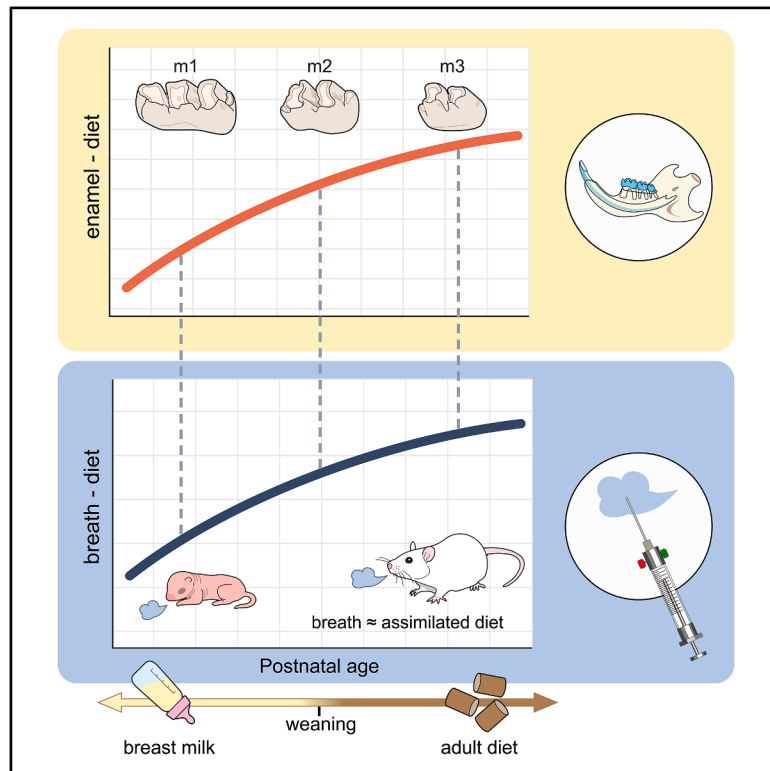


Dynamic tracking of breastfeeding and weaning in carbon isotopes of tooth enamel

Graphical abstract



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In brief

Biological sciences; Biochemistry;
Animal physiology; Paleobiochemistry

Highlights

- Dynamic isotope tracking quantifies breastfeeding effects on enamel $\delta^{13}\text{C}$
- Breast milk induces up to $\sim 4\text{‰}$ ^{13}C depletion in early-forming teeth
- Enamel-diet enrichment ($\sim 12\text{‰}$) remains constant through growth and weaning



Article

Dynamic tracking of breastfeeding and weaning in carbon isotopes of tooth enamel

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SUMMARY

Stable carbon isotopes are fundamental for dietary and life-history reconstructions across living and fossil mammals. However, commonly used enamel-diet isotopic offsets are primarily derived from large herbivores and rarely account for breastfeeding in developing teeth. We present controlled feeding experiments using mother-pup pairs of four rodent species to quantify dynamic change in the carbon isotope composition of tooth enamel during early development. Breastfeeding induces a ^{13}C depletion of up to 4 per mil in early-forming teeth. The weaning signal is propagated from the first molar (m1) through m3 with a progressive change from milk to a fully adult diet, with a “half-life” of about 20 days for the rodents. Despite lipid-rich dietary influence, the offset between enamel and progressively changing diet stabilizes at 12.1 per mil in small mammals, consistent with enrichment values measured in later-forming teeth. This framework advances our understanding of life-history strategies and the ecology of mammals in the past.

INTRODUCTION

Geochemical approaches have been developed to understand physiological and ecological traits of mammals over deep time, including ancient humans. Bone collagen and tooth enamel are often sampled due to high resistance against decay and diagenesis, whereas hair and tooth enamel are of special interest because sequential measurements of these materials can provide chronological records of elemental (e.g., Sr/Ca and Ba/Ca) and isotopic (e.g., $\delta^{15}\text{N}$, $\delta^{44/42}\text{Ca}$, and $\delta^{13}\text{C}$) trajectories in life history. Such trajectories are often related to a nutrient transition from exclusive breastfeeding to non-milk solid diet during weaning^{1–5} and a change of dietary input due to various reasons, including seasonal migration and food availability in home ranges.^{6–9} Expanding to evolutionary perspectives, these proxies have addressed the long-term process and tempo of dietary shift and evolution of life-history strategies in mammalian lineages due to changes in resource availability caused by climatic factors.^{10–21}

Isotopically distinct dietary sources incorporated into animal tissues go through biochemical processes, causing isotope fractionation between tissues and consumed products. Thus, a

reasonable estimate of isotopic offsets between targeted tissues of an individual and its food (breast milk vs. post-weaning, non-milk diet) is necessary to translate isotope ratios to paleoecological signatures. In carbon isotopes, which primarily distinguish different photosynthetic pathways of plants (C_3 vs. C_4 plants), an enrichment of 14‰ between enamel and diet ($\epsilon^*_{\text{enamel-diet}}$) is used as a standard protocol for paleoecological reconstruction from tooth enamel in mammals.²² Interspecific variation of the enrichment has been recognized by field and experimental studies, ranging from 9‰ to 11.5‰ for laboratory rodents^{23–28} to 12‰–14‰ for medium-to large-sized mammals.^{22,27,29–32} This variation is attributed to exhaled ^{13}C -depleted methane gas³³ produced by symbiotic microbial activities in the digestive systems of herbivorous mammals and to the differential digestion of food.^{27,34} Different plant tissues show different $\delta^{13}\text{C}$ values relative to “bulk leaf,” with lipids and lignin showing more depleted values by –3 to –4‰ on average, and sugars, starches, and cellulose exhibiting more enriched values by +1.5 to +2‰.³⁵ Given that lignin is poorly digestible, selective digestion of plant components is therefore expected.

