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Carbon isotope fractionation between diet, breath CO_2, and bioapatite in different mammals

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Abstract

The carbon isotope fractionation between tooth enamel bioapatite, breath CO_2, and diet was measured for voles, rabbits, pigs, and cattle on controlled diets. The measured fractionation (expressed as isotope enrichment ε^*) between enamel and diet was $11.5 \pm 0.3‰$, $12.8 \pm 0.7‰$, $13.3 \pm 0.3‰$, and $14.6 \pm 0.3‰$ for these respective species. There is a 1:1 correlation between $\varepsilon^*_{breath-diet}$ and $\varepsilon^*_{enamel-diet}$ ($r^2 = 0.94$, $p < 0.01$), whereas our data do not resolve significant inter-species differences in $\varepsilon^*_{enamel-breath}$. These findings are consistent with the hypothesis that inter-species differences in $\varepsilon^*_{bioapatite-diet}$ are primarily a result of differences in digestive physiology, rather than differences in the magnitude of fractionation between mineral and body fluid.

Keywords: Stable isotopes; Diet; Dietary reconstruction; Mammal; Carbon; Methane

1. Introduction

The carbon isotope composition of carbonate in mammalian bioapatite is related to diet, is preserved on archaeological and geological time-scales, and is widely used for reconstructing dietary preferences and availability of different food resources to mammals [19,21,32]. Specific applications include estimating the amount of C_4 plant food in diet [33] (including agricultural maize [1]), identifying closed-canopy habitats [10,26], evaluation of herd-management strategies of ancient pastoralists [6], and estimating age at weaning [40]. Many of these applications require a precise knowledge of the isotopic enrichment between diet and bioapatite. To this end, we present the results of controlled feeding experiments where the isotopic enrichment between diet, breath CO_2, and bioapatite was measured for several animal species.

Bioapatite carbonate is enriched in ^{13}C by several per mil relative to diet. This fractionation has been measured or estimated in several previous controlled feeding experiments and field studies, with results ranging between about 6‰ and 15‰ [9,36], but with consistently reproducible values of 9–11‰ for lab rodents [3,12,18,36], and 12–14‰ for herbivorous ungulates [5,7,9,24,34].

Several workers have theorized that inter-species differences in bioapatite–diet carbon isotope fractionation might arise primarily from differences in digestive physiology between species [2,9,16,19]. This entails that the isotopic fractionation between dissolved inorganic carbon in blood (blood DIC, which includes

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CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}) and carbonate in precipitating bioapatite mineral is similar between species (sensu Krueger and Sullivan [22]), whereas the fractionation between bulk dietary carbon and metabolic CO_2 (including CO_2 produced by the animal and by microorganisms inside the animal) differs between species. Variability in the latter component would lead to variability in bioapatite–diet spacing, because metabolic CO_2 is the input into blood DIC, which is in isotopic equilibrium with bioapatite.

The factor most often implicated for causing inter-species differences in the fractionation between diet and metabolic CO_2 is inter-species differences in the amount of methane production by microorganisms in the digestive tract [2,9,16,19]. Methanogens produce CH_4 using H_2 and CO_2 as substrates; the methane is depleted in ^{13}C by more than 30‰ relative to food [30], and the CO_2 is correspondingly enriched in ^{13}C [31]. This ^{13}C-enriched CO_2 may then enter the blood stream, equilibrate with the blood DIC pool, and impart its signature into developing bioapatite [27]. Because the residual CO_2 from methanogenesis is highly fractionated in ^{13}C compared to diet, even a small amount of methane production may result in significant changes in bioapatite–diet carbon isotope spacing, given that the CO_2 is able to enter the blood stream in sufficient quantities [16]. Ruminants are known to produce amounts of methane that constitute several percent of ingested energy [14,37,38], whereas mice and rats are thought to produce significantly smaller, if not insignificant, quantities [13,23]. Animals known to be highly methanogenic, including cattle, have bioapatite–diet spacings of 12–14‰ [9], while those producing little or no methane have smaller spacings of ~9‰ [3,36]. Expected differences in methane production between species are consistent with observed differences in bioapatite–diet spacing, and this lends credence to the model outlined above.

Several aspects of this model are currently untested or inadequately explored. Aside from presenting new estimates of bioapatite–diet carbon isotopic fractionation, this study seeks to evaluate the hypothesis that fractionation between bioapatite and blood DIC is similar between species, and that variation in bioapatite–diet spacing is mirrored by variations in DIC–diet spacing that are presumably caused by differences in digestive physiology. This study uses the isotopic composition of respired CO_2 as a proxy for the isotopic composition of blood DIC. Previous studies indicate that respired CO_2 and bioapatite are in isotopic equilibrium [36], and that the isotopic composition of respired CO_2 closely tracks changes in the isotopic composition of food [4].

We present results from controlled feeding studies of four species: *Bos taurus* (domestic cattle), *Sus scrofa* (domestic pig), *Oryctolagus cuniculus* (rabbit), and *Microtus ochrogaster* (Prairie vole). In addition to presenting data for breath CO_2 and being the first multiple taxon experimental study of diet–bioapatite fractionation, this study is unique in that it includes animals (cattle and rabbits) fed single plant species diets; this eliminates the possibility of preferential digestion of isotopically disparate components in mixed diets, and so avoids the spurious estimates of bioapatite–diet fractionation that this leads to. This study also differs from most previous experimental studies of bioapatite–diet fractionation in that it is restricted to tooth enamel bioapatite formed entirely during the periods of controlled diet. Previous controlled feeding studies [3,12,17,18,36] have analyzed bone, which contains mineral deposited throughout life, and has a very low mineral turnover rate. Therefore, bone records previous diets, including nursing, and is not an ideal material for the study of diet–bioapatite fractionation.

2. Materials and methods

2.1. Nomenclature

Many papers dealing with isotopic spacing between diet and animal tissues use 'big delta' values:

$$\Delta_{A-B} = \delta^{13}C_A - \delta^{13}C_B \quad (1)$$

This value is convenient to calculate, and is an approximation that is accurate when isotopic differences between two phases are less than about 10‰ [35]. When isotopic differences are greater than this value, the Δ value becomes specific to the region of the isotopic scale (e.g. PDB, SMOW) in which it was calculated, and loses its meaning elsewhere. The fractionation factor α, and the isotope enrichment ε, are mathematically correct expressions of isotopic fractionation or difference between two phases, and they are not specific to the isotopic scale on which they were originally calculated. These values are defined as

$$\alpha_{A-B} = \frac{R_A}{R_B} = \frac{1000 + \delta_A}{1000 + \delta_B} \quad (2)$$

and (Ref. [11]).

$$\varepsilon_{A-B} = [\alpha_{A-B} - 1]1000 \quad (3)$$

Cerling and Harris [9] show that the difference between using Δ and ε is small but significant (0.2–0.3‰) for bioapatite–diet spacing calculated for the same Δ at the C_4 end of the scale versus the C_3 end. It may be convenient to use Δ values, but we make the argument that it is worth the effort of a few more algebraic steps if the result is a correct and universal value rather than an approximate value. In this paper we

use the notation ε^*, which is the same as the ε defined above, but indicates a fractionation not associated with chemical equilibrium [9].

