



# CH<sub>4</sub>/CO<sub>2</sub> Ratios and Carbon Isotope Enrichment Between Diet and Breath in Herbivorous Mammals

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Breath and diet samples were collected from 29 taxa of animals at the Zurich and Basel Zoos to characterize the carbon isotope enrichment between breath and diet. Diet samples were measured for  $\delta^{13}\text{C}$  and breath samples for CH<sub>4</sub>/CO<sub>2</sub> ratios and for the respiration component of  $\delta^{13}\text{C}$  using the Keeling plot approach. Different digestive physiologies included coprophagous and non-coprophagous hindgut fermenters, and non-ruminant and ruminant foregut fermenters. Isotope enrichments from diet to breath were  $0.8 \pm 0.9\text{‰}$ ,  $3.5 \pm 0.8\text{‰}$ ,  $2.3 \pm 0.4\text{‰}$ , and  $4.1 \pm 1.0\text{‰}$ , respectively. CH<sub>4</sub>/CO<sub>2</sub> ratios were strongly correlated with isotope enrichments for both hindgut and foregut digestive strategies, although CH<sub>4</sub> production was not the sole reason for isotope enrichment. Average CH<sub>4</sub>/CO<sub>2</sub> ratios per taxon ranged over several orders of magnitude from  $10^{-5}$  to  $10^{-1}$ . The isotope enrichment values for diet-breath can be used to further estimate the isotope enrichment from diet-enamel because Passey et al. (2005b) found a nearly constant isotope enrichment for breath-enamel for diverse mammalian taxa. The understanding of isotope enrichment factors from diet to breath and diet to enamel will have important applications in the field of animal physiology, and possibly also for wildlife ecology and paleontology.

**Keywords:** stable isotopes, diet, methane, carbon dioxide, physiology

## INTRODUCTION

The carbon-13 isotope enrichment from diet to tissue is key for using stable isotopes in diet studies, and the enrichment from diet to enamel for both modern and fossil diet studies. Lee-Thorp and Van der Merwe (1991) and Wang and Cerling (1994) showed that bioapatite as tooth enamel was far less susceptible to diagenesis than bioapatite as bone, and thus enamel can be used for paleodiet studies. A few studies have evaluated the enrichment from diet to bioapatite (either as bone or as tooth enamel) using modern mammals and the published range of values is from 9 to 16‰.

DeNiro and Epstein (1978) evaluated enrichments to a variety of tissues including bioapatite (bone) for a laboratory mouse (*Mus musculus*) on several formulated diets; they found an enrichment of about 10‰ from bulk diet to bone (bioapatite). Krueger and Sullivan (1984)

suggested that the isotope enrichment from diet to bioapatite in herbivores should be about 12‰; their reasoning was based on the oxidation of carbohydrates to energy during respiration ( $\text{CH}_2\text{O} = \text{CO}_2 + \text{H}_2\text{O}$  with complete isotope transfer from substrate ( $\text{CH}_2\text{O}$ ) to product ( $\text{CO}_2$ ) and the known equilibrium isotope fractionations in the  $\text{CO}_2\text{-H}_2\text{CO}_3^\circ\text{-HCO}_3^-$  system. Ambrose and Norr (1993), also working with rodents (*Rattus norvegicus*), found the diet to bioapatite enrichment to be 9.4‰ across a variety of carbohydrate—protein mixtures and concluded that bioapatite faithfully records the  $\delta^{13}\text{C}$  of bulk diet and is not skewed toward the carbohydrate component. Tieszen and Fagre (1993) also used laboratory mice (*Mus musculus*) on a formulated laboratory diet and found an isotope enrichment of 9.1‰ for the  $\delta^{13}\text{C}$  value of diet to bone; they also measured breath  $\text{CO}_2$  and found an isotope enrichment of -1.2‰ from diet to breath.

Lee-Thorp and Van der Merwe (1987) and Lee-Thorp et al. (1989) found that large African herbivores had an isotope enrichment of 12‰ for diet to bioapatite based on comparison of collagen to bioapatite and on assumed  $\delta^{13}\text{C}$  value for  $\text{C}_3$  and  $\text{C}_4$  vegetation. Cerling and Harris (1999) studied a variety of ecosystems in East Africa and concluded that ruminant herbivores had an isotope enrichment of 14‰ for diet to bioapatite (enamel) and suggested that large non-ruminant herbivores may have a somewhat smaller enrichment. Following on these studies, Passey et al. (2005b) conducted feeding trials of herbivores over a range of sizes and digestive physiologies in which they measured the  $\delta^{13}\text{C}$  of diet, breath  $\text{CO}_2$ , and tooth enamel; they concluded that the isotope enrichment between breath and enamel was constant at about 11.5‰ across different taxa, but that the diet-breath enrichment varied from -1 to +3‰. Passey et al. (2005b) suggested that methane production, such as in ruminants with the largest diet-breath enrichment, should be considered as a possible reason for different diet-breath enrichment values. Recently, Tejada-Lara et al. (2018) showed correlations between body size and isotope enrichment from diet to enamel, and Codron et al. (2018) showed that shifts in collagen-carbonate spacing in herbivores are consistent with higher  $\text{CH}_4$  emissions for ruminants compared to non-ruminants.

Taken together, there are very few explicit studies to determine carbon isotope enrichment measurements for breath-diet or enamel-diet. Those studies include several rodents, one lagomorph, two sloths, domestic cattle, and domestic pigs. The purpose of this paper is to further evaluate the isotope enrichment in mammals by considering digestive physiology as a possible factor in understanding the diet to enamel isotope enrichment, and especially to see whether methane production has a significant role in determining the diet to enamel isotope enrichment factors for different mammals.

In this study we evaluate herbivores of different digestive physiologies for diet to breath isotope enrichment as related to methane/carbon dioxide ( $\text{CH}_4/\text{CO}_2$ ) ratios in breath. We studied the following digestive physiologies: ruminant foregut fermenter, non-ruminant foregut fermenter, coprophagous hindgut fermenter, and non-coprophagous hindgut fermenter. We compare isotope enrichment values to  $\text{CH}_4/\text{CO}_2$  production

ratios and also compare the enrichment to body size for each of the digestive physiologies.

## MATERIALS AND METHODS

Breath and atmosphere samples were collected in 60 mL syringes with a valve to prevent atmosphere exchange after collection. Forty mL samples were injected into the in-line gas flushing a Picarro G2131-i High Concentration Isotopic Carbon Analyzer and analyzed for concentrations of  $\text{CO}_2$  and  $\text{CH}_4$ , and for the  $\delta^{13}\text{C}$  of  $\text{CO}_2$ .  $\delta^{13}\text{C}$  values were linear between 300 and 7,500 ppmV  $\text{CO}_2$ ; above 7,500 ppm  $\text{CO}_2$  the  $\delta^{13}\text{C}$  measurements were not linear and those  $\delta^{13}\text{C}$  values were not used.  $\text{CH}_4/\text{CO}_2$  ratios were linear to concentrations of  $\text{CH}_4 > 10,000$  ppmV and  $\text{CO}_2$  concentrations  $> 40,000$  ppmV, which was the range of our measurements in this experiment. Thus, some samples were suitable for  $\text{CH}_4/\text{CO}_2$  measurements but not for  $\delta^{13}\text{C}$  measurements. For calibration of the  $\delta^{13}\text{C}$  we used 3 internal laboratory gas standards whose composition was determined by mass spectrometry on a ThermoFisher MAT 253 IRMS calibrated against the reference materials RM8562, 8563, and 8564 distributed by the IAEA. A secondary reference material SIRM consisted of a single large  $\text{CO}_2$  tank which was analyzed through the study on the Picarro G2131-i High Concentration Isotopic Carbon Analyzer; within-run precision for SIRM was  $\pm 0.19\text{\%}$  and overall precision was  $\pm 0.33\text{\%}$ .