Carbon isotope compositions further act as biomarkers in weaning practice because milk lipids are more depleted in ^{13}C



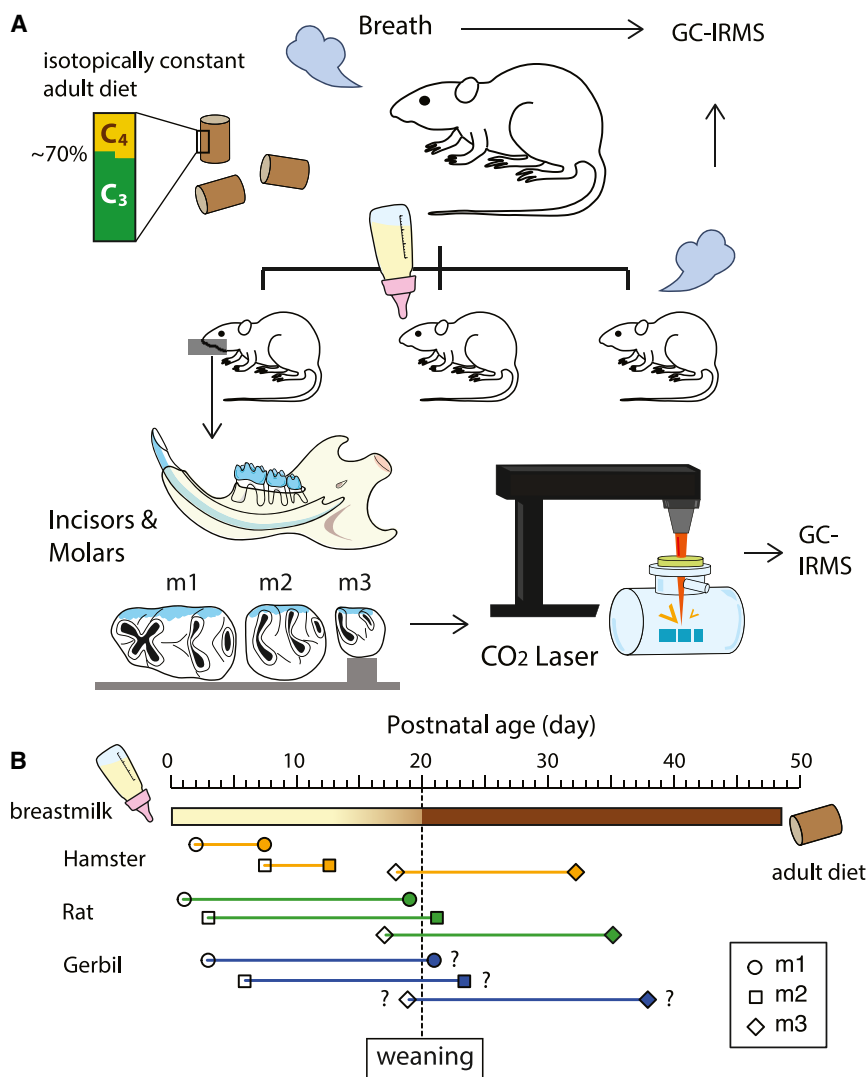


Figure 1. Experimental design with isotopically constant diet and timing of molar enamel formation

(A) Schematic representation of the experiments in which stable carbon isotopes ($\delta^{13}\text{C}$) in the breath of mothers and pups and $\delta^{13}\text{C}$ in molar enamel of pups were measured.

(B) Main dietary sources and timing (days) of enamel formation in the analyzed rodents (Table S1). Open and filled symbols indicate the beginning of the enamel apposition stage and timing of tooth eruption into the oral cavity of each of the three molar teeth. The proportion of C₃ and C₄ components in the chow diet was estimated, assuming $\delta^{13}\text{C}$ of C₃ = -26.5‰ and $\delta^{13}\text{C}$ of C₄ plants = -12.5‰ in a two-source mixing model.^{10,11,43,44}

is attenuated by the introduction of supplementary foods during the weaning period and time-averaged isotopic record in tooth enamel, as well as interspecific variation in the enamel-diet enrichment. No controlled study has demonstrated the degree of the breastfeeding effect on $\delta^{13}\text{C}$ in tooth enamel using an isotopically constant diet in pairs of mothers and offspring.

Here, we evaluate the dynamic change of carbon isotope compositions ($\delta^{13}\text{C}$) in tooth enamel from birth to adulthood and quantify the breastfeeding signal through feeding experiments on four species of laboratory rodents—brown rat (*Rattus norvegicus*), golden hamster (*Mesocricetus auratus*), Mongolian gerbil (*Meriones unguiculatus*), and house mouse (*Mus musculus*). These animals were chosen to include a variety of tooth

eruption timing (Figure 1B and Table S1), water intake, and body mass. *Rattus* and *Mus* are members of the subfamily Murinae, and *Meriones* belongs to the Gerbillinae (both subfamilies nested within the family Muridae). *Mesocricetus* is classified in the sister family Cricetidae. The isotopic contribution of breastfeeding was progressively monitored in $\delta^{13}\text{C}$ of breath CO₂ and that of molars that crystallize rapidly and sequentially throughout a dietary shift from exclusive breastfeeding to adult diet (Figure 1A). In previous studies, fully adult teeth of individuals that were fed on an isotopically controlled diet were used to derive isotope enrichment between tooth enamel and bulk diet ($\epsilon^*_{\text{enamel-diet}}$). That method gives a single enrichment value for each animal. In our study, we designed the experiment to dynamically track the isotope enrichment between tooth enamel and progressively changing diet at any growth stage ($\epsilon^*_{\text{enamel-progressive diet}}$). This was achieved by selecting a set of breast milk-influenced molars whose eruption timings cover the duration of the dietary shift from breast milk to adult diet (Figure 1B and Table S1), and by measuring the carbon isotope composition of breath as an