2.2. Animal rearing and tooth selection

The basic design of this experiment was maintenance of each animal on an unchanging, homogeneous diet for a set period of time, sampling breath CO_2 at regular intervals during this time, and sampling well-developed tooth enamel that formed exclusively during the period of controlled diet. The young cattle and piglets required 'rearing diets' consisting of milk formula and other dietary supplements until they were several months old, and these were selected as much as possible to be similar in isotopic composition to the experimental diets used in the main phases of the experiments. Ayliffe et al. [4] show that the carbon isotopic composition of blood DIC in horses, as measured by breath CO_2, closely tracks changes in dietary isotopic composition, with ~85% of the blood DIC pool turning over with a half life of less than 3 days. Similarly rapid DIC turnover has also been demonstrated for lactating cows [27], and rabbits [29]. Therefore, the isotopic influence of the 'rearing diets' on breath samples collected (and tooth enamel formed) after the change to homogeneous diets is expected to be small, and there is no clear evidence of this influence in the time-series breath data or sequential enamel data given in Table 1. Details of the dietary histories of each group are outlined below.

Two Holstein cattle (Co1 and Ed1) raised on milk formula ($\delta^{13}C = -17.4‰$, $n = 1$) and C_4 feeds (C_4 grass + corn supplement; $\delta^{13}C = -12.8 \pm 0.4‰$, $n = 6$) for the first 2–3 months of life, and then grass + corn supplement from months 3–4 to 10, were switched in November 2001 at the age of 10 months to a mono-specific diet of *Cynodon dactylon* (bermuda-grass; $\delta^{13}C = -13.7 \pm 0.3‰$, $n = 8$). This diet continued unchanged until the animals were euthanized at the age of 20 months. Two other Holstein cattle (Ma1 and As1) reared on the same milk formula and C_3 feeds (C_3 grass + barley supplement; $\delta^{13}C = -25.9 \pm 0.5‰$, $n = 5$) for the first 2–3 months of life, and then grass + barley supplement from months 3–4 to 8, were switched in November 2001 at the age of 8 months to a mono-specific diet of *Medicago sativa* (alfalfa; $\delta^{13}C = -27.3 \pm 0.6‰$, $n = 7$). This diet continued unchanged until euthanization at the age of 17 months. Lower second molars were dissected from the jaws, cleaned, and analyzed for this study. Tooth enamel was sampled in a series of 5–6 samples beginning distally near the occlusal surface and moving proximally to the cervical margin. Data from Balasse [5] shows that the distal portion of the m2 is well-mineralized by 9–10 months age (i.e., prior to the period of controlled diet for our animals), whereas the proximal half to third of the tooth appears to form entirely after this age. Therefore, the distal portions of m2 teeth from cattle in this study may record feeds given prior to the implementation of constant diets. In light of this, we avoid the distal portion of the m2, and for epsilon calculations only use data for enamel between 0 and 20 mm distance from the cervical margin for the older cattle (Co1 and Ed1), and 0 and 30 mm for the younger cattle (As1 and Ma1). The 10 mm offset compensates for a slight difference between the two age groups in the timing of second molar development relative to the onset of the target diet.

Two *S. scrofa* piglets (Ki1 and Et1) reared on mothers' milk (mothers' diet unknown), C_3-based pelleted feed ($\delta^{13}C = -23.6 \pm 0.2‰$, $n = 12$), and soy ($\delta^{13}C = -24.6$, $n = 1$) were switched in September 2001 at the age of 5 months to the C_3 pelleted feed only, and continued on this diet until euthanization at the age of 16 months. The pelleted diet was mixed in a single batch by Intermountain Farmers Association, Draper, Utah, and consisted of 50% ground barley, 41% millrun bran from unknown plant sources, and 9% binders and mineral supplements. Two other piglets (Ge1 and Sq1) reared on mothers' milk, C_4-based pelleted feed ($\delta^{13}C = -17.1 \pm 0.5‰$, $n = 9$), and the same soy were switched in September 2001 at the age of 5 months to the C_4 pelleted feed only, and continued on this diet until euthanization at the age of 16 months. This diet was mixed in a single batch by the manufacturer listed above at the same time that the C_3 diet was made, and consisted of 50% corn, 43% millrun bran, and 7% binders and supplements. It was later discovered that the C_4 pigs were accidentally fed the C_3-based pig feed for at least one week in late March and early April 2002. This is apparent in the some of the breath and tooth enamel data; these data are presented in this paper for completeness, but are not used for calculating average epsilon values for pigs. Mandibular X-rays and teeth dissected from other animals showed that the lower second incisors formed entirely during the period from September 2001 to August 2002 while the animals had constant diets. Tooth enamel was sampled in a series of 5–8 samples along the growth axes of these teeth.

Three adolescent *Oryctolagus cuniculus* (rabbits) were acquired in April–May 2001 and put on an alfalfa diet ($\delta^{13}C = -27.0 \pm 0.4‰$, $n = 17$). These animals were part of a study investigating how dietary changes are recorded in evergrowing teeth. One animal (R1) continued on the alfalfa diet until euthanization in Feb 2002, while the two others (R2, R4) were switched to other diets prior to euthanization, thus precluding use of their teeth for estimating $\varepsilon^*_{enamel-diet}$. Lower incisors were selected for isotopic analysis; these teeth are evergrowing, are about 30 mm long, and were observed to have a growth rate of ~0.3 mm/day. Therefore, the time required for replacement of an entire tooth is ~3