A single cow was sampled to determine variation in  $\delta^{13}\text{C}$  intercept and  $\text{CH}_4/\text{CO}_2$  ratios between individual breaths of an animal in close succession; characterization of seven distinct single breaths was evaluated using a sample suite from this cow. Seven breath sample suites were collected from the cow over a period of about 30 min. Each sample suite comprised a background syringe sample, and 5 syringe samples collected simultaneously from a feeding bowl into which the animal had exhaled; the 5 syringe intakes were approximately within 3 cm of each other but pointed in various directions in the bowl. After each sampling event, the bowl was removed from the vicinity of the animal, turned upside down and flushed with ambient air for about a minute to clear it from remains of the previous breath.

Mammal breath was sampled from 29 different taxon groups at the Zurich and Basel Zoos (we consider the same taxon sampled at different zoos as different taxon groups), including both domestic and non-domestic species (**Supplementary Data Set 2**). Due to permitting restrictions, only individual breaths were allowed to be sampled. When possible, we collected between 5 and 8 different breaths from each animal, and 3–8 different individuals per taxon per zoo. For each individual, the different samples were taken within approximately 15 min. All animals had access to food in the hour prior to breath sampling. Breath was collected within a few cm of the nostrils of each animal, judging for the opportune moment during exhalation, during routine feeding procedures. Samples were collected in 60 mL valved syringes. Mammals were considered according to four different digestive physiologies: ruminant foregut fermenter; non-ruminant foregut fermenter; coprophagous hindgut fermenter; non-coprophagous hindgut fermenter.

fermenter (Müller et al., 2013). Body masses representative for the species were taken from Nowak and Walker (1999) or from estimates of individual animals in each study group.

$\delta^{13}\text{C}$  intercepts using Keeling plots ( $\delta^{13}\text{C}$  vs.  $1/\text{CO}_2$ ) were determined for each animal using all samples in the linear portion of the  $\delta^{13}\text{C}$  vs.  $\text{CO}_2$  range (i.e.,  $<7,500$  ppmV  $\text{CO}_2$ ) because variations in methane concentrations did not affect the  $\delta^{13}\text{C}$  intercept.  $\text{CH}_4/\text{CO}_2$  ratios were determined for each breath sample by comparison to a background sample, where the  $\text{CO}_2$  concentration range (sample–background) was  $>200$  ppmV.

The animals were fed typical zoo diets that consisted of combinations of forages, such as hay, a pelleted compound feed, and different individual items such as fruits and vegetables, and are detailed in **Supplementary Material**. Because individual intake recording was beyond the logistical scope of this study, the ratios of the individual diet components of the overall diet were estimated based on information of the respective zoo. Samples of diet items being consumed on the day of sampling were collected for each animal group at each zoo; these food items for all taxa were the long-term diet of each respective taxon. In the scope of this study, it was not possible to determine the exact food intake of the individual animals or animal groups; therefore, diet composition was estimated based on information given by the zoo. Approximately 20 g of each diet item was homogenized and ground to fine powder. Samples were analyzed in duplicate on a ThermoFisher Flash-EA coupled to a ThermoFisher DeltaV mass spectrometer, and proportions of items were weighted to give the average  $\delta^{13}\text{C}$  of the total diet (**Supplementary Data Set 1**). The method was calibrated using the international standards NBS22 ( $\delta^{13}\text{C} = -30.03$ ) and IAEA CH-6 ( $\delta^{13}\text{C} = -10.46$ ).

Epsilon ( $\epsilon$ ) is the isotope enrichment between related phases, originally defined as under isotope equilibrium conditions; we use  $\epsilon^*$  in this paper to remind ourselves that the breath-diet isotope relationship is not an equilibrium reversible reaction. Thus, we calculate:

$$\epsilon^*_{\text{breath-diet}} = [(1,000 + \delta^{13}\text{CO}_2\text{-breath}) / (1,000 + \delta^{13}\text{C}_{\text{diet}}) - 1]^*1,000$$

for the isotope enrichment between diet and breath, and use an analogous expression for  $\epsilon^*_{\text{enamel-diet}}$ .  $\epsilon^*_{\text{CH}_4}$  is the estimated component of  $\epsilon^*_{\text{breath-diet}}$  assuming equilibrium fractionation between breath  $\text{CH}_4$  and  $\text{CO}_2$  for individual taxa studied using the fractionation observed for bovids (Klevenhusen et al., 2010); and  $\epsilon^*_{\text{other}}$  is the remaining isotope enrichment remaining after attribution of the  $\epsilon^*_{\text{CH}_4}$  component.

Statistical analysis was performed using JMP 15.0.0 (SAS Institute, 2019), and consisted of parametric correlations and linear regressions. If required, data were ln-transformed to meet requirements of normal distribution. The significance level was set to 0.05. For Keeling Plots, Pataki et al. (2003) showed that for  $r^2 > 0.95$  the intercepts using the standard linear regression method and geometric mean linear regression method were indistinguishable; therefore, we use the standard linear regression in this study.

## RESULTS

### Single Breath Samples— $\delta^{13}\text{C}$ Intercept and $\text{CH}_4/\text{CO}_2$ Ratios

A single cow was sampled to determine the variability of  $\delta^{13}\text{C}$  values and  $\text{CH}_4/\text{CO}_2$  ratios in individual breaths. **Supplementary Figure 1** shows that the  $\delta^{13}\text{C}$  of respired  $\text{CO}_2$  was very similar between breaths, with an average of  $-20.4 \pm 0.3\text{\%}$  determined by using a Keeling plot ( $\delta^{13}\text{C}$  vs.  $1/\text{CO}_2$ ) using standard linear regression (**Supplementary Table 1**). Correlation coefficients ( $r^2$ ) were all  $> 0.98$ .  $\text{CH}_4/\text{CO}_2$  ratios were measured in the same samples, and ranged over an order of magnitude: from 0.025 to 0.28 for the different breaths (**Supplementary Figure 2**), with an average value of  $0.12 \pm 0.9$ ; yet, correlation coefficients ( $r^2$ ) within each breath sample were all  $> 0.99$ . The average  $\delta^{13}\text{C}$  intercept for the 7 samples was  $-20.4 \pm 0.3\text{\%}$  and the average  $\text{CH}_4/\text{CO}_2$  ratio was  $0.129 \pm 0.92$ .

### Diets of Study Groups

The  $\delta^{13}\text{C}$  of diets for both the Zurich and Basel Zoo animals have a restricted range and were mainly principally, or wholly, comprised of  $\text{C}_3$  plants. Therefore, differential digestibilities of  $\text{C}_3$  and  $\text{C}_4$  plants, such as may be found in formulated diets, was largely eliminated. **Supplementary Data Set 1** gives the average diets for each animal group as the weighted  $\delta^{13}\text{C}$  value ( $\delta^{13}\text{C}_{\text{diet}}$ ) from the proportions as consumed on a long-term basis for each animal group.

### Comparison of Taxa for $\delta^{13}\text{C}$ of Breath and Diet, and Comparison to $\text{CH}_4/\text{CO}_2$ Ratios

$\delta^{13}\text{C}$  and  $\text{CH}_4/\text{CO}_2$  data for all taxon groups is given in **Supplementary Data Set 2** where methane is reported as  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  in percent. The different digestive physiologies each had a characteristic relationship for the breath-diet enrichment and methane production and therefore each is discussed separately. **Table 1** summarizes the relationships for each digestive physiology.