than the maternal solid diet.^{23–25,36} Maternal milk has an average $\delta^{13}\text{C}$ depletion of $-2.1 \pm 0.9\text{‰}$ relative to lactating maternal blood plasma for various non-human mammals.³⁷ In weaning studies of humans, $\delta^{13}\text{C}$ values, however, do not show a clear trophic-level fractionation, in contrast to nitrogen isotopes that track a change of protein sources toward a lower trophic level,^{2,38–41} but instead show a decrease of 1‰ or less toward a non-milk diet in sequential profile sampling.^{2,4,42} No $\delta^{13}\text{C}$ difference was reported between maternal plasma and that of nursing offspring in non-human mammals.³⁷ Nevertheless, a general age-related trend toward more positive $\delta^{13}\text{C}$ values has been assumed to be linked with a change in proportions of breast milk to non-milk diet. For this reason, many paleoecological studies have used post-weaning teeth to avoid an undefined isotopic influence of breast milk.

The breastfeeding signal detectable by $\delta^{13}\text{C}$ values is important because $\delta^{13}\text{C}$ values recorded in tooth enamel are more readily trackable in deeper time than $\delta^{15}\text{N}$ in bone collagen and trace element analyses. However, a true breastfeeding signal

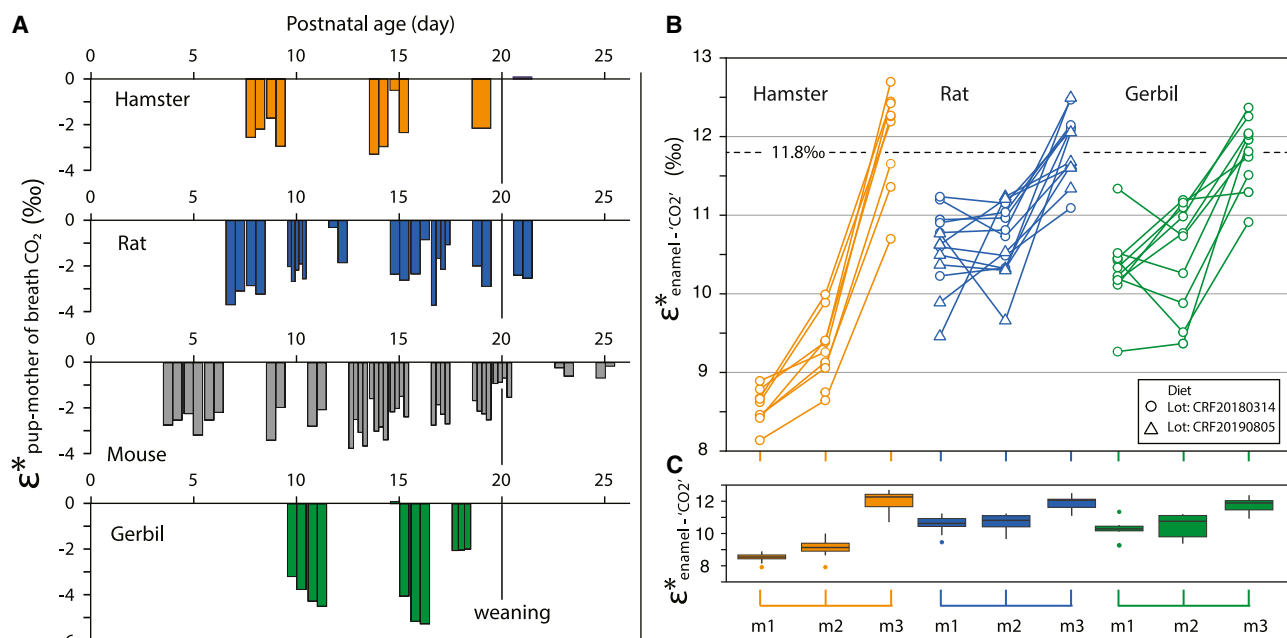


Figure 2. ^{13}C enrichment, measured for breath CO_2 and molars of rodents

(A) ϵ^* of breath CO_2 in pups relative to their mothers.

(B) ϵ^* between enamel bioapatite of molars (m1, m2, m3) and ‘ CO_2 ’.

(C) Boxplot of ϵ^* enamel-‘ CO_2 ’ for each tooth (bottom). The term ‘ CO_2 ’ indicates the assimilated portion of the adult diet, whose $\delta^{13}\text{C}$ value was approximated by the $\delta^{13}\text{C}$ of adult breath CO_2 (-19.7‰ , Figure S1) with a 0‰ offset. Each of the analyzed individuals is connected by a line. Tables 1, 2, and S2 for summary statistics.

analogue to progressively changing diet. Key to this experiment is that a full set of analyzed rodent molars records the entire period of the dietary turnover, while the rapid enamel maturation of each tooth minimizes the attenuation of the isotopic signal.

RESULTS

Stable carbon isotope ratios are reported in δ notation as parts per thousand (‰) on the Vienna Pee Dee Belemnite (VPDB) scale, where $\delta^{13}\text{C}_{\text{sample}} = (\text{R}_{\text{sample}}/\text{R}_{\text{standard}} - 1) \times 1000$; $\text{R} = ^{13}\text{C}/^{12}\text{C}$.