Table 1
Breath and tooth enamel carbon isotope data

Animal ID/ species/tooth sampled	Breath collection date	$\delta^{13}C$ breath	Enamel position (mm)[a]	$\delta^{13}C$ enamel	Animal ID/ species/tooth sampled	Breath collection date	$\delta^{13}C$ breath	Enamel position (mm)[a]	$\delta^{13}C$ enamel
As1	9/6/01	−21.8[b]	44	−11.9[b]	Et1	9/6/01	−22.1	40	−10.0
B. taurus	9/20/01	−20.8[b]	36	−12.2[b]	*S. scrofa*	11/16/01	−22.8	35	−10.3
M_2	11/16/01	−23.4	26	−13.1	I_2	3/6/02	−22.7	28	−10.2
	1/24/02	−24.7	18	−12.9		4/2/02	−21.5	23	−10.7
	2/5/02	−23.8	10	−12.1		6/14/02	−20.8	18	−10.8
	3/6/02	−24.4	4	−12.5		8/9/02	−22.9	12	−11.0
	4/2/02	−25.1						4	−10.5
	6/14/02	−24.0							
	7/29/02	−24.3			Ge1[c]	8/23/01	−15.8	35	−5.2
					S. scrofa	11/16/01	−16.1	29	−5.5
Ma1	9/6/01	−21.6[b]	38	−12.6[b]	I_2	1/24/02	−15.4	24	−4.8
B. taurus	9/20/01	−21.9[b]	29	−13.1		3/6/02	−16.3	16	−4.5
M_2	11/16/01	−23.4	20	−13.3		4/2/02	−21.3[c]	8	−4.0
	1/24/02	−25.8	13	−13.4		6/14/02	−14.8		
	3/6/02	−24.2	5	−13.1		8/9/02	−16.4		
	4/2/02	−25.2							
	6/14/02	−24.1			Sq1[c]	8/23/01	−15.5	28	−5.8
	7/29/02	−24.6			*S. scrofa*	11/16/01	−15.1	24	−5.5
					I_2	1/24/02	−15.4	18	−4.9
Co1	9/20/01	−10.4[b]	39	0.8[b]		3/6/02	−15.2	14	−4.8
B. taurus	11/16/01	−10.1	30	0.2[b]		4/2/02	−21.4[c]	3	−4.1
M_2	1/24/02	−10.8	19	0.2		6/14/02	−14.0		
	3/6/02	−11.1	11	0.1		8/9/02	−16.1		
	4/2/02	−11.3	5	0.0					
	6/14/02	−11.4			R1	1/24/02	−26.0	28.0	−13.7
	7/29/02	−11.3			*O. cuniculus*	1/28/02	−25.6	25.8	−14.2
					I_1	1/30/02	−26.2	23.5	−14.8
Ed1	9/6/01	−9.8[b]	40	0.8[b]		2/1/02	−26.1	20.9	−15.2
B. taurus	9/20/01	−9.3[b]	31	−0.4[b]		2/4/02	−26.0	18.5	−14.9
M_2	11/16/01	−10.9	21	1.0		2/5/02	−26.2		
	1/24/02	−10.0	13	1.3					
	2/5/02	−9.5	5	1.2	R2	1/24/02	−26.1	–	–
	3/6/02	−10.2			*O. cuniculus*	1/28/02	−26.1	–	–
	4/2/02	−11.1			I_1	1/30/02	−26.8	–	–
	6/14/02	−11.1				2/1/02	−25.9	–	–
	7/29/02	−11.0				2/4/02	−26.2	–	–
						2/5/02	−26.3	–	–
Ki1	9/6/01	−22.2	36	−10.3					
S. scrofa	11/16/01	−23.0	28	−10.2	R4	1/24/02	−25.7	–	–
I_2	1/24/02	−21.9	21	−10.8	*O. cuniculus*	1/28/02	−25.8	–	–
	3/6/02	−20.9	13	−11.0	I_1	1/30/02	−26.3	–	–
	4/2/02	−21.4	6	−10.6		2/1/02	−25.6	–	–
	6/14/02	−19.9				2/4/02	−25.7	–	–
	8/9/02	−21.6				2/5/02	−25.5	–	–
Lab Vole #45	10/2/02	−24.9[d]	–	–	Lab Vole #24	–	–	right I_1	−14.9
M. ochrogaster	10/10/02	−27.7[d]	–	–	*M. ochrogaster*	–	–	left I_1	−14.9
	10/26/02	−24.5[d]	–	–	I_1	–	–	enamel + dentine	−14.5
	10/26/02	−27.5[d,e]	–	–					
Lab Vole #20	10/2/02	−23.9[d]	–	–					
M. ochrogaster	10/9/02	−25.6[d]	–	–	Lab Vole #79	–	–	right I_1	−15.4
	10/10/03	−27.2[d]	–	–	*M. ochrogaster*	–	–	left I_1	−15.0
	10/26/02	−26.7[d]	–	–	I_1	–	–	enamel + dentine	−14.7
	10/26/02	−27.4[d,e]	–	–					
Lab Vole #87	10/9/02	−26.8[d]	–	–	Lab Vole #65	–	–	enamel + dentine	−14.8
M. ochrogaster	10/10/02	−27.0[d]	–	–	*M. ochrogaster*				
	10/26/02	−24.9[d]	–	–	I_1				
	10/26/02	−26.9[d,e]	–	–					
					Lab Vole #88	–	–	right I_1	−14.8
Lab Vole #29	10/9/02	−23.1[d]	–	–	*M. ochrogaster*	–	–	left I_1	−15.5
M. ochrogaster	10/10/02	−26.5[d]	–	–	I_1	–	–	enamel + dentine	−15.1

Table 1 (*continued*)

Animal ID/ species/tooth sampled	Breath collection date	$\delta^{13}C$ breath	Enamel position (mm)[a]	$\delta^{13}C$ enamel	Animal ID/ species/tooth sampled	Breath collection date	$\delta^{13}C$ breath	Enamel position (mm)[a]	$\delta^{13}C$ enamel
Lab Vole #63	10/26/02	−26.5[d]	–	–					
M. ochrogaster	10/26/02	−27.0	–	–					

Note: No attempt was made to temporally correlate individual breath and tooth enamel samples, because breath provides an instantaneous signal, whereas tooth enamel is a time-averaged signal (Balasse [5]). Presentation of breath and enamel data in the same row of this table does not imply a temporal correlation.

[a] Distance from cervical margin.

[b] These values not included in the individual and species summary calculations in Table 2 because they precede the period of constant dietary isotopic composition (see text).

[c] These animals were inadvertently fed the C_3-based pig feed for at least one week in late March and early April 2002, and enamel values and the 4/2/02 breath values are not used in the species summary calculations in Table 2.

[d] These values are averages of duplicate or triplicate breath samples collected within a few minutes time; the average reproducibility of these samples was 0.2‰.

[e] Breath was sampled after 4 h of food deprivation; values are not used in the individual and species summary calculations of Table 2.

months, and it is likely that lower incisors from R1 formed entirely when the animal had an alfalfa diet. Breath was sampled from all three animals while they were in equilibrium with the alfalfa diet.

M. ochrogaster (vole) offspring were acquired from mothers that were fed Harland Teklad 2031 high-fiber rabbit chow ($\delta^{13}C = -26.1 \pm 0.6‰$, $n = 15$), which consists primarily of alfalfa, soy, oats, wheat, and corn. The corn component is apparently very minor based on the carbon isotopic composition of the bulk feed compared to the expected value for corn (~−13‰). Offspring continued on the same diet following weaning, and the individuals used in this study were adults several months post-weaning. Lower incisors were selected for isotopic analysis; these teeth are evergrowing and are the most likely teeth to have formed post-weaning. Due to their small size, only one enamel sample per tooth could be obtained for isotopic analysis, and mixed enamel/dentine samples were also analyzed.