*Non-coprophagous hindgut fermenter* included several Perissodactyla (four equids, rhinoceros, tapir), Proboscidea (elephant), Cetartiodactyla (pig), and Diprotodontia (koala) and data are presented in **Supplementary Data Set 2**. Percent methane as  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  in breath samples ranged from 0.5 to 1.4% with an average value of about 0.9%. Isotope enrichment  $\epsilon^*_{\text{breath-diet}}$  was  $3.5 \pm 0.8\text{\%}$ .  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  values had variation between individual breaths (**Supplementary Data Set 2**), but the absolute range was smaller than ruminants.

*Coprophagous hindgut fermenter* included several Rodentia (four rodents) and Lagomorpha (rabbit) and data are presented in **Supplementary Data Set 2**. Percent methane as  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  in breath samples ranged from 0.0 to 0.5% with an average value of about 0.2%. Isotope enrichment  $\epsilon^*_{\text{breath-diet}}$  was  $0.8 \pm 0.9\text{\%}$  but ranged from  $-0.1$  to  $2.2\text{\%}$ ; there was a significant correlation of  $\epsilon^*_{\text{breath-diet}}$  with percent methane (**Figure 1** and **Supplementary Table 2**), but not with ln(body mass) (**Supplementary Figure 3** and **Supplementary Table 2**).

**TABLE 1** | Percent methane as  $(\text{CO}_2 + \text{CH}_4)$  and  $\varepsilon^*_{\text{breath-diet}}$ ,  $\varepsilon^*_{\text{CH}_4}$ ,  $\varepsilon^*_{\text{other}}$  for this study for different digestive physiologies.

Digestive physiology	n (taxa)	$\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$	$\varepsilon^*_{\text{breath-diet}}$	$\varepsilon^*_{\text{CH}_4}$	$\varepsilon^*_{\text{other}}$
		$\pm 1\sigma$ %	$\varepsilon^* \pm 1\sigma$	$\varepsilon^* \pm 1\sigma$	$\varepsilon^* \pm 1\sigma$
Ruminant foregut fermenter	11	$5.43 \pm 2.30$	$4.1 \pm 1.1$	$2.6 \pm 1.1$	$1.5 \pm 1.1$
Non-ruminant foregut fermenter	4	$1.10 \pm 0.92$	$2.3 \pm 0.4$	$0.5 \pm 0.4$	$1.8 \pm 0.5$
Coprophagous hindgut fermenter	5	$0.20 \pm 0.22$	$0.8 \pm 0.9$	$0.1 \pm 0.1$	$0.8 \pm 0.8$
Non-coprophagous hindgut fermenter	9	$0.86 \pm 0.30$	$3.5 \pm 0.8$	$0.4 \pm 0.1$	$3.1 \pm 0.7$

*Ruminant foregut fermenters: giraffe, okapi, reindeer, bison, yak, zebu, nilgai, goat, Bactrian camel, llama, alpaca.*

*Non-ruminant foregut fermenter: common hippo, pygmy hippo, kangaroo, and sloth.*

*Coprophagous hindgut fermenter: rabbit, mole rat, guinea pig, nutria, and porcupine.*

*Non-coprophagous hindgut fermenter: koala, wild ass, donkey, pony Zurich, pony Basel, rhinoceros, tapir, and elephant.*

For details see **Supplementary Materials**.

With the small sample size ( $n = 5$ ) we do not speculate on the relationship between  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  and  $\varepsilon^*_{\text{breath-diet}}$ .  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  values had some variation between individual breaths (**Supplementary Data Set 2**), but as with other hindgut fermenters, the absolute range was small compared to ruminants.

*Non-ruminant* foregut fermenters included Cetartiodactyla (2 hippos), Diprotodontia (kangaroo), and Xenarthra (sloth) and data are presented in **Supplementary Data Set 2**. Percent methane as  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  in breath samples ranged from 0.4 to 2.4% with an average value of about 1.1%. Isotope enrichment  $\varepsilon^*_{\text{breath-diet}}$  was  $2.3 \pm 0.4\text{‰}$ .  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  values had some variation between individual breaths (**Supplementary Data Set 2**), but the absolute range was small compared to ruminants.

*Ruminant foregut fermenters* were all Cetartiodactyla (5 Bovidae, 3 Camelidae, 1 Cervidae, and 2 Giraffidae) and data are presented in **Supplementary Data Set 2**. Percent methane as  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  in breath samples ranged from 1.9 to 9.8% with an average value of about 5.4%. Isotope enrichment  $\varepsilon^*_{\text{breath-diet}}$  was  $4.1 \pm 1.1\text{‰}$  (**Figure 1** and **Table 1**).  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  values were quite variable between individual breaths so that the reported values are only an approximation of the true integrated  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  percent values. Of the digestive groups studied, ruminants had the highest variation in  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  ratios between individual breath samples (e.g., see **Supplementary Figure 2**).

## $\varepsilon^*_{\text{CO}_2-\text{CH}_4}$ Contribution to the Total

### $\varepsilon^*_{\text{breath-diet}}$

Using the isotope enrichment  $\varepsilon^*_{\text{CO}_2-\text{CH}_4}$  of  $50.1\text{‰}$  for cattle as determined by Klevenhusen et al. (2010), it is possible to use the  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  values to estimate the contribution of  $\varepsilon^*_{\text{CH}_4}$  component to the total  $\varepsilon^*_{\text{breath-diet}}$ , leaving the residual  $\varepsilon^*_{\text{other}}$  component which represents the differential isotope digestibility of the diet. Previous studies (e.g., Sponheimer et al., 2003) have shown that mammalian feces are  $^{13}\text{C}$  depleted compared to bulk diet by 0 to  $1.5\text{‰}$ , suggesting the differential digestion occurs in many taxa with the digested component being enriched in  $^{13}\text{C}$  compared to diet. **Figure 1** shows that  $\varepsilon^*_{\text{breath-diet}}$  values were greater than, or the same as, the  $\varepsilon^*_{\text{CH}_4}$  value based on the enrichment of  $^{13}\text{C}$  in  $\text{CO}_2$  due to  $\text{CH}_4$  production. A few coprophagous hindgut fermenters had  $\varepsilon^*_{\text{breath-diet}}$  values near  $0\text{‰}$  or  $< 0\text{‰}$  (see **Supplementary Data Set 3**).