The $\delta^{13}\text{C}$ of breath CO_2 is enriched relative to the bulk $\delta^{13}\text{C}$ of the adult diet. The commercial rat chow used in this study has a $\delta^{13}\text{C}$ value of $-22.1 \pm 0.4\text{‰}$; 22 samples of breath CO_2 of adult mice had $-19.7 \pm 0.5\text{‰}$ (Figure S1 and Data S3), suggesting ^{13}C enrichment in breath CO_2 relative to the bulk diet by $2.4 \pm 0.6\text{‰}$. Previous studies have shown that $\delta^{13}\text{C}$ values of breath CO_2 reflect those of the bulk diet with minimal offset for small coprophagous hindgut digesters (e.g., rabbits) with negligible methanogenesis³⁴ because more complete digestion is expected compared to larger hindgut fermenters.^{45,46} Thus, the larger breath-diet offset observed in this study may be derived from the differential digestion of the commercial chow as a similar observation had been reported for a diet-feces offset for rodents on pelleted lab diets.⁴⁷ Due to the selective digestion, we considered the assimilated portion of the adult diet rather than the bulk diet of the commercial chow.

Apparent carbon isotope enrichment, $\epsilon^*_{\text{enamel-‘CO}_2\text{’}} = [(1000 + \delta^{13}\text{C}_{\text{enamel}})/(1000 + \delta^{13}\text{C}_{\text{assimilated portion of adult diet}}) - 1] \times 1000$, was

calculated,⁴⁸ approximating $\delta^{13}\text{C}$ of the assimilated portion of adult diet by $\delta^{13}\text{C}$ of breath CO_2 in non-pregnant adult mice ($\delta^{13}\text{C} = -19.66\text{‰}$) with a 0‰ offset (see STAR Methods). The breath CO_2 -based approximation for $\delta^{13}\text{C}$ of assimilated diet (=‘ CO_2 ’) is necessary because we need to monitor a progressive isotopic change of assimilated nutrients from exclusive breast milk through varying mixing degrees to the fully adult diet. To clarify which values are being compared in our experimental data, $\epsilon^*_{\text{enamel-‘CO}_2\text{’}}$ describes ^{13}C enrichment in tooth enamel relative to breathe CO_2 -approximated assimilated portion of the adult diet, whereas $\epsilon^*_{\text{enamel-progressive diet}}$ expresses the enrichment relative to progressively changing diet (also breath CO_2 -based approximation) during a transition from milk to adult diet.

Breastfeeding signal on $\delta^{13}\text{C}$ values in breath CO_2

Carbon isotope compositions of breath CO_2 ($\delta^{13}\text{C}_{\text{breath}}$) were measured for pairwise comparisons between mothers and their pups to detect isotopic milk signal from birth to weaning (Figure S2). The mother-pup pairwise comparisons ensure the detection of isotopic signals of maternal milk in breath CO_2 .

Mother-pup pairwise comparisons show that $\delta^{13}\text{C}$ of breath CO_2 in pups is consistently more negative by a few permil than that of mothers during lactation (Figure 2A, Table 1, and Data S1–S2). A breastfeeding signal (= more negative $\delta^{13}\text{C}$ in pups than in their mother due to lipid-rich milk diet) was detected consistently until weaning. The isotope enrichment of pups relative to their breastfeeding mothers ($\epsilon^*_{\text{pup-mother of breath CO}_2}$) is -3.9‰ (from days 0 to 15) in Mongolian gerbils, which is

Table 1. Descriptive summary of carbon isotope enrichment and isotopic spacing between breath of pups and that of their mother, corresponding to Figure 2A

Taxa	ϵ^* pup-mother of breath CO ₂		$\Delta^{13}\text{C}$ (= $\delta^{13}\text{C}$ pup of breath CO ₂ - $\delta^{13}\text{C}$ mother of breath CO ₂)	n
	Mean	SD	Mean	
Hamster (<i>Mesocricetus auratus</i>)	-2.6	0.54	-2.52	7
Rat (<i>Rattus norvegicus</i>)	-2.6	0.57	-2.52	12
Mouse (<i>Mus musculus</i>)	-2.6	0.63	-2.55	22
Gerbil (<i>Meriones unguiculatus</i>)	-3.9	0.51	-3.87	5

In the calculation, pups at $\leq$ day 15 or younger, and ϵ^* pup-mother of breath ≤ -0.5 are considered. SD: standard deviation.

significantly more negative than the average of (-2.6‰) in the other studied rodents (Table S2).

Tracking breastfeeding in tooth enamel

Along the molar series (m1, m2, m3), $\delta^{13}\text{C}$ values are most depleted in m1 and are sequentially more enriched from m1 to m3, which is reflected in ϵ^* enamel- CO_2 (Figure 2B, Table 2, and Data S4) where 'CO₂' is the measured $\delta^{13}\text{C}$ of adult breath CO₂ (-19.7‰ , Figure S1) and represents the assimilated portion of adult diet (commercial chow), and thus is measured in the same way as breath CO₂-based dietary proxy for progressively changing diet (see STAR Methods and supplemental information).

In m1, ϵ^* enamel- CO_2 of hamsters is more negative than Mongolian gerbils by -1.7‰ and rats by -2.1‰ , respectively, the opposite ranking to mother-pup pairwise $\delta^{13}\text{C}$ of breath CO₂, where the Mongolian gerbil is the most depleted. The ϵ^* enamel- CO_2 of hamsters is more depleted than that of rats and gerbils for m2, whereas the ϵ^* enamel- CO_2 values are consistent among the studied species for m3, converging toward the empirical average of $11.8 \pm 0.1\text{‰}$ ($p = 0.54$, ANOVA). First molars show the widest range of ϵ^* enamel- CO_2 values, whereas third molars show the smallest range (Figure 2B and Table 2). Thus, the isotopic influence of milk consumption clearly depends on the timing of tooth development relative to the intake of milk.