2.3. Breath collection and analysis

Breath samples of cattle, pigs, and rabbits were collected by placing a flexible plastic cup over the muzzle. Attached to the base of the cup was a 60 ml syringe into which a mixture of respired and atmospheric gas was collected over a period of about 10–20 s. Vole breath was sampled by placing each animal in a 600 ml beaker covered with a watchglass, allowing 1–2 min for CO_2 levels to increase, and sampling the gas into a 60 ml syringe. Immediately after collection into the syringe, the gas was forced through a stainless steel tube immersed in cold ethanol (−50 °C) to remove water vapor, and the effluent was collected into 6 ml headspace vials that were then quickly capped with Hycar septa (Alltech, Deerfield, Illinois) held in place with crimped aluminum seals. The samples were analyzed within 2–3 weeks of collection by injecting ~100–500 μl of the sample gas onto a gas chromatography column (Varian Poraplot Q®, 25 m length, 0.32 mm i.d.) coupled to a Finnigan MAT 252 mass spectrometer operating in the continuous flow mode. Samples typically had CO_2 concentrations between 10,000 and 30,000 ppm. Results were calibrated to the PDB scale using in house CO_2 standards calibrated versus NBS-19 ($\delta^{13}C = 1.95‰$). These standards were diluted in ultra high purity N_2 to 3% vol. CO_2, and were analyzed in the same manner as the breath samples. Reproducibility of these standards is better than 0.1‰, and that of multiple breath samples collected from the same animal over a narrow period of time (~5 min) is better than 0.3‰.

The carbon isotope data from the breath samples were corrected for atmospheric contamination using a mass balance approach:

$$\delta^{13}C_{meas} = f_{amb}\delta^{13}C_{amb} + f_{breath}\delta^{13}C_{breath} \quad (4)$$

where

$$f_{amb} + f_{breath} = 1 \quad (5)$$

and

$$f_{amb} = \frac{[CO_2]_{amb}}{[CO_2]_{sample}} \quad (6)$$

In these equations, $\delta^{13}C_{meas}$, $\delta^{13}C_{amb}$, and $\delta^{13}C_{breath}$ are the carbon isotopic compositions of the sample CO_2, ambient CO_2, and pure breath CO_2, respectively, f_{amb} and f_{breath} are the fractions of CO_2 coming from ambient gas and breath gas, and $[CO_2]_{amb}$ and $[CO_2]_{sample}$ are the CO_2 concentrations of ambient gas and breath gas. These equations can be solved for $\delta^{13}C_{breath}$ if values are estimated for $\delta^{13}C_{amb}$ and $[CO_2]_{amb}$. This method differs from the Keeling plot approach of Carleton et al. [8] in that it requires independent estimates for $\delta^{13}C_{amb}$ and $[CO_2]_{amb}$, and that a single gas sample is needed as opposed to several gas samples needed for Keeling plots.

For animals housed in outdoor pens (cattle and pigs), atmospheric CO_2 values of $\delta^{13}C_{amb} = -8‰$ and $[CO_2]_{amb} = 400$ ppm were used. These numbers led to average correction factors ($\Delta_{corrected-uncorrected} = \delta^{13}C_{corrected} - \delta^{13}C_{uncorrected}$) of $-0.4 \pm 0.1‰$ and $0.0 \pm 0.0‰$ for C_3 and C_4 steers, respectively, and $-0.3 \pm 0.2‰$ and $-0.1 \pm 0.1‰$ for C_3 and C_4 pigs, respectively. The rabbits were housed indoors in a small closed room along with several other rabbits. The isotopic composition and concentration of ambient CO_2 in this room was not measured, but was estimated to range between $-20‰$ and 800 ppm (values reflective of poor air circulation), and $-8‰$ and 400 ppm (values reflective of equilibrium with atmospheric CO_2). The average correction factor for rabbit breath samples was $-0.30 \pm 0.2‰$. Vole breath was sampled inside a laboratory where ambient CO_2 was about $-14‰$ and 800 ppm. The average correction factor for voles was $-0.58 \pm 0.4‰$.

2.4. Isotopic analysis of feeds and bioapatite

Feed samples were collected on the dates of breath sampling (see Table 1). These were ground to <40 mesh size using a Wiley Mill, and were analyzed for $\delta^{13}C$ using a Carlo-Erba Elemental Analyzer coupled to a Finnigan Delta-S mass spectrometer, or using a Costech Elemental Combustion System coupled to a Finnigan MAT 252 mass spectrometer. Both mass spectrometers operated in the continuous flow mode. Reproducibility was better than 0.15‰ (1σ) for internal standards run along with these samples.

Tooth enamel was sampled using diamond-impregnated abrasive burrs. Cattle and pig enamel was treated for 5–15 min in 0.1 M CH_3COOH, and 20–30 min in 3% H_2O_2. The powders were rinsed in distilled water several times between and after these treatments, and were oven dried at 50–70 °C. Rabbit and vole enamel was not treated because of sample size limitations. A comparison for modern tooth enamel of the treatment described above versus no treatment shows little isotopic difference, and no consistent isotopic offset between the two [28]. Koch et al. [20] report a treated versus untreated offset of up to 0.3‰ for enamel treated using a slightly different method (2% NaOCl in place of 3% H_2O_2). Bioapatite (400–700 μg) was reacted in a 100% phosphoric acid common acid bath held at 90 °C. Evolved CO_2 was cryogenically purified, and was analyzed through the dual inlet and micro-volume coldfinger on a Finnigan MAT 252 mass spectrometer. Isotope results were standardized to the PDB scale by running the samples along with tooth enamel standards calibrated using NBS-19 ($\delta^{13}C = 1.95‰$). Reproducibility was better than 0.1‰ for all samples. Steer and pig enamel samples were analyzed in duplicate, while those from rabbits and voles were analyzed only once because of small sample sizes.

2.5. Calculations and statistics

Individual animal and species summary and epsilon values reported in Table 2 are calculated using Eqs. (2) and (3), where δ_A is the mean of delta values from phase A, and δ_B is the mean of delta values from phase B. For animals eating the same diets, the significance of differences in enamel and breath values was evaluated using both *t*-tests and Mann–Whitney rank sum tests, and are noted in the text as 'significantly different' when *p*-values are less than 0.05 for both tests. Kruskal–Wallis rank sums, and ANOVA followed by Tukey's tests, were used to evaluate the significance of interspecies differences in fractionation between diet, breath CO_2, and enamel bioapatite.

3. Results

3.1. Cattle

Isotope data for breath and bioapatite are presented in Table 1, and summary data and epsilon values are presented in Table 2 and Figs. 1 and 2. Breath values for the C_3 cattle As1 and Ma1 give $\varepsilon^*_{breath-diet}$ values of $4.5 \pm 0.5‰$ for the C_3 grass + barley diet, and $3.0 \pm 0.7‰$ for the alfalfa diet. The greater spacing for the mixed diet may reflect preferential digestion of the barley component ($\delta^{13}C = -24.4 \pm 0.2$, $n = 3$) over the grass component ($\delta^{13}C = -26.7$, $n = 1$), although other possibilities such as changes in gut microbial composition cannot be ruled out. Breath data for the C_4 cattle Co1 and Ed1 give $\varepsilon^*_{breath-diet}$ values of $3.0 \pm 0.6‰$ and $2.9 \pm 0.6‰$ for the post-weaning grass + corn supplemented diet, and the pure bermudagrass diet, respectively. There is no significant difference in breath $\delta^{13}C$ between the C_4 steers Co1 and Ed1 while on the bermudagrass diet, nor is there a significant difference in breath $\delta^{13}C$ between the C_3 steers As1 and Ma1 while on alfalfa diet.