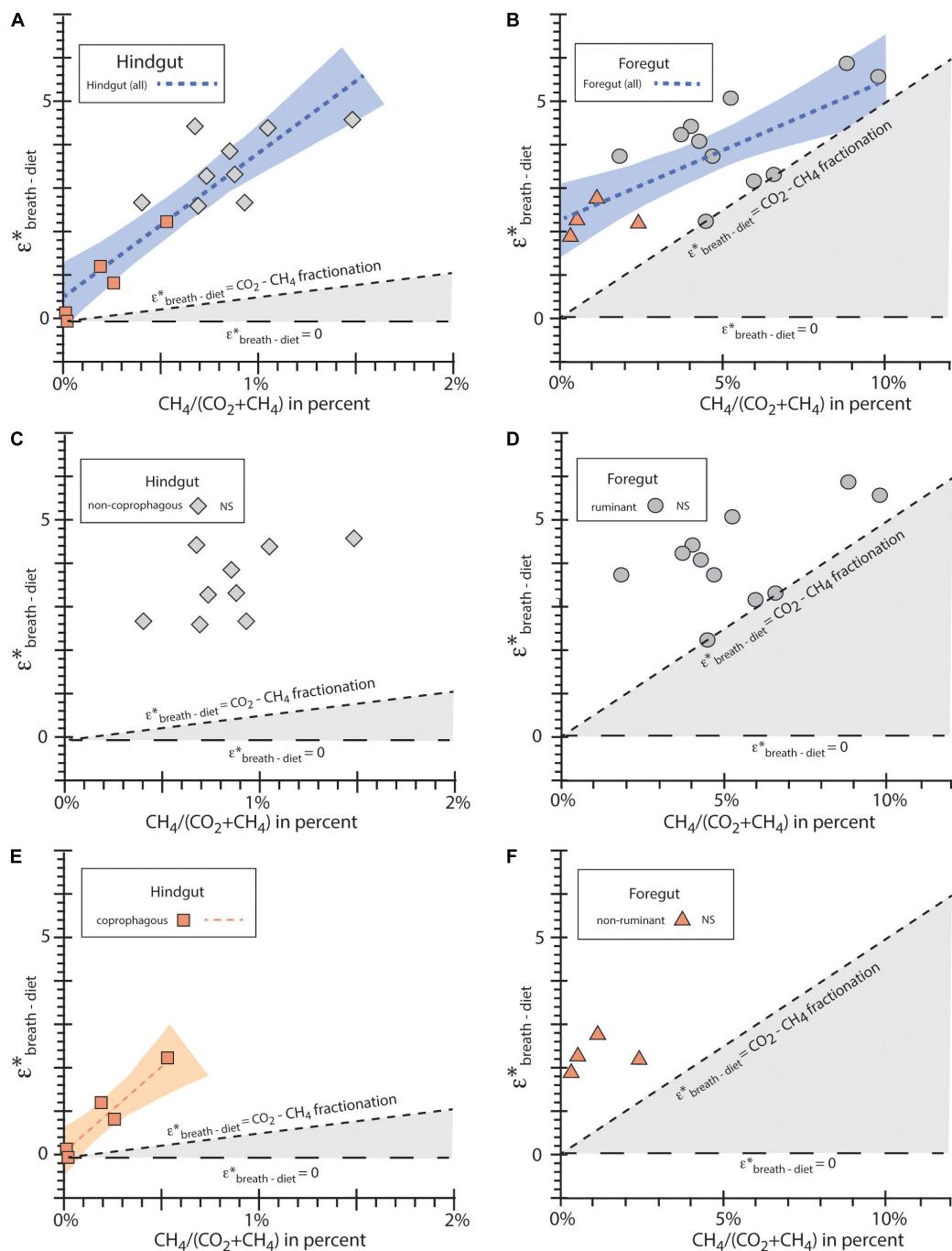
## DISCUSSION

The results of this study show that methane production is an important factor for all digestive strategies in considering isotope enrichment of breath compared to diet, but in particular for ruminants;  $\text{CH}_4-\text{CO}_2$  fractionation in isotope enrichment in  $\text{CO}_2$  from methane production can have a significant effect on the overall isotope enrichment of breath. Using the methane-related isotope enrichment of  $\text{CO}_2$ , we can evaluate differential digestion as an isotope enrichment factor. We compare our isotope enrichment results to previous controlled diet studies which, to date, are few (e.g., DeNiro and Epstein, 1978; Ambrose and Norr, 1993; Tieszen and Fagre, 1993; Passey et al., 2005b; Podlesak et al., 2008); several of those studies showed a small range of isotope  $\varepsilon^*_{\text{enamel}-\text{CO}_2}$ , and thus we discuss isotope enrichment  $\varepsilon^*_{\text{enamel-diet}}$ , which has important applications in paleoecology. We compare controlled feeding studies with previously published data for natural ecosystems to estimate enamel-diet enrichment in natural ecosystems. This comparison provides a basis for recommendations for using tooth enamel to estimate diet isotope values in natural ecosystems, both modern and fossil. In the final section we provide some recommendations for additional studies related diet, breath, and tooth enamel.

## Repeated Breath Samples of an Individual Cow

The experiment with a single cow showed up constraints when sampling breath for our analyses. Although the 5 samples from a single breath were collected within approximately 3 cm of each other, the  $\text{CO}_2$  concentration from those single breaths could vary by  $> 4\text{X}$  (**Supplementary Figure 1**), which underlines that even when sampling the same breath, uncontrolled variation in the positioning of the sampling equipment can lead to differences in dilution with ambient air. Nevertheless, within the samples of a single breath, the ratio of  $\text{CH}_4/\text{CO}_2$  remained constant. This approach gave a robust value for the  $\delta^{13}\text{C}$  of respired  $\text{CO}_2$ . However, different breaths varied distinctively in the  $\text{CH}_4/\text{CO}_2$  ratio.

It is known that  $\text{CH}_4$  emissions can show strong variation across the day, which is, in respiration chamber experiments in cattle, particularly linked to feeding activity (Crompton et al., 2011). Our results from the single cow experiment suggest that in addition to these intra-diurnal fluctuations,



**FIGURE 1 |**  $\epsilon^*$  breath-diet vs. percent  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  for taxa studied for **(A)** all hindgut fermenters; **(B)** all foregut fermenters; **(C)** non-coprophagous hindgut fermenters; **(D)** ruminant foregut fermenters; **(E)** coprophagous hindgut fermenters; **(F)** non-ruminant foregut fermenters. The blue dotted lines in **(A,B)** show the best fit lines for all hindgut taxa and foregut taxa, respectively. The orange dotted line in **(E)** shows the best fit lines for coprophagous hindgut digestive physiology. No significant correlations (NS) were observed for ruminant foregut, non-ruminant foregut, and non-coprophagous hindgut fermenters. Blue and orange shaded areas represent the confidence intervals for the best fit lines as computed by JMP (SAS Institute, 2019). Horizontal dashed line shows  $\epsilon^*$  breath-diet = 0 for reference; sloped dashed line shows  $\epsilon^*$  breath-diet fractionation due to  $\text{CO}_2\text{-CH}_4$  isotope equilibrium; the gray shaded area represents the region with no differential digestion ( $\epsilon^*$  breath-diet = 0) and where the total isotope enrichment  $\epsilon^*$  breath-diet is smaller than  $\epsilon^*$   $\text{CH}_4$  due to  $\text{CH}_4$ .

individual breaths can also vary in the amount of  $\text{CH}_4$  exhaled. Possibly, this is linked to a rhythm of rumen gas eructation that is of a lesser frequency than the animal's regular

breathing frequency. Whether similar between-breath variation should be expected for other foregut fermenters in addition to demonstrated diurnal irregularities (Vendl et al., 2015), and to

what degree  $\text{CH}_4$  produced in the hindgut will be excreted by flatulence vs. absorption in the blood and exhalation, remains to be investigated. Although it is well established that  $\text{CH}_4$  produced in the hindgut is also exhaled in breath (McKay et al., 1985; Sasaki et al., 1999), the contribution of flatulence to overall  $\text{CH}_4$  remains unexplored in non-ruminants, and even in respiration chambers.

Therefore, an average  $\text{CH}_4/\text{CO}_2$  ratio representative for the individual or species should be integrated over a longer period than the 15 min of our sampling intervals, and single-breath data should not be used as a surrogate for respiration chamber measurements. Future studies should better integrate long-term  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  concentrations than we were able to do in this preliminary survey. Although our preference would have been to use respiration chambers for this study, this was not possible given our IACUC (Institutional Animal Care and Use Committee) permissions. Yet, the long exchange time for isotope equilibrium between  $\text{CO}_2$  and  $\text{CH}_4$  is also evident in the single cow experiment:  $\delta^{13}\text{C}$  values are essentially constant for all samples in spite of a 10-fold change in the  $\text{CH}_4/\text{CO}_2$  ratio of the samples we collected.