These values of molars were arranged by the timing of tooth eruption into the oral cavity as days after birth (Figure 3A). In the three species combined, an increasing trend of ϵ^* enamel- CO_2 is well-fitted to an exponential decay curve in an increasing form as in breath CO₂ (Figure 3B). The half-life of ϵ^* enamel- CO_2 , that is, time duration necessary to incorporate 50% of carbon atoms from the adult diet into pup's metabolic reserve, was calculated to be 19.8 days (Figure 3C), which is well tracked by long-term monitoring of breath CO₂ (Figures 3B and 3D) and consistent with carbon turnover of whole blood in *Rattus* and *Mus*.⁵⁰ This half-life represents the progressive increase in solid food as the pups approach a fully adult diet.

Long-term monitoring of breath CO₂

$\delta^{13}\text{C}_{\text{breath}}$ was monitored from birth to the subadult stage (day 47) in mice (Figure 3B and Table S3). $\delta^{13}\text{C}_{\text{breath}}$ recorded an isotopic turnover associated with the incorporation of more enriched food into the metabolic reserve. The breath data captured the initial phase of weaning (i.e., onset of adult diet consumption)

prior to the physical separation of pups from their mothers (forced weaning) at day 20. This pattern likely reflects the actual feeding behavior of the pups. From around day 10 onward, when their eyes were open, pups were observed nibbling crumbled chow diet that had fallen from the food container mesh, although they continued to demand milk, as evidenced by ϵ^* pup-mother of breath CO₂ in pup-mother pairs (Figure 2A). In contrast, the completion of isotopically observable weaning lagged behind the forced weaning date by 20–30 days, reflecting complete turnover of the metabolic reserve. The Bayes-estimated initial value [$=\epsilon^*$ breath at day $t-\text{CO}_2$ (0)] is -3.8‰ at day 0. Increasing $\delta^{13}\text{C}_{\text{breath}}$ of monitored mice reaches an upper limit with the estimated asymptotic value [$=\delta^{13}\text{C}$ (eq)] nearly 0‰ as expected, which ensures that the subadults fully metabolize the adult diet.

DISCUSSION

Breastfeeding signals in the mother-pup pair of breath CO₂

Mother-pup pairwise comparisons of breath samples allow us to detect an isotopic signal of lactation as carbon isotope enrichment between breast milk and mothers' assimilated diet (Figure 2A). A pairwise comparison is important to detect the signal in breath CO₂ because $\delta^{13}\text{C}$ values of breath CO₂ in pups are highly variable, influenced by their fasting time and the daily isotopic change of the mother's metabolic pool. In fact, based on carbon isotopes of breath CO₂, fat turnover is rapidly completed within several hours in small mammals with high metabolism.⁵¹

The stronger lactation signal in gerbils would suggest that gerbils have higher lipid content in breast milk (Figure 2A) than any other taxon tested, which would be related to desert-adapted fat metabolism,⁵² although milk composition data are not available for gerbils. In the other three species, ϵ^* pup-mother values (carbon isotope enrichment in pups relative to their mother) of breath CO₂ are not significantly different from each other (Table S2), whereas the reported fat content of milk greatly varies from 4.9% in *Mesocricetus auratus* (golden hamster) and 8.8% in *Rattus norvegicus* (Norway rat) to 27% in *Mus musculus* (summarized in^{53,54}). This falsely suggests that there is no apparent correlation between ϵ^* pup-mother of breath CO₂ and milk fat content. However, Oftedal and Iverson⁵³ pointed out that the reported nutrient data of rodent milk lack standardized protocols, and that the values of milk fat percentage are, for example,

Table 2. Descriptive summary of $\delta^{13}\text{C}$ values in tooth enamel and carbon isotope enrichment between tooth enamel and the assimilated part of the commercial chow consumed by adults, corresponding to Figure 2B

	Incisor						m1						m2						m3					
	$\delta^{13}\text{C}$ enamel			ϵ^* enamel - 'CO2'			$\delta^{13}\text{C}$ enamel			ϵ^* enamel - 'CO2'			$\delta^{13}\text{C}$ enamel			ϵ^* enamel - 'CO2'			$\delta^{13}\text{C}$ enamel			ϵ^* enamel - 'CO2'		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Hamster	-12.1	0.36	13	7.8	0.36	13	-11.4	8.5	10	-10.7	9.1	11	-7.9	12.0	9	-7.9	12.0	9	-7.9	12.0	9	-7.9	12.0	9
Rat	-11.7	0.35	21	8.1	0.35	21	-9.3	10.6	15	-9.2	10.7	15	-8.1	11.8	15	-8.1	11.8	15	-8.1	11.8	15	-8.1	11.8	15
Gerbil	-11.6	0.45	9	8.2	0.45	9	-9.7	10.2	12	-9.4	10.4	12	-8.2	11.7	12	-8.2	11.7	12	-8.2	11.7	12	-8.2	11.7	12
Average	8.0			8.0			9.7			10.1			11.8			11.8			11.8			11.8		
SD	0.2			0.2			1.1			0.9			0.1			0.1			0.1			0.1		

Note: 'CO2' = assimilated portion of adult diet (commercial chow), whose $\delta^{13}\text{C}$ value was approximated by $\delta^{13}\text{C}$ of adult breath CO_2 (-19.66‰, Figure S1) with a 0‰ offset. SD: standard deviation.

substantially different in various publications, ranging from 8% to 19% for the rat and 13.1%–41.6% for the house mouse.