Tooth enamel values are $-12.7 \pm 0.4‰$ for As1, and $-13.2 \pm 0.1‰$ for Ma1, and are significantly different despite the fact that the animals consumed exactly the same C_3 alfalfa feed. Likewise, tooth enamel values are significantly different between the two C_4 steers, with Co1 averaging $0.1 \pm 0.1‰$, and Ed1 averaging $1.2 \pm 0.2‰$. The C_3 cattle As1 and Ma1 give $\varepsilon^*_{enamel-diet}$ values of $15.0 \pm 0.7‰$ and $14.4 \pm 0.6‰$, respectively, and the C_4 steers Co1 and Ed1 give values of $14.0 \pm 0.3‰$ and $15.0 \pm 0.3‰$. The enamel–breath spacings for the C_3 steers are $11.9 \pm 0.7‰$ (As1) and $11.6 \pm 0.6‰$ (Ma1), and for the C_4 steers are $11.2 \pm 0.5‰$ (Co1) and $11.8 \pm 0.7‰$ (Ed1).

Table 2
Summary data and epsilon values for carbon isotope spacing between diet, breath, and bioapatite

Animal	Species	$\delta^{13}C_{diet}$	$\delta^{13}C_{breath}$	$\delta^{13}C_{enamel}$	$\varepsilon^*_{breath-diet}$	$\varepsilon^*_{enamel-breath}$	$\varepsilon^*_{enamel-diet}$
Individual animals summary							
As1	*B. taurus*	−27.3 ± 0.6	−24.2 ± 0.6	−12.7 ± 0.4	3.1 ± 0.8	11.9 ± 0.7	15.0 ± 0.7
Ma1	*B. taurus*	−27.3 ± 0.6	−24.6 ± 0.6	−13.2 ± 0.1	2.8 ± 0.8	11.6 ± 0.6	14.4 ± 0.6
Co1	*B. taurus*	−13.7 ± 0.3	−11.0 ± 0.5	0.1 ± 0.1	2.7 ± 0.6	11.2 ± 0.5	14.0 ± 0.3
Ed1	*B. taurus*	−13.7 ± 0.3	−10.5 ± 0.6	1.2 ± 0.2	3.2 ± 0.7	11.8 ± 0.7	15.0 ± 0.3
Ki1	*S. scrofa*	−23.6 ± 0.2	−21.6 ± 0.8	−10.6 ± 0.3	2.1 ± 0.8	11.2 ± 0.8	13.3 ± 0.4
Et1	*S. scrofa*	−23.6 ± 0.2	−22.1 ± 1.0	−10.5 ± 0.4	1.5 ± 1.0	11.9 ± 1.1	13.4 ± 0.4
Ge1	*S. scrofa*	−17.1 ± 0.2	−15.8 ± 0.6	−4.8 ± 0.4	1.3 ± 0.8	11.2 ± 0.7[a]	12.5 ± 0.6[a]
Sq1	*S. scrofa*	−17.1 ± 0.2	−15.2 ± 0.7	−5.0 ± 0.5	1.9 ± 0.8	10.4 ± 0.9[a]	12.3 ± 0.4[a]
R1	*O. cuniculus*	−27.0 ± 0.4	−26.0 ± 0.2	−14.6 ± 0.6	1.0 ± 0.4	11.8 ± 0.6	12.8 ± 0.7
R2	*O. cuniculus*	−27.0 ± 0.4	−26.2 ± 0.3	–	0.8 ± 0.5	–	–
R4	*O. cuniculus*	−27.0 ± 0.4	−25.8 ± 0.3	–	1.3 ± 0.5	–	–
lv#24	*M. ochrogaster*	−26.1 ± 0.6	–	−14.8 ± 0.2	–	–	11.6 ± 0.6
lv#79	*M. ochrogaster*	−26.1 ± 0.6	–	−15.0 ± 0.3	–	–	11.4 ± 0.7
lv#65	*M. ochrogaster*	−26.1 ± 0.6	–	−14.8	–	–	11.6 ± 0.6
lv#88	*M. ochrogaster*	−26.1 ± 0.6	–	−15.1 ± 0.4	–	–	11.3 ± 0.7
lv#45	*M. ochrogaster*	−26.1 ± 0.6	−25.7 ± 1.7	–	0.4 ± 1.8	–	–
lv#20	*M. ochrogaster*	−26.1 ± 0.6	−25.9 ± 1.5	–	0.2 ± 1.6	–	–
lv#87	*M. ochrogaster*	−26.1 ± 0.6	−26.2 ± 1.2	–	−0.1 ± 1.3	–	–
lv#29	*M. ochrogaster*	−26.1 ± 0.6	−24.8 ± 2.4	–	1.3 ± 2.5	–	–
lv#63	*M. ochrogaster*	−26.1 ± 0.6	−26.5	–	−0.4 ± 0.6	–	–
Species summary							
Steers	*B. taurus*	–	–	–	2.9 ± 0.4	11.6 ± 0.3	14.6 ± 0.3
Pigs	*S. scrofa*	–	–	–	1.8 ± 0.6	11.6 ± 0.7	13.3 ± 0.3
Rabbit	*O. cuniculus*	−27.0 ± 0.4	−26.0 ± 0.2	−14.6 ± 0.6	1.0 ± 0.4	11.8 ± 0.6	12.8 ± 0.7
Voles	*M. ochrogaster*	−26.1 ± 0.6	−25.8 ± 0.9	−14.9 ± 0.1	0.3 ± 0.8	11.2 ± 0.9	11.5 ± 0.3
Mice	*M. mus*	(data from Tieszen and Fagre [36])			−1.6 ± 1.8	10.7 ± 0.8	9.1 ± 1.6

Note: Plus or minus values are one standard deviation for $\delta^{13}C$ values, and are 'random walk' error propagation (square root of the sum of squared standard deviations) for ε^* values, except for the mice data from Tieszen and Fagre [36], which are one standard deviation.
[a] Values are influenced by inadvertent feeding of C_3 feed and are not used in species summary calculations.

3.2. Pigs

Breath values for the C_3 pigs Ki1 and Et1 average −21.6 ± 0.8‰ and −22.1 ± 1.0‰ and are not significantly different from each other. These data give $\varepsilon^*_{breath-diet}$ values of 2.1 ± 0.8‰ and 1.5 ± 1.0‰ for Ki1 and Et1, respectively. Breath values for the C_4 pigs Ge1 and Sq1 average −15.8 ± 0.6‰ and −15.2 ± 0.7‰, respectively, and are also not significantly different from each other. These data give $\varepsilon^*_{breath-diet}$ values of 1.3 ± 0.8‰ and 1.9 ± 0.8‰ for the two animals. These calculations exclude the 4/2/02 breath data for the C_4 pigs because these reflect the inadvertent feeding of the C_3-based feed to these animals; this mistake was also verified by carbon isotope analysis of feed collected from the C_4 pen on the same day ($\delta^{13}C = -23.8‰$). It is unknown how long the animals were given C_3-based feed, but as each food bag lasted approximately one week, it is assumed that the interval was at least one week.