A different source for variation of  $\delta^{13}\text{C}$  values, which was not addressed in the present study, could be related to diurnal differences in the use of carbohydrates, fats or protein for energy production (McCue and Pollock, 2013; Whigham et al., 2014). This will depend critically on the time lag between feeding and measuring breath samples, and on the individual's activity during that period (e.g., if it was highly active or at rest; Gautier et al., 1996; McCue et al., 2015). Although we collected breath during a short 15-min interval per animal, and always after the animals had had access to food, differences between individuals with respect to food intake and activity were beyond the control logically feasible in our study. Yet, they might affect the variability of our  $\delta^{13}\text{C}$  readings between individuals, and hence groups. Additional controlled breath collection experiments with defined time periods since feeding represent an important future step.

## CH<sub>4</sub>/CO<sub>2</sub> Production Ratios

Clauss et al. (2020) recently reviewed the literature for methane emissions from a wide variety of mammalian herbivore taxa. They found methane production in herbivores to be ubiquitous, and that no general rules related to digestive strategy explained the observations made in live animals in respiration experiments. When evaluating measured  $\text{CH}_4:\text{CO}_2$  ratios, they found patterns similar to that in the present study, with an overall increase of the ratio with body mass due to the inclusion of (small) coprophagous hindgut fermenters (rodents) in the dataset. In their dataset, being a ruminant was the main predictor or having a high  $\text{CH}_4:\text{CO}_2$  ratio, similar to our own findings where ruminants had generally higher values than other groups. Below we discuss relationships between  $\epsilon^{\text{breath-diet}}$ ,  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$ , and body mass.

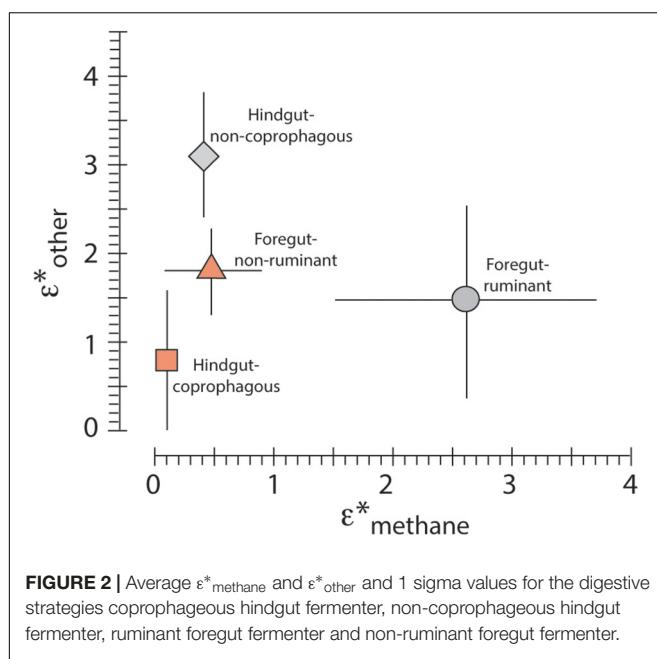
Ruminant foregut fermenters have the highest  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  production ratios, and range from 1.9 to 9.8%, with an average value of  $5.4 \pm 2.3\%$  (Table 1 and

Supplementary Data Set 2). Our data set is too small to speculate on the differences between Bovidae, Cervidae, Giraffidae and Camelidae. Respiration measurements in live animals do not suggest such a difference beyond a systematic difference due to the generally lower food intake in camelids as compared to ruminants, which, however, does not affect the  $\text{CH}_4:\text{CO}_2$  ratio (Clauss et al., 2020).

Non-ruminant foregut fermenters are represented by a limited number of taxa in this study (4) and range in the  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  production ratios from 0.4 to 2.4%. The position of the sloth with a ratio in the range of ruminants is in accord with a previous measurement (Vendl et al., 2016b), as is the low ratio in kangaroos and the higher value in pygmy hippos (Clauss et al., 2020). The most surprising result of the present study were the low values of the common hippos, which differed distinctively from those of the pygmy hippos in spite of the generally similar digestive anatomy and physiology of these two species (Clauss et al., 2004). The reason for the difference between these two species remains to be investigated, but the common hippo values match the observation by Codron et al. (2018) that collagen-carbonate  $^{13}\text{C}$  fractionation of free-ranging common hippos did not resemble that of ruminants, but of other non-ruminant grazers, which also suggests low  $\text{CH}_4$  production. The great variation in the foregut non-ruminants warns against using this descriptor of digestive strategy as a proxy for  $\text{CH}_4$  emissions.

The coprophagous hindgut fermenters had the lowest methane yield [as  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$ ] and two taxa [*Oryctolagus cuniculus* (domestic rabbit) and *Fukomys anselli* (mole rat)] had  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  production ratios indistinguishable from 0 (Supplementary Data Set 2). This confirms previously reported low  $\text{CH}_4$  emission values for the rabbit (Clauss et al., 2020). Other members of this group also have low  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  production ratios (range 0.2–0.5%, Supplementary Data Set 2), and the average  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  production ratio for the 5 taxa studied is  $0.2 \pm 0.2\%$ . The main discrepancy to previous data is the lower ratios in nutrias and porcupines in this study; for these taxa methane emissions in the range of ruminants had been previously reported, albeit on diets of higher fiber content (Clauss et al., 2020). Again, verification of these species-specific values by further respiration chamber work is desirable.

The non-coprophagous hindgut fermenters have  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  production ratios from 0.4 to 1.5% with an average value of  $0.9 \pm 0.3\%$  (Table 1 and Supplementary Data 2), with the highest and lowest groups studied being equids. The generally low ratio of the group corresponds to previous observations in equids (Clauss et al., 2020). Among the species not investigated for their  $\text{CH}_4$  emissions so far, results for the other perissodactyls tapirs and rhinos were similar to those of equids, and to elephants, which matches the sparse observations in the latter. The koala corresponded to the other non-coprophagous hindgut fermenters. The wild pig species also resembled the other non-coprophagous hindgut fermenters; it has previously been suggested that the lower  $\text{CH}_4$  emissions measured in domestic pigs as compared to equids or guinea pigs were caused by the low-fiber diets on which these production animals are typically kept (Vendl et al., 2016a).



**FIGURE 2 |** Average  $\epsilon^*$ methane and  $\epsilon^*$ other and 1 sigma values for the digestive strategies coprophagous hindgut fermenter, non-coprophagous hindgut fermenter, ruminant foregut fermenter and non-ruminant foregut fermenter.

### $\epsilon^*$ CH<sub>4</sub> Related to Digestive Strategies

We calculated the enrichment for each taxon that is due to methane production ( $\epsilon^*$ CH<sub>4</sub>) and find that the isotope enrichment in breath of foregut ruminants due to methane production is, on average, 2.6‰ (Table 1 and Figure 2). The other three digestive strategies have isotope enrichments  $\epsilon^*$ CH<sub>4</sub> < 0.5‰.

Passey et al. (2005b) suggested that methane production could be an important factor to consider in evaluating the isotope enrichment values  $\epsilon^*$ breath-diet and  $\epsilon^*$ enamel-diet. Figure 1 shows that almost all taxa have a component of isotope enrichment in addition to  $\epsilon^*$ CH<sub>4</sub>; this is  $\epsilon^*$ other in the terminology of this paper (Figure 1, Table 1 and Supplementary Data Set 2) and is discussed in the section below.

### $\epsilon^*$ other: Differential Digestion

Individual plant parts (seeds, leaves, roots) within single plants have a small range of isotope ratios whereas the different diet components (fats, proteins, carbohydrates) within individual plants have isotope ratios with a larger range as compared to the bulk plant value (Park and Epstein, 1961; Cernusak et al., 2009). It is expected that differential digestion of plant components could result in  $\delta^{13}\text{C}$  values of respiration CO<sub>2</sub> to have a value different than the bulk plant: thus, differential digestion can have a significant isotope effect in diet studies. Studies of herbivore feces on controlled diets shows that the feces are usually depleted in <sup>13</sup>C compared to the bulk diet (e.g., Sponheimer et al., 2003; Codron et al., 2005) by up to 2‰; the implication of the observation is that the digested component is enriched in <sup>13</sup>C compared to the bulk diet.

