissue δ^2H and $\delta^{18}O$ offset by diet type is possibly mitigated by the fact that many species have rather consistent dietary habits. However, in the case of omnivores/generalists, humans, and species that may vary their diet type (e.g., seasonally or through ontogeny), the differences in diet-tissue isotopic offsets may add extra variability in tissue isotopic values.

4.5 Implications for palaeoecology and archaeology

The $\Delta\delta^2H$ difference in dentine collagen with the meat-based diet has interesting implications for archaeological or palaeoecological samples where bone or dentine collagen is analyzed – that diet is an important factor to consider in resultant collagen δ^2H values, particularly in omnivores such as humans. The effect of diet type is supported by studies on archaeological samples: in dentine collagen from human first molars δ^2H varied significantly along incrementally sampled

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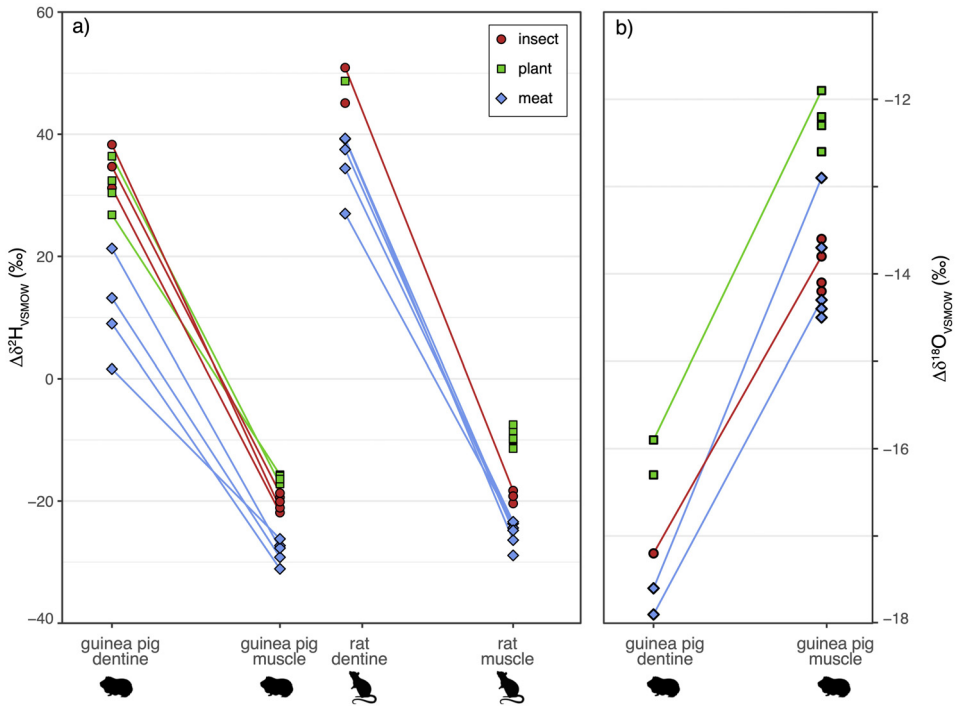

FIGURE 7
(A) $\Delta\delta^2H$ for guinea pig and rat dentine collagen and muscle, by diet; **(B)** $\Delta\delta^{18}O$ for guinea pig dentine collagen and muscle, by diet. Tie lines join data points for individual animals.

TABLE 6 ANOVA and Tukey's honest significant differences for muscle $\delta^{18}O$ by species.

	plant[a]			insect[b]			meat[c]		
	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p
quail-guinea pig	-0.2	± 0.6	0.51	-0.4	± 0.4	0.028	-0.6	± 0.9	0.26
rat-guinea pig	0.8	± 0.5	0.0029	0.3	± 0.3	0.077	0.9	± 0.8	0.019
rat-quail	1.1	± 0.6	0.0013	0.7	± 0.4	< 0.001	1.5	± 0.9	0.0022

[a]ANOVA: F-statistic: 16.17 on 2 and 10 DF, p-value: < 0.001.
[b]ANOVA: F-statistic: 13.78 on 2 and 10 DF, p-value: 0.0013.
[c]ANOVA: F-statistic: 11.6 on 2 and 11 DF, p-value: 0.0020.