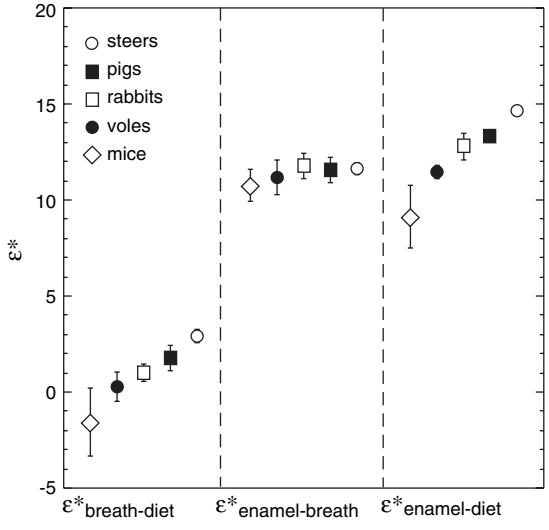

Fig. 1. Single axis plot of epsilon values for different species. Mice data are from Tieszen and Fagre [36].

Enamel values for the C_3 pigs Ki1 and Et1 are −10.6 ± 0.3‰ and −10.5 ± 0.4‰, respectively, and are not significantly different. Ki1 and Et1 give $\varepsilon^*_{enamel-breath}$ values of 11.2 ± 0.8‰ and 11.9 ± 1.1‰. Enamel values for the C_4 pigs Ge1 and Sq1 are −4.8 ± 0.4‰ and −5.0 ± 0.5‰, respectively, and are not significantly different. Ge1 and Sq1 give $\varepsilon^*_{enamel-breath}$ values of 11.2 ± 0.7‰ and 10.4 ± 0.9‰. The average

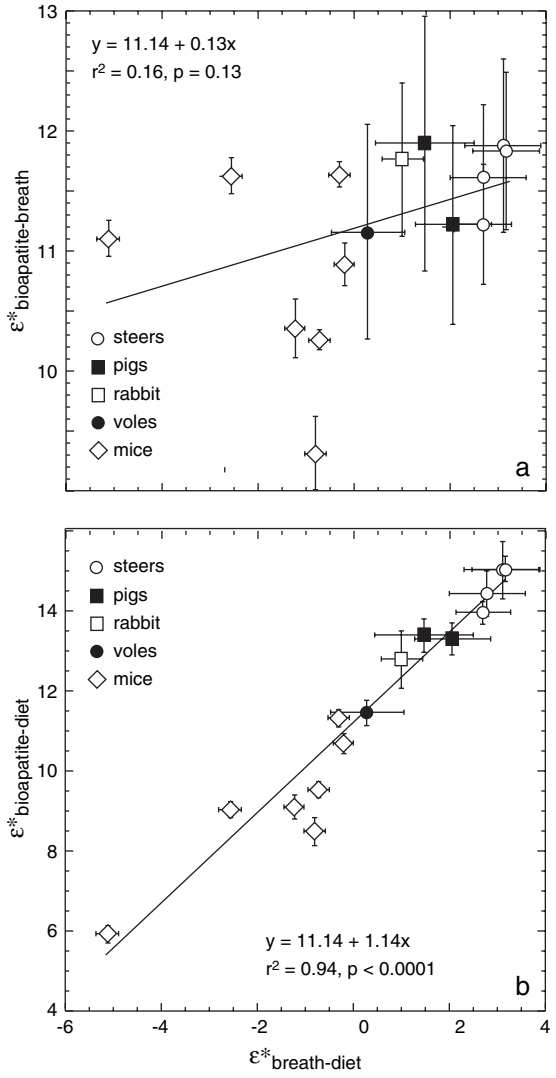

Fig. 2. (a) Bivariate plot of $\varepsilon^*_{\text{bioapatite-breath}}$ versus $\varepsilon^*_{\text{breath-diet}}$ showing no correlation between the two variables. (b) Bivariate plot of $\varepsilon^*_{\text{bioapatite-diet}}$ versus $\varepsilon^*_{\text{breath-diet}}$ showing a strong correlation between the two variables. Mice data are from Tieszen and Fagre [36].

value of $\varepsilon^*_{\text{enamel-breath}}$ for the C_3 pigs is $11.6 \pm 0.5‰$, while that for the C_4 pigs is $10.8 \pm 0.7‰$. The enamel–diet spacing for the C_3 pigs is $13.3 \pm 0.4‰$ for Ki1 and $13.4 \pm 0.4‰$ for Et1, and for the C_4 pigs is $12.5 \pm 0.6‰$ for Ge1 and $12.3 \pm 0.4‰$ for Sq1. The mean difference between the C_3 and C_4 pigs is 1.0‰ and is in the direction expected for the C_4 pigs being inadvertently fed C_3-based feed. Excluding the data from the C_4 pigs, the mean $\varepsilon^*_{\text{enamel-diet}}$ for pigs is $13.3 \pm 0.3‰$.

3.3. Rabbits

Breath values for the rabbits R1, R2, and R4 are $-26.0 \pm 0.2‰$, $-26.2 \pm 0.3‰$, and $-25.8 \pm 0.3‰$, respectively, and give $\varepsilon^*_{\text{breath-diet}}$ values of $1.0 \pm 0.4‰$, $0.8 \pm 0.5‰$, and $1.3 \pm 0.5‰$; of these, only the difference between R2 and R4 is significant. Enamel values for R1 average $-14.6 \pm 0.6‰$ and give an $\varepsilon^*_{\text{enamel-diet}}$ value of $12.8 \pm 0.7‰$. The isotopic spacing between enamel bioapatite and breath for R1 is $11.8 \pm 0.6‰$.