All digestive strategies have, on average, a non-zero  $\epsilon^*$ other isotope enrichment. The hindgut coprophagous has the smallest value with an average  $\epsilon^*$ other value of 0.8‰; however, within this

group two taxa (rabbit: *Oryctolagus cuniculus*, and the mole rat: *Fukomys anselli*) have near 0 values for both methane production and  $\epsilon^*$ other. One possible reason for difference in this effect might result from chewing efficiency: animals that comminute their diet more homogenously into very fine particles, such as ruminants or small hindgut fermenters (Fritz et al., 2009) might show less differential digestion than animals where the digesta remains more heterogeneous due to less efficient chewing. However, in order to address this question systematically, measurements would have to be repeated with animals on a consistent diet and with a parallel recording of food intake and digestibility.

### $\epsilon^*$ breath-diet Related to Digestive Strategies

Previously, only a few studies have measured the  $\delta^{13}\text{C}$  of diet and the  $\delta^{13}\text{C}$  of breath for isotope fractionation studies to determine  $\epsilon^*$ breath-diet (e.g., Metges et al., 1990; Tieszen and Fagre, 1993; Passey et al., 2005b; Podlesak et al., 2008; Klevenhusen et al., 2010). A few other studies have data that give additional  $\epsilon^*$ breath-diet values but were not explicitly determined in the original paper (Ayliffe et al., 2004; Sponheimer et al., 2006) and are included in this analysis (Dataset 3). Thus, our study expands the number of taxa with  $\epsilon^*$ breath-diet measurements, even though measurements on individual diet item intake and an assessment of digestibility was beyond the scope of the approach.

We find that the overall  $\epsilon^*$ breath-diet is related to digestive strategy: the coprophagous hindgut fermenters have the smallest average  $\epsilon^*$ breath-diet value ( $0.8 \pm 0.9\text{‰}$ ); followed by the non-ruminant foregut fermenters ( $2.3 \pm 0.4\text{‰}$ ); followed by non-coprophagous hindgut fermenters ( $3.5 \pm 0.8\text{‰}$ ); with the ruminant foregut fermenters having the highest  $\epsilon^*$ breath-diet value ( $4.1 \pm 1.1\text{‰}$ ). The  $\epsilon^*$ breath-diet values of this study can be used to greatly expand the understanding of  $\epsilon^*$ enamel-diet values which are widely used in modern- and paleo-ecology studies. This will be discussed below.

### $\epsilon^*$ breath-diet vs. CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>)

We evaluated the measured  $\epsilon^*$ breath-diet vs. CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>) for the taxa studied. Neither of the foregut fermenter groups had a significant correlation in itself, but the all the foregut taxa together had a significant relationship for  $\epsilon^*$ breath-diet vs. CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>) (Figure 1 and Supplementary Table 2), corroborating a general  $\epsilon^*$ breath-diet vs. CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>) relationship for foregut taxa.

The coprophagous hindgut fermenters had the lowest CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>) values, but had the highest correlation coefficients for  $\epsilon^*$ breath-diet vs. CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>) with a significant relationship, although the number of total taxa considered is small ( $n = 5$ ). The non-coprophagous hindgut fermenters did not have a significant relationship. Taken together, all hindgut fermenters had the highest significant relationship (Figure 1 and Supplementary Table 2), again corroborating that CH<sub>4</sub> production is a major factor in determining  $\epsilon^*$ breath-diet values.

Including all taxa in this study also gives a significant correlations between  $\epsilon^*$ breath-diet and CH<sub>4</sub>/(CO<sub>2</sub> + CH<sub>4</sub>) but

with a lower  $r^2$  than then either hindgut fermenters or foregut fermenters considered separately (Supplementary Table 2).

### $\varepsilon^*$ breath-diet vs. Body Mass (BM)

Tejada-Lara et al. (2018) found a correlation between  $\varepsilon^*$  enamel-diet and  $\ln(\text{BM})$  for both hindgut and foregut digestive strategies. In the present study, we have further sub-divided these categories to coprophagous and non-coprophagous hindgut, and ruminant and non-ruminant foregut fermenters. Only hindgut coprophagous fermenters, of the individual digestive strategies, had a significant correlation for  $\varepsilon^*$  breath-diet vs.  $\ln(\text{BM})$ . Including all the hindgut fermenters together also yielded a significant relationship for  $\varepsilon^*$  breath-diet vs.  $\ln(\text{BM})$  (Supplementary Figure 4 and Supplementary Table 4).

### $\varepsilon^*$ enamel-breath: Results From Controlled and Restricted Feeding Studies

There are very few direct measurements for the isotope enrichment between breath and enamel ( $\varepsilon^*$  breath-enamel). Such studies require a long-term constant diet over the length of tooth enamel formation including the period of tooth maturation, measurements of breath, and measurements of tooth enamel after tooth extraction or after death of the animal; the measurements of diet, breath, and enamel must all be related temporally to each other. Tooth enamel maturation varies from weeks for incisors of small mammals (e.g., 18 days for lagomorphs (Passey et al., 2005a) to several years for the teeth of some large mammals; e.g., three or more years for hippo canines (maturation length and from Passey and Cerling, 2002 and growth rates from Passey et al., 2005a) and elephant molars (growth rate from Uno et al., 2013, and maturation length from Uno et al., 2020). Although bioapatite, such as bone, has a similar (and likely identical) carbon isotope value as tooth enamel if formed at the same time in the same individual, it is more readily altered and, relevant to this discussion, especially by methods used to “pre-treat” samples for isotope analysis. The typical methods include treatment with either sodium hypochlorite or hydrogen peroxide followed by treated with acetic acid (either 0.1 M or 1.0 M; either buffered or non-buffered). The treatment of bone by these methods results to changes the measured  $\delta^{13}\text{C}$  values of bone (Koch et al., 1997), but not enamel (Koch et al., 1997; Passey et al., 2002). Therefore, we do not consider in this analysis the data of Tieszen and Fagre (1993) for the evaluation of  $\varepsilon^*$  breath-enamel. Likewise, Passey et al. (2005b) noted that two individuals in their study had inadvertently been fed the wrong diet for a short time during the period of tooth enamel maturation, and therefore suggested that those individuals should not be used in the analysis for  $\varepsilon^*$  breath-enamel.

Thus, the number of taxa for evaluation of  $\varepsilon^*$  breath-enamel is limited to the studies of Passey et al. (2005b) and Podlesak et al. (2008; see Supplementary Data Set 5). These taxa represent a range of body size and digestive physiologies: domestic cattle, domestic pig, domestic rabbit, vole, and wood rat. Despite the differences in body size and physiology, the five-taxa average for the combined Passey et al. (2005b) and Podlesak et al. (2008) studies is  $\varepsilon^*$  breath-enamel =  $11.6 \pm 0.2\text{‰}$  with a range

from 11.2 to 11.8‰; the total of the 15 individual values has  $\varepsilon^*$  breath-enamel =  $11.7 \pm 0.4\text{‰}$  with a range for individuals ( $n = 15$ ) from 11.2 to 12.7‰. Presumably, breath is in isotope equilibrium with dissolved bicarbonate, and if enamel forms in isotope equilibrium with blood bicarbonate at the similar body temperatures for most mammals (ca. 37°C), then a near constant value for  $\varepsilon^*$  breath-enamel for most mammal species is expected. The observations of Yang et al. (2020) concerning ever-growing and non-ever growing teeth, presumably formed at the same time in the same individual, having different  $\delta^{13}\text{C}$  values suggests additional work needs to be done on understanding isotope signals in teeth of different growth and maturation patterns. The value for  $\varepsilon^*$  breath-enamel =  $11.6 \pm 0.2\text{‰}$  is very similar to the 12‰ value proposed by Krueger and Sullivan (1984) based on isotope considerations in the  $\text{H}_2\text{O}-\text{CO}_2$  system and assuming complete conversion of diet (as carbohydrates) to  $\text{CO}_2$ . Note that our present study does not add to this data base, but the above discussion is context for the application of determining additional  $\varepsilon^*$  enamel-diet values relevant to ecologic and paleo-ecologic studies.