TABLE 7 ANOVA and Tukey's honest significant differences for muscle δ^2H by species.

	plant[a]			insect[b]			meat[c]		
	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p	mean difference (‰)	95% conf.	p
quail-guinea pig	-0.4	± 2.5	0.92	-2.3	± 2.3	0.048	-2.6	± 3.9	0.22
rat-guinea pig	7.0	± 2.2	< 0.001	1.2	± 2.0	0.29	3.0	± 3.2	0.067
rat-quail	7.4	± 2.5	< 0.001	3.5	± 2.3	0.0051	5.6	± 3.8	0.0052

[a]ANOVA: F-statistic: 48.79 on 2 and 10 DF, p-value: < 0.001.
[b]ANOVA: F-statistic: 8.616 on 2 and 10 DF, p-value: 0.0067.
[c]ANOVA: F-statistic: 8.648 on 2 and 11 DF, p-value: 0.0055.

sections (up to 30 ‰ range), corresponding to changes over the first few years of life (Ryan et al., 2020). These variations were likely dietary in nature as juveniles changed their diets from mother's milk to possibly plant-food gruel (cooked) and further towards an adult diet (as represented by adult bone isotopic values).

There are limited studies on $\delta^{18}O$ in archaeological protein (collagen), but our results in dentine collagen suggest diet type will be an important consideration in interpreting $\delta^{18}O$ values, among other parameters such as water flux, metabolism, and geographic locality. Data thus far are mixed: medieval bone collagen $\delta^{18}O$ from the same site shows considerable differences between species (up to 3 ‰), including humans (Ryan et al., 2018); in contrast, little inter-species $\delta^{18}O$ variation is seen in bone collagen in Bronze Age archaeological material but a large intra-species range is noted (Reynard et al., 2020). Magozzi et al. (2019) predict lowering of $\delta^{18}O$ in tissue protein with trophic level in a strictly-modelled trophic system where the diet input is the protein from the previous trophic level.

There is no *a priori* expectation for the effect of protein type or amount on resultant tissue isotopic values. Generally the isotopic composition of macronutrients in a given plant rank from $\delta^{2}H_{carbohydrate} > \delta^{2}H_{protein} > \delta^{2}H_{fat}$ (Estep and Hoering, 1980; da Silveira Lobo Sternberg, 1989), $\delta^{18}O_{carbohydrate} > \delta^{18}O_{protein}$ and $\delta^{18}O_{carbohydrate} > \delta^{18}O_{fat}$ (Silva et al., 2015), and in animal muscle $\delta^{2}H_{protein} > \delta^{2}H_{fat}$ (Supplementary Table 9) so that the result of any change in protein routing to tissue (and thus over- or under-representation) is not straightforward to predict.

Acknowledging the small number of dentine samples, our results suggest for both $\delta^{2}H$ and $\delta^{18}O$ in bone or dentine collagen that there are important inter-species differences to consider when interpreting archaeological material, as well as consideration of variation due to changes in diet. For example, inter-site or temporal comparisons should involve the same or very similar species (in diet and physiology). In species with varied diets, e.g. humans, care is needed in interpretations of small magnitude $\delta^{2}H$ and $\delta^{18}O$ variations, as diet may have played a role in the noted bone or tooth isotopic variability.

5 Conclusions

The three diets consumed (plant-, insect-, or meat-based) by the three different animals (guinea pigs, rats, and quail) result in different diet-tissue offsets; variation in hydrogen ($\Delta\delta^{2}H$) and oxygen ($\Delta\delta^{18}O$) isotope offsets are small but greater than intra-group variances, and thus important to consider in ecological and archaeological studies. Controlling for diet, there are also $\delta^{2}H$ and $\delta^{18}O$ variations noted between tissues and between species.

Consequently, the effect of diet type, tissue type, and species on diet-tissue isotopic offsets should be considered when using proteinaceous tissues for ecological, environmental, and diet reconstruction or migration studies. When the same species and tissue is under study, and if the diet type is relatively consistent, then the consequences of these isotopic variations may be mitigated. In addition, these results reinforce that caution is warranted in making inter-species H and O isotope comparisons using proteinaceous tissues.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Swiss Cantonal Animal Care and Use Committee, Zurich. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LR: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. JL: Project administration, Resources, Writing – review & editing. DW: Resources, Writing – review & editing. MC: Methodology, Resources, Writing – review & editing. TT: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2025.1516786/full#supplementary-material

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