3.4. Voles

Vole breath showed a large amount of variability in $\delta^{13}C$, and ranged between $-23.1‰$ (LV#29, 10/9/02) and $-27.7‰$ (LV#45, 10/10/02) for animals eating the same diet. Because CO_2 samples were collected from chamber air, it is possible that CO_2 coming directly from the digestive tract via eructation or flatulence was also sampled. Animals that were deprived of food for 4 h had values that were consistently depleted in ^{13}C compared to values from the same animals prior to food deprivation ($-27.3 \pm 0.3‰$ versus $-25.4 \pm 1.2‰$), although the sample size is limited and the difference is not significant at the 95% level. Food deprived values were not included in summary and epsilon calculations. Several breath samples collected at other times had values indistinguishable from the food deprived values, and the average within-animal range (excluding the food deprived and LV#63 data) was 3.0‰. This range is about 1.5–2 times larger than that observed for steers and pigs fed constant diets (excluding the 11/16/01 pig breath value for Ki1), and is several times larger than the range observed for rabbits. Because of the variability in vole breath $\delta^{13}C$, and because samples were only collected during daylight hours, it is possible that the average vole breath $\delta^{13}C$ values presented here are not representative of the time-integrated $\delta^{13}C$ signal. Breath–diet isotopic spacing is correspondingly variable, and ranges from $-0.4 \pm 0.6‰$ for LV#63 to $1.3 \pm 2.5‰$ for LV#29. In contrast, bioapatite $\delta^{13}C$ values do not show increased variability: they range from $-15.5‰$ to $-14.5‰$, and average $-14.8 \pm 0.2‰$, $-15.0 \pm 0.3‰$, $-14.8‰$, and $-15.1 \pm 0.4‰$ for the four different animals that were analyzed. These give $\varepsilon^*_{\text{enamel-diet}}$ values of $11.6 \pm 0.6‰$, $11.4 \pm 0.7‰$, $11.6 \pm 0.6‰$, and $11.3 \pm 0.7‰$. None of the animals were analyzed for both breath and enamel, but the pooled breath and enamel data give an estimate for $\varepsilon^*_{\text{enamel-breath}}$ of $11.2 \pm 0.9‰$. This value suffers from the same uncertainty as the average breath data, and is lower than the average values observed for steers, C_3 pigs, and the R1 rabbit, but is the same as the estimate for the steer Co1 and the pig Ki1.

3.5. Inter-species relationships

There is a large inter-species range in the estimates for $\varepsilon^*_{\text{breath-diet}}$ and $\varepsilon^*_{\text{enamel-diet}}$ (Fig. 1). Including data for mice from Tieszen and Fagre [36], $\varepsilon^*_{\text{breath-diet}}$ values

show a total range of 4.5‰, from -1.6 ± 1.8‰ for mice to 2.9 ± 0.4‰ for steers. The total range in $\varepsilon^*_{\text{enamel-diet}}$ is 5.5‰, from 9.1 ± 1.6 for mice to 14.6 ± 0.3 for cattle. Enamel–breath spacing has a range of 1.1‰, with the smallest value observed for mice (10.7 ± 0.8‰), and the largest value observed for the rabbit (11.8 ± 0.6‰). A simple regression between $\varepsilon^*_{\text{enamel-breath}}$ and $\varepsilon^*_{\text{breath-diet}}$ shows a poor correlation and no significant regression slope (Fig. 2; $r^2 = 0.16$, $p = 0.13$). The same regression between $\varepsilon^*_{\text{enamel-diet}}$ and $\varepsilon^*_{\text{breath-diet}}$ shows a high degree of correlation and a slope that is significantly different than zero ($r^2 = 0.94$, $p < 0.01$).

There are significant differences among species for $\varepsilon^*_{\text{enamel-diet}}$ (ANOVA, $F = 16.9$, $p < 0.01$; Kruskal–Wallis, $\chi^2 = 19.69$, $p < 0.01$) and $\varepsilon^*_{\text{breath-diet}}$ (ANOVA, $F = 13.1$, $p < 0.01$; Kruskal–Wallis, $\chi^2 = 16.00$, $p < 0.01$), whereas no significant differences can be resolved among species for $\varepsilon^*_{\text{enamel-breath}}$ (ANOVA, $F = 1.98$, $p = 0.18$; Kruskal–Wallis, $\chi^2 = 5.75$, $p = 0.124$). For $\varepsilon^*_{\text{enamel-diet}}$, Tukey's tests suggest that there are significant differences between cattle and voles, cattle and mice, and voles and mice. For $\varepsilon^*_{\text{breath-diet}}$, Tukey's tests suggest significant differences between cattle and voles, cattle and mice, and rabbits and mice.

4. Discussion

4.1. Comparison of bioapatite–diet spacing with previous results

This is the first experimental study to present results for a ruminant herbivore fed a single plant species diet. The $\varepsilon^*_{\text{enamel-diet}}$ spacing for cattle is 14.6 ± 0.3‰ and is slightly greater than the value obtained by Balasse [5] for steers fed mixed diets (13.7 ± 0.7‰). Preferential digestion of the C_3 soy and wheat components over the C_4 maize components of the diets fed to the animals in Ref. [5] may account for this difference. Cerling and Harris [9] observed a value of 14.1 ± 0.5‰ for a variety of wild ruminant species, which is consistent with the value observed in this study for steers. The value of 12–13‰ for wild ungulates that is commonly attributed to Lee-Thorp and van der Merwe [24] is seemingly at odds with the results presented in this study, Balasse [5], and Cerling and Harris [9]. However, Lee-Thorp and van der Merwe [24] did not present plant $\delta^{13}C$ values from the ecosystems from which animals were sampled, and they analyzed primarily bone rather than tooth enamel, so their results are not necessarily comparable with the results from this study.

The $\varepsilon^*_{\text{bioapatite-diet}}$ value of 13.3 ± 0.3‰ for pig tooth enamel in this study compares with values for pig bone ranging between 8.9‰ and 12.4‰ for pigs fed six different diets in the study by Howland et al. [17]. The pigs in the present study and those in Ref. [17] were fed mixed diets, so preferential digestion of isotopically different dietary components may influence $\varepsilon^*_{\text{bioapatite-diet}}$ values, and inter-comparisons are accordingly less meaningful. This is further exacerbated by the fact that this study analyzes enamel, whereas Howland et al. [17] analyzed bone. It is noted, however, that the animals with the most pure C_3 and C_4 end-member diets in Ref. [17] also had the largest $\varepsilon^*_{\text{bioapatite-diet}}$ values (12.4‰ and 11.4‰, respectively). Because of their greater isotopic homogeneity, these diets reduce the influence of preferential digestion of isotopically different dietary components on the isotopic composition of bone, and $\varepsilon^*_{\text{bioapatite-diet}}$ values obtained from them should be the most representative of animals eating pure diets.

Measured carbon isotope values for tooth enamel from warthogs in east Africa average -0.5 ± 1.2‰, and range between −4.0‰ and 1.6‰ [15]. If the enamel values greater than 1.0‰ are assumed to represent the animals with the least amount of C_3 vegetation in diet, then $\varepsilon^*_{\text{bioapatite-diet}}$ values are at least 12.9‰ for animals eating NADP C_4 grasses, which are the most ^{13}C-enriched C_4 grasses, and have been measured at -11.7 ± 0.7‰ [9]. Animals eating other C_4 grasses or a small fraction of C_3 vegetation would have even larger $\varepsilon^*_{\text{enamel-diet}}$ values, and these would be consistent with the values measured in this study.