### $\varepsilon^*$ enamel-diet: Direct Results From Controlled and Restricted Feeding Studies

There are few direct measurements of  $\varepsilon^*$  enamel-diet whereby both a prolonged controlled diet and tooth enamel were studied; the above referenced studies of Passey et al. (2005b) and Podlesak et al. (2008) have such data for  $\varepsilon^*$  enamel-diet. The earlier estimates of Lee-Thorp and Van der Merwe (1987) and Cerling and Harris (1999) had only estimates of the  $\delta^{13}\text{C}$  value of diet, so they did not make direct measurements on the taxa considered in those studies. DeNiro and Epstein (1978); Tieszen and Fagre (1993), and Ambrose and Norr (1993), determined  $\varepsilon^*$  bioapatite-diet values for *Mus musculus*, *Mus musculus*, and *Rattus norvegicus*, respectively, all analyzing bone bioapatite; and Tejada-Lara et al. (2018) determined  $\varepsilon^*$  bioapatite-diet values for *Bradypus variegatus* and *Choloepus hoffmanni* using dentine bioapatite. Taken together, there are few taxa that have direct measurements for  $\varepsilon^*$  enamel-diet or  $\varepsilon^*$  bioapatite-diet with prolonged diet control and measurement of tissues. Explicit  $\varepsilon^*$  enamel-diet determinations include domestic cattle, domestic pig, rabbit, vole, wood rat; explicit  $\varepsilon^*$  bone-diet determinations include laboratory mice and laboratory rats; explicit  $\varepsilon^*$  dentine-diet measurements exist for two species of sloths.

### $\varepsilon^*$ enamel-diet: Inferred Results From Controlled and Restricted Feeding Studies

Zoo and lab studies with  $\delta^{13}\text{C}$ -measured diets can provide additional data for  $\varepsilon^*$  enamel-diet. Passey et al. (2005b) and Podlesak et al. (2008) found a near constant isotope enrichment factor  $\varepsilon^*$  enamel-breath of  $11.6 \pm 0.2\text{‰}$  for 5 different taxa (see discussion above).

Using that enrichment and the  $\varepsilon^*$  breath-diet we calculate the enrichment  $\varepsilon^*$  enamel-diet for our study animals in Supplementary Data Set 2; results are tabulated in Supplementary Data Set 3.

This analysis does not provide any additional information for  $\text{CH}_4/\text{CO}_2$  vs.  $\varepsilon^*_{\text{breath-diet}}$  than described previously, but would conclude that the isotope enrichments  $\varepsilon^*_{\text{enamel-diet}}$  is related to  $\text{CH}_4$  production: the coprophagous hindgut fermenters have the smallest average  $\varepsilon^*_{\text{enamel-diet}}$  value ( $12.4 \pm 0.9\%$ ;  $n = 5$ ); followed by the non-ruminant foregut fermenters ( $13.9 \pm 0.5\%$ ;  $n = 4$ ) and the non-coprophagous hindgut fermenters ( $14.1 \pm 0.8\%$ ;  $n = 9$ ), with the ruminant foregut fermenters having the highest  $\varepsilon^*_{\text{enamel-diet}}$  value ( $15.7 \pm 1.1\%$ ;  $n = 11$ ).

We can also include previous studies of controlled diets or zoo-diet studies (**Supplementary Data Set 3** and references therein). This analysis gives similar results with coprophagous hindgut fermenters having the smallest average  $\varepsilon^*_{\text{enamel-diet}}$  value ( $11.5 \pm 1.5\%$ ;  $n = 11$ ), followed by the non-ruminant foregut fermenters ( $13.3 \pm 1.4\%$ ;  $n = 7$ ) and the non-coprophagous hindgut fermenters ( $14.8 \pm 1.0\%$ ;  $n = 13$ ), with ruminant foregut fermenters having the highest  $\varepsilon^*_{\text{enamel-diet}}$  value ( $15.2 \pm 1.3\%$ ;  $n = 19$ ) (**Supplementary Table 1**).

Notably, the only mammals with an  $\varepsilon^*_{\text{enamel-diet}}$  value of  $11\%$  or less, and therefore an apparent negative enrichment factor for  $\varepsilon^*_{\text{breath-diet}}$ , were murids or cricetids of the Rodentia Family and one species of sloth: *Mus musculus* (DeNiro and Epstein, 1978; Tieszen and Fagre, 1993), *Rattus norvegicus* (Ambrose and Norr, 1993), *Neotoma cinerea* (Podlesak et al., 2008), and *Bradypus variegatus* (Tejada-Lara et al., 2018). The study of Passey et al. (2005b), using *Mus ochrogaster*, found  $\varepsilon^*_{\text{breath-diet}}$  of  $0.3 \pm 0.8\%$ , indistinguishable from 0. We note that all of murids and cricetid studies used commercial formulated (mixed) diets. Additional studies are needed to resolve whether murids and cricetids have a negative enrichment factor for  $\varepsilon^*_{\text{breath-diet}}$ , different than other mammals, or if this is related to using commercial formulated diets.

Tejada-Lara et al. (2018) previously found correlations within the hindgut and foregut digestive physiologies; we also find a significant correlation between  $\ln(\text{BM})$  and  $\varepsilon^*_{\text{enamel-diet}}$  for hindgut and foregut physiologies (**Supplementary Figures 5A,B** and **Supplementary Table 5**). However, when we look at the four digestive physiologies individually, we find that only the coprophagous hindgut fermenters have a significant relationship ( $p = 0.005$ ; **Supplementary Figure 5E** and **Supplementary Table 5**). Thus, the body mass to  $\varepsilon^*_{\text{enamel-diet}}$  (Tejada-Lara et al., 2018) seems to be related to grouping different physiologies with different body masses and different  $\varepsilon^*_{\text{enamel-diet}}$  characteristic to those physiologies including  $\text{CH}_4$  production.

## Comparison of Phylogenetically Related Species

This study provides the opportunity to compare the same, or related, species for methane production and for isotope enrichment values. In all cases, diets have been homogeneous and well characterized for stable isotope but not for fiber or for other factors that might affect differential digestion or methane production, and therefore, net isotope enrichment between diet and breath, or diet and enamel. Taking all studies together, Equidae have a range in  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  ratios from 0.4 to 1.5% (average  $0.9 \pm 0.4\%$ ,  $n = 4$ ); have  $\varepsilon^*_{\text{breath-diet}}$  values

from 2.1 to 4.6% (average  $3.4 \pm 1.0\%$ ,  $n = 5$ ); and  $\varepsilon^*_{\text{enamel-diet}}$  values 13.6 to 16.2 (average have a range in  $\varepsilon^*_{\text{enamel-diet}}$  from  $14.9 \pm 1.0\%$ ,  $n = 6$ ). The family Bovidae provide a similar comparison: bovid taxa have a range in  $\text{CH}_4/(\text{CO}_2 + \text{CH}_4)$  ratios from 4.0 to 9.8% (average  $6.2 \pm 2.2\%$ ,  $n = 6$ ); have  $\varepsilon^*_{\text{breath-diet}}$  values from 2.9 to 5.4 (average  $4.2 \pm 0.9\%$ ,  $n = 8$ ); and have a range in  $\varepsilon^*_{\text{enamel-diet}}$  from 14.6 to 17.21 (average  $15.8 \pm 0.8\%$ ,  $n = 9$ ). Thus, phylogenetically related taxa have broad similarities in methane production,  $\varepsilon^*_{\text{breath-diet}}$ , and  $\varepsilon^*_{\text{enamel-diet}}$  values, but additional studies are needed to determine further sub-divisions within any particular digestive physiology.