Breastfeeding effect in ϵ^* as inter-tooth and interspecific variations

In mammals, $\delta^{13}\text{C}$ of m1, an early-erupting tooth, is typically more depleted than that of later-erupting teeth^{30,55,56} because lipid-rich breast milk is isotopically depleted in ^{13}C compared to the whole diet.^{23,57–59} Lower ϵ^* enamel-'CO2' in hamsters compared to rats and gerbils reflects the rapid enamel formation of m1 and m2 in hamsters (Table S1). ϵ^* enamel-'CO2' of m1 and m2 in hamsters (cricetid rodents) are significantly different from those in rats and gerbils (murid rodents) by -1.9‰ for m1 and -1.5‰ for m2, respectively (Tables 2 and S2). Our study found that the offset of ϵ^* enamel-'CO2' between m2 and m1 is negligible for rats (0.1‰) and gerbils (0.2‰), in which the enamel formation of both tooth loci happens at about the same time (as opposed to 0.6‰ offset in hamsters). This is consistent with the only study on the inter-tooth $\delta^{13}\text{C}$ variation of rooted rodent molars,⁶⁰ which also found no offset between m2 and m1 in *Meriones* gerbils.

Interspecific comparison of carbon isotopic values of breath and molar enamel results in an apparent discrepancy in that breast milk-influenced m1 shows more negative ϵ^* enamel-'CO2' values in hamsters, whereas the lactation signal in breath ($=\epsilon^*$ pup-mother of breath CO_2) is greater in gerbils than in other species. This discrepancy is well explained when ϵ^* enamel-'CO2' values of molars are rearranged based on the timing of tooth eruption into the oral cavity as an approximation of the completion of enamel formation (Figure 3A).

The largest inter-tooth variation (3.5‰) of the mean ϵ^* enamel-'CO2' is found between m3 and m1 in the hamster, the degree of which is consistent with the Bayesian-estimated breastfeeding signal (~4‰) in breath CO_2 (Tables 2 and S2). In contrast to the almost full range of breastfeeding signals in the hamster, the m3-m1 offset in rats and gerbils is only 1.4‰ on average (Figure 2B and Table S2) because enamel formation completes later in the murid rodents (Figure 1B).

The m3-m1 offset in the hamster corresponds with that in the kangaroo (3.5‰, *Macropus* spp.),⁶¹ while fat% of breast milk is 4.9% and 6.1%, respectively.⁵³ The fat % of human breast milk (3.6%)⁶² is equivalent to that of the hamster, whereas a smaller offset (<1‰) is generally observed for ϵ^* enamel-diet of humans.⁵⁶ When the duration of the completion of m3 formation in humans is scaled to that of hamsters (Figure 3E), the relative weaning age is younger in humans. In fact, early weaning is characteristic in humans compared to non-human great apes.⁶³ Our empirical model suggests that a breastfeeding signal in humans is expected to be around 3‰, comparable to that of the hamster, but it cannot be observed across the full range in teeth because weaning occurs earlier than the completion of m1 enamel formation.

Empirical enrichment values also serve as correction factors for inter-tooth isotopic variation. The reported offsets between m1 and m3 are as small as -0.5‰ in humans⁵⁶ and -0.8‰ in female chimpanzees⁶⁴ and vary from ~ -1.0‰ (*Tragoportax*, an extinct bovid)²⁰ to -1.7‰ (tapir)⁶⁵ and -3.5‰ (kangaroo)⁶¹ in non-primate mammals. In isotopic ecology, third molars and canines are preferentially selected over first and second molars for medium-sized to large mammals because the isotopic

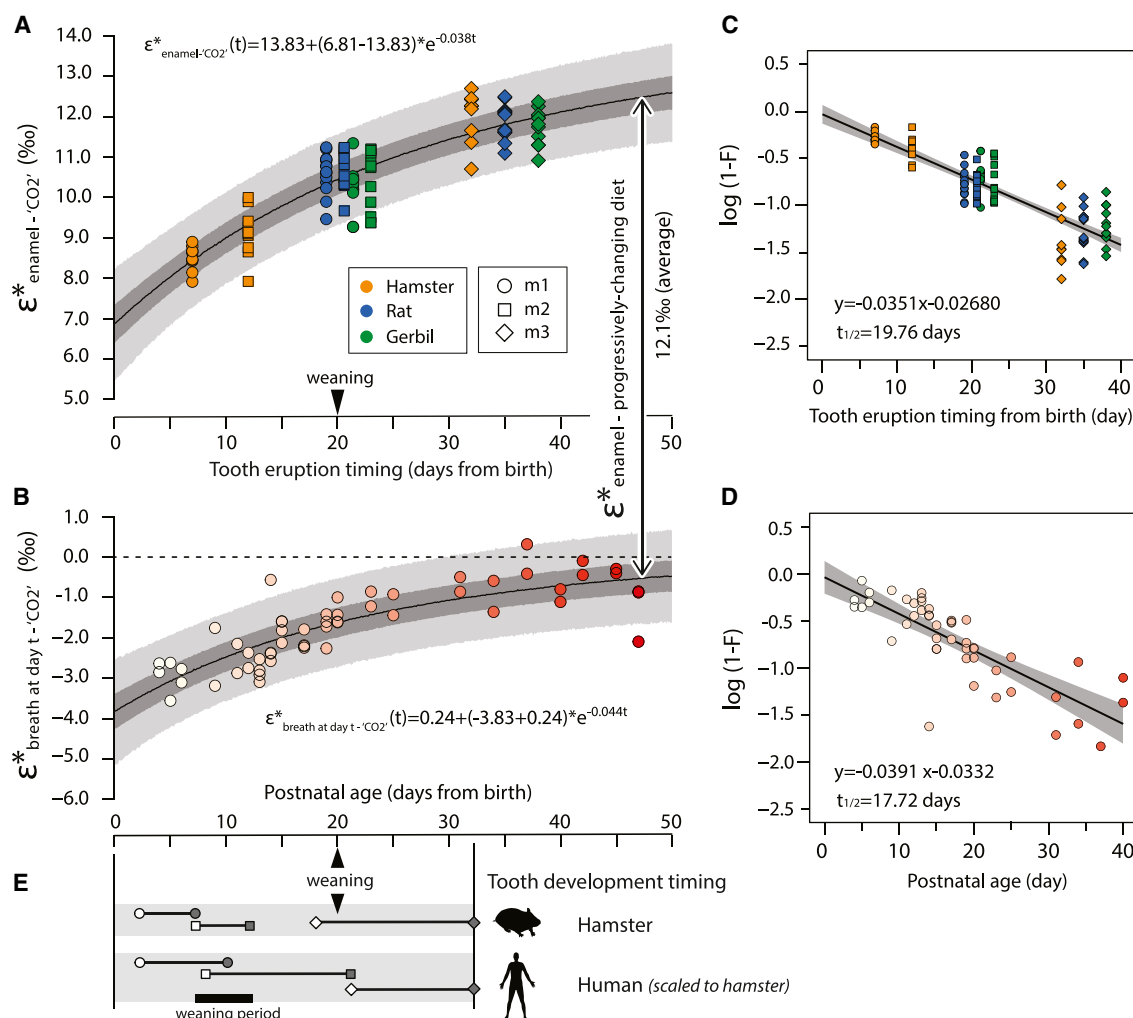


Figure 3. Dynamic monitoring of ϵ^* between tooth enamel and progressively changing diet along the growth timeline of pups

(A) ϵ^* between tooth enamel and breath-approximated assimilated portion of adult diet ($^{13}\text{CO}_2$).

(B) ϵ^* between breath CO_2 at day t and adult breath CO_2 ($^{13}\text{CO}_2$). The isotopic spacing between corresponding data points along the two exponential decay curves indicates ϵ^* between enamel bioapatite and progressively changing diet along growth. Solid lines are the estimates of the 50% conditional quantile of the Bayesian prediction interval. Likewise, the dark gray shadow indicates the area between the 25% and 75% conditional quantiles, and the light gray shadow indicates the area between 2.5% and 97.5%.

(C) Reaction progress variable, $\ln(1-F)$, calculated from the data (A) of tooth enamel.

(D) Reaction progress variable, $\ln(1-F)$, calculated from the data (B) of breath.

(E) Timing of enamel formation. Symbols are the same as in Figure 1, from the beginning of the enamel apposition stage to eruption timing into the oral cavity. For the human, the interval is approximated based on modified Moorrees' stages,⁴⁹ from the cusp coalescence stage (Cco) to the root quarter stage ($R_{1/4}$) and is fitted to the interval of the hamster (Table S1) by scaling the age at the $R_{1/4}$ stage (14.5 years old) of human m3 to that of eruption timing (32 days) of hamster m3. Applying the scaling factor (0.006568144) isometrically, the typical weaning age (2.4–2.7 years; summarized in⁴) in non-industrialized human societies corresponds to approximately 6 days in the “hamster scale.”

variation of breast milk-influenced teeth has been poorly documented. However, in many rodent clades, m1 is preferred over m3 because it preserves more taxonomic characters and offers higher resolution at the species level (e.g.,^{66,67}). Its larger size facilitates isotope analysis when m3 is too small to be used.⁶⁰ Because inter-tooth variations of rodent molars come from tooth development, a choice of correction values for inter-tooth variations would be “molar-specific” and highly dependent on phylogenetic relatedness.

In paleoecological and paleoenvironmental reconstruction using small mammals with rooted molars, the milk-influenced inter-tooth isotopic variation must be taken into account, especially when the $\delta^{13}\text{C}$ of dietary sources is compared among distantly related species with different tooth development timing. Conversely, inter-tooth variation of $\delta^{13}\text{C}$ reflecting a breastfeeding signal is useful for providing evolutionary perspectives on breastfeeding and weaning across mammalian lineages in deep time.

$\epsilon^*_{\text{enamel-}^{13}\text{C}} \text{ of molars and incisors}$

In contrast to the trend in molars, the $\epsilon^*_{\text{enamel-}^{13}\text{C}}$ values of the ever-growing incisor are even more negative than those of m1 in all species (Figure S3, Table 2, and Data S5). The average $\epsilon^*_{\text{enamel-}^{13}\text{C}}$ of the incisor is $8.0 \pm 0.3\text{‰}$, more depleted than that of m1, whereas the standard deviation of the incisor is as small as that of m3 (Table 2), suggesting that the incisor is heavily milk-influenced even in subadults (>day 60) among all studied species.

The incisor begins initial development *in utero* and erupts into the oral cavity on the 1st or 2nd days after birth in cricetids, on the 10th days in murines, and between the 4th and 10th days in gerbillines.^{68,69} Assuming the eruption rates of the incisors are 0.1 mm/day as measured in immature rats⁷⁰ and 0.8 mm/day in subadult rats,⁷¹ the breastfeeding period would overlap with the increment of a few mm of the incisor formation. A further 30 mm or less would be added post-weaning to the mandibular incisor in the examined subadults (>day 60), presumably making up most of incisor length at that age. However, slower eruption rates during early ages would partially explain the more negative $\epsilon^*_{\text{enamel-}^{13}\text{C}}$ values in the incisor compared to rapidly mineralized m1 in the same subadults. In addition, the outer and middle enamel layers—typically targeted by CO_2 -laser ablation—mineralize more slowly than the innermost layer in the incisor.^{72–74} Finally, we analyzed mature enamel located several millimeters posterior to the apical end but anterior to newly formed enamel (i.e., near the zone of white opaque enamel *sensu* Hiller et al.⁷⁵ to avoid organic inclusion from Ca-depleted immature enamel. All the above would contribute to a strong breastfeeding signal in the incisors of the subadults.

Although an isotopic turnover of incisors, apparently slower than that of molars, is beyond the scope of our research, our experimental observation that the completion of the enamel maturation in ever-growing incisors appears slower than in rooted molars may be a significant target of investigation to integrate isotopic signals with enamel maturation during tooth development.^{74,76,77} When rodent incisors are used in paleoecological analyses, those from older individuals are preferred to avoid unwanted breastfeeding signals.

Dynamic monitoring of constant $\epsilon^*_{\text{enamel}}$ -progressive diet throughout growth

We modeled the dietary transition from breast milk to adult diet as a one-compartment process, using standard equations (see STAR Methods).^{48,50,78–80} Weaning is considered a transitional dietary switch from lipid-rich milk to a non-milk solid diet.

The exponential decay model of $\delta^{13}\text{C}_{\text{enamel}}$ is nearly parallel to that of $\delta^{13}\text{C}_{\text{breath}}$ from birth to subadults due to the similarly rapid turnover rates in both substances (Figures 3A and 3B). The $\delta^{13}\text{C}$ difference between molar enamel and breath CO_2 is considered as isotopic spacing between tooth enamel and progressively changing diet ($=\epsilon^*_{\text{enamel-progressive diet}}$) during growth. Based on Bayesian-estimated coefficients, the simulated $\epsilon^*_{\text{enamel-progressive diet}}$ for hypothetical individuals between days 0 and 60 is $12.1 \pm 0.7\text{‰}$, consistent with the empirical values measured on m3 ($11.8 \pm 0.1\text{‰}$). Thus, the $\epsilon^*_{\text{enamel-progressive diet}}$ does not change with growth from infancy to adulthood. With multiple lines of evidence, we confirmed that the theoretical value

($\sim 12\text{‰}$) proposed by Krueger and Sullivan,⁵⁸ derived based on an assumption of a complete conversion of carbohydrates in the diet to carbon in blood bicarbonate, is correct regardless of growth.

In herbivores, $\epsilon^*_{\text{enamel-diet}}$ is in a linear relationship with $\epsilon^*_{\text{breath-diet}}$ ²⁷ because $\epsilon^*_{\text{breath-diet}}$ and methanogenesis are positively correlated separately in hindgut (such as horses) and foregut (ruminants) fermenters.³⁴ A linear regression of $\epsilon^*_{\text{enamel-diet}}$ on body mass³¹ is only secondarily established because a strong positive correlation between respiratory CH_4 emission and body mass is recognized across mammals.⁸¹ When body mass is not correlated with methanogenesis as in primates ($\epsilon^*_{\text{enamel-diet}}$ of the chimpanzee, 11.8‰ ³²), the body-mass regression inevitably leads to an overestimation of $\epsilon^*_{\text{enamel-diet}}$.

Combining the above, the $\sim 12\text{‰}$ enamel-diet offset— 11.8‰ , calculated as the four-point average of the m3 values from the three species analyzed in this study ($n = 36$; Table 2) together with a previously reported value for the prairie vole *Microtus ochrogaster* (11.5‰)²⁷—represents a baseline $\epsilon^*_{\text{enamel-diet}}$ for mammals with minimal respiratory methane emission, such as small mammals and primates. Herbivorous mammals exhibit physiologically dependent positive offsets of up to $+3\text{‰}$ from this baseline value, driven primarily by digestive methane production (i.e., exhalation of ^{13}C -depleted methane produced during methanogenesis³³) and secondarily by body mass. By establishing the developmental stability in $\epsilon^*_{\text{enamel-diet}}$, our controlled feeding experiments characterized the carbon isotopic influence of breastfeeding, showing that early-erupting teeth record a distinct $\delta^{13}\text{C}$ depletion due to lipid-rich milk consumption. Conversely, despite this early dietary effect, the enrichment between enamel and diet—whether maternal milk or non-milk diet—remains developmentally stable at 11.8‰ in homeothermic herbivorous mammals with minimal methane production.

In this study, we demonstrated how nursing and weaning are recorded in enamel and resolved a long-standing uncertainty in carbon isotope ecology by experimentally isolating the breastfeeding effect in tooth enamel. Although milk consumption drives substantial ^{13}C depletion in early-forming teeth, enamel-diet enrichment remains constant at $\sim 12\text{‰}$ across ontogeny, based on model-derived averages (11.8‰ measured from m3). Thus, developmental changes in $\delta^{13}\text{C}_{\text{enamel}}$ reflect dietary turnover rather than physiological shifts in fractionation. This 11.8‰ value should serve as a baseline enrichment for herbivorous mammals with minimal respiratory methane emission. Differential digestion and CH_4 production act as additional modifiers, explaining enrichment variation across herbivorous mammalian groups. Our findings strengthen the use of enamel $\delta^{13}\text{C}$ as a life-history proxy in deep time. Accounting for milk-induced inter-tooth variation refines paleoecological reconstructions and opens new opportunities to investigate life-history evolution in fossil mammals. By establishing the developmental stability of enamel-diet enrichment and quantifying milk effect, we provide carbon isotopes as an interpretable tool