This study presents the first $\varepsilon^*_{\text{enamel-diet}}$ estimates for rabbits (12.8 ± 0.7‰) and voles (11.5 ± 0.3‰). We are unaware of any published carbon isotope data from these species, so these data stand alone at present. Voles may have greater physiological affinity with mice and rats than with the other species in this study. The average $\varepsilon^*_{\text{bioapatite-diet}}$ for the mice in DeNiro and Epstein [12] and Tieszen and Fagre [36] is 9.3 ± 1.4‰, and that for rats in Ambrose and Norr [3], and Jim et al. [18] is 9.7 ± 0.6‰. At present we cannot account for the difference between voles and mice + rats, and the possibilities include physiological differences, artifacts associated with preferential digestion of mixed diets, or the fact that this study analyzed primarily enamel, whereas the other studies analyzed bone mineral.

4.2. The digestive physiology signal in bioapatite $\delta^{13}C$

An end-member scenario that attributes all inter-species variation in $\varepsilon^*_{\text{bioapatite-diet}}$ to differences in digestive physiology requires no inter-species variation in $\varepsilon^*_{\text{bioapatite-breath}}$ (assuming that there is no inter-species variation in the fractionation between breath and blood DIC). The data presented in this paper, together with data from Tieszen and Fagre [36] show a 1.1‰ range in $\varepsilon^*_{\text{bioapatite-breath}}$ across the five species of animals, and suggest that this end-member situation is nearly but not

completely met. However, ANOVA and Kruskal–Wallis tests indicate no significant difference among these species for $\varepsilon^*_{\text{bioapatite-breath}}$, and half of the 1.1‰ range is due to the mouse data [36].

Tieszen and Fagre [36] analyzed mouse bioapatite as bone, whereas this study examined tooth enamel. Data in Ref. [36] show that collagen of suckling offspring was 2.1‰ depleted in ^{13}C relative to mothers, and muscle was 0.8‰ depleted. No data were given for bioapatite of suckling offspring relative to adults, but bioapatite values for adult animals were positively correlated with values for collagen ($r^2 = 0.73$) and muscle ($r^2 = 0.77$) and it is possible that bioapatite of suckling offspring is similarly depleted in ^{13}C relative to mothers. Weight data in Ref. [36] show that offspring are at least 50% of their adult weight at the time of weaning. Therefore, even with mineral turnover from bone remodeling, the bone apatite likely contains a significant fraction of mineral deposited during suckling, and this might account for the smaller $\varepsilon^*_{\text{bioapatite-breath}}$ values observed for these animals.

Despite the apparent, but statistically insignificant, difference in $\varepsilon^*_{\text{bioapatite-breath}}$ between mice and the other species, data in this paper show that most of the inter-species variability in $\varepsilon^*_{\text{bioapatite-diet}}$ is accounted for by variability in $\varepsilon^*_{\text{breath-diet}}$. This finding is consistent with inter-species differences in digestive physiology, including differences in methane production, being the major determinant of $\varepsilon^*_{\text{bioapatite-diet}}$ for an individual or species when preferential digestion of isotopically disparate components of mixed diets is not a factor.

If the fraction of ingested carbon lost as methane is the only factor influencing $\varepsilon^*_{\text{bioapatite-diet}}$, then the data in this paper suggest that fractional methane production is greatest in cattle followed by pigs followed by rabbits followed by voles. This is generally consistent with studies of methane production in these animals [13,14,23,37]. However, the pigs and voles had mixed diets, so preferential digestion cannot be ruled out as a factor influencing $\varepsilon^*_{\text{bioapatite-diet}}$. More importantly, as Hedges [16] points out, the degree to which $\varepsilon^*_{\text{bioapatite-diet}}$ will track methane production depends on the fraction of ^{13}C-enriched CO_2 generated during methanogenesis that enters the body, versus the fraction expelled directly from the digestive tract. It is possible, for example, that enriched CO_2 produced in the foregut has a different absorbed/expelled ratio than that produced in the hindgut. A mass balance for carbon would also require data from feces and perhaps minor sources of carbon loss, so it is premature at this point to estimate methane production based solely on $\varepsilon^*_{\text{bioapatite-diet}}$ values. Nonetheless, the data in this paper, combined with data in Tieszen and Fagre [36], indicate that there is a strong physiological signal in $\varepsilon^*_{\text{bioapatite-diet}}$. The role of methane production versus other factors in generating this signal warrants further investigation.

4.3. Bioapatite–collagen spacing

Previous studies have shown that herbivores have larger carbon isotope spacings between bioapatite and collagen than carnivores (~7‰ versus ~4‰), with omnivores having intermediate values [22,25]. This pattern has been partly attributed to higher dietary intake and metabolism of lipids (which are depleted in ^{13}C; Vogel [39]) for carnivores and omnivores relative to herbivores (Lee-Thorp et al. [25]). That is, metabolism of ^{13}C-depleted lipids to CO_2 would result in ^{13}C-depleted bioapatite because of isotopic equilibrium within the bicarbonate buffer system, whereas lipid metabolism would affect collagen isotopic composition very little because proteins, and not lipids, are the primary carbon source for collagen (especially for carnivores and omnivores with protein-rich diets). However, mass balance calculations by Hedges [16] suggest that dietary differences between carnivores, omnivores, and herbivores could only account for about half of the observed difference in $\varepsilon^*_{\text{bioapatite-collagen}}$ between these groups of animals. Methanogenesis is an additional, and independent, mechanism that would lead to greater $\varepsilon^*_{\text{bioapatite-collagen}}$ values in methane-producing animals compared to non-methane producing animals. Methanogenesis produces isotopically enriched CO_2 that will influence the isotopic composition of bioapatite, but does not lead to significant fractionation in fermentation-produced volatile fatty acids [27]. Therefore, collagen should not be enriched in ^{13}C as a result of methanogenesis, even if it contains carbon atoms ultimately derived from fatty acids produced during fermentation. Thus, to the extent that carnivores and omnivores have lower methane production than herbivores, methanogenesis is a valid explanation for observed $\varepsilon^*_{\text{bioapatite-collagen}}$ patterns among these groups of animals. For the same reasons, methanogenesis is not expected to greatly influence $\varepsilon^*_{\text{collagen-diet}}$ values.

5. Conclusions

Study of the carbon isotope composition of bioapatite is extremely useful as a recorder of the isotopic composition of diet. Breath and bioapatite data presented in this paper are consistent with the idea that digestive physiology also has a strong influence on the carbon isotope ratio of bioapatite. So far, a ~5‰ range in bioapatite–diet spacing has been identified for species ranging from mice to cows. There are no data for animals with very different physiologies, such as mammalian carnivores and insectivores, or reptiles, and these might augment the observed variability. Bioapatite data are commonly interpreted in the context of isotopic mixing lines between C_3 and C_4 vegetation; the isotopic difference between these plant types is ~14‰, so a 5‰ physiological

signal is very important in this context if animals with disparate physiologies are being compared. A physiological signal will also be very important when investigating isotopic variation within C_3-dominated ecosystems. The role of carbon isotopes in breath and bioapatite as a physiological record has been largely unutilized, and might find useful application in a variety of fields.

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