## $\varepsilon^*_{\text{enamel-diet}}$ : Comparison of Controlled/Restricted Diet Studies to Field Observations

The recent large dataset of Cerling et al. (2015) provides the opportunity to compare the results from this analysis to field observations of diet enrichment factors; that study uses all of the data from Africa of the specimens of Cerling and Harris (1999). All tooth enamel and plant samples were corrected to the same baseline atmospheric value for the pre-Industrial atmosphere ( $\delta^{13}\text{C}_{1750}$  following Long et al. (2005) and Cerling et al. (2015). We consider taxa from Africa with  $n = 1 > 5$  from an individual locality having a standard deviation  $< 1.0\%$  for that locality, implying a constant diet for that population of animals in that taxa (**Supplementary Data Set 4**). The rationale is that the small standard deviation of  $< 1.0\%$  for enamel values for a taxon in a particular locality implies a consistent diet for all specimens, and therefore that taxon likely has either a pure- $\text{C}_3$  or a pure- $\text{C}_4$  diet with little admixture of the minor component, making it suitable for determination of  $\varepsilon^*_{\text{enamel-diet}}$ .  $\delta^{13}\text{C}_{1750}$  values for vegetation was estimated using published  $\delta^{13}\text{C}$  values for plants (Cerling and Harris, 1999; Cerling et al., 2003, 2004, 2018; Blumenthal et al., 2016) using the corrections from  $\delta^{13}\text{C}_{\text{modern}}$  to  $\delta^{13}\text{C}_{1750}$ , again following Long et al. (2005) and Cerling et al. (2015). For forest, mesic savanna, and xeric savanna sites we get average  $\delta^{13}\text{C}_{1750}$  values for  $\text{C}_3$  plants of  $-26.7$ ,  $-26.7$ , and  $-25.4\%$ , respectively; and for forest, mesic savanna, and xeric savanna sites we get average  $\delta^{13}\text{C}_{1750}$  values for  $\text{C}_4$  plants of  $-9.7$ ,  $-10.3$ , and  $-11.5\%$ , respectively. Sites were classified as forest, mesic savanna, or xeric savanna and the  $\delta^{13}\text{C}_{1750}$  values for  $\text{C}_3$  plants and  $\text{C}_4$  plants as above were used with  $\delta^{13}\text{C}_{1750}$  values for the African taxa from **Supplementary Data Set 4** to calculate  $\varepsilon^*_{\text{enamel-diet}}$  values for field observations.

This analysis of gives  $\varepsilon^*_{\text{enamel-diet}}$  of  $14.3 \pm 0.8\%$  ( $n = 13$ ) for ruminant foregut fermenters, and  $13.2 \pm 1.1$  ( $n = 13$ ) for the non-coprophagous hindgut fermenters under field conditions. Both of these values are about 1% smaller than determined above for the zoo study ( $15.7 \pm 1.1\%$  and  $14.1 \pm 0.8\%$ , respectively), or for the larger data set using all previously published data for controlled and restricted diet studies ( $15.2 \pm 1.3\%$ , and  $14.8 \pm 1.0\%$ , respectively). It is possible that this offset could be due to difference in  $\text{CH}_4$  production:  $\text{CH}_4$  methane production generally increases with increasing fiber content of a diet. Natural diets are typically of a higher fiber content than those used in captivity or in experimental conditions, but note that this effect is

in the opposite direction as observed here. It is also possible that differences in differential digestion between the field conditions and the controlled diets could have a significant isotope effect. Until more controlled studies are available that compare the effects of different diets, such as zoo- or farm-type diets vs. natural diets, the issue remains unresolved.

## CONCLUSION AND RECOMMENDATIONS

We studied 29 taxa from the Basel and Zurich Zoos having four different digestive strategies for herbivorous mammals. We measured CH<sub>4</sub>/CO<sub>2</sub> ratios in breath,  $\delta^{13}\text{C}$  of food, and  $\delta^{13}\text{C}$  of respired CO<sub>2</sub>. Keeling plots proved valuable for determining the  $\delta^{13}\text{C}$  of respired CO<sub>2</sub> in these studies and will be a valuable addition to the methodology of isotope and animal physiology studies. CH<sub>4</sub>/(CH<sub>4</sub> + CO<sub>2</sub>) ratios were correlated with isotope enrichment in breath ( $\epsilon^*_{\text{breath-diet}}$ ) for both foregut and hindgut digestion, but the total  $\epsilon^*_{\text{breath-diet}}$  was related to both CH<sub>4</sub> production ( $\epsilon^*_{\text{CH4-CO2}}$ ) and differential digestion ( $\epsilon^*_{\text{other}}$ ). Previous correlations of  $\epsilon^*_{\text{enamel-diet}}$  with body mass (Tejada-Lara et al., 2018) may be related to differences in CH<sub>4</sub> production, with the smaller hindgut coprophagous fermenters being both smaller in size and have intrinsic lower CH<sub>4</sub> production than larger hindgut non-coprophagous fermenters. Likewise, foregut fermenters have a large range in CH<sub>4</sub>/CO<sub>2</sub> production ratios and any observed correlations between  $\epsilon^*_{\text{enamel-diet}}$  and body mass may also be related to CH<sub>4</sub>/CO<sub>2</sub> production ratios.

Estimates of the  $\epsilon^*_{\text{enamel-diet}}$  isotope enrichment value is very important in ecological and paleontological studies. However, the determination of precise  $\epsilon^*_{\text{enamel-diet}}$  values is elusive: with the species measured to date, the groups of ruminant fermenters, non-coprophagous hindgut fermenters, non-ruminant foregut fermenters, and coprophagous hindgut fermenters have successively smaller  $\epsilon^*_{\text{enamel-diet}}$  values; we recommend using  $14.5 \pm 1\%$ ,  $13.5 \pm 1\%$ ,  $12.5 \pm 1\%$ , and  $12 \pm 1\%$ , respectively, for these categories for field conditions, but nevertheless remain cautious about potential species differences within these groups. Ruminants had the highest CH<sub>4</sub>/CO<sub>2</sub> ratios and were the only group showing an average  $> 1\%$  isotope enrichment due to CH<sub>4</sub> production. Non-coprophagous hindgut fermenters had the highest apparent isotope enrichment due to differential digestion.

This study suggests that additional work on controlled diet studies in mammals will yield important insights into animal physiology that will have important applications in domestic livestock, wildlife ecology, and paleontology. The Keeling plot approach will have validity in both single breath sampling, as we have done here, or in respiration chamber-like experiments where

better estimates of the integrated CH<sub>4</sub>/CO<sub>2</sub> values are obtained by integrating over longer time periods for each taxon. Since only a few 100 ppmV range is needed for a high-quality Keeling plot (Pataki et al., 2003), only an hour or so should suffice for open respiration chamber (stable, room, or enclosure scaled to the size of the study animal) studies to obtain high quality values of  $\delta^{13}\text{C}_{\text{breath-diet}}$  studies and to determine integrated CH<sub>4</sub>/CO<sub>2</sub> ratios which could be compared for diurnal variations.

## 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 author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of Utah IACUC. The collection of breath was permitted by the Swiss Cantonal Veterinary Authorities under license no. ZH017/19+.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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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.2021.638568/full#supplementary-material>

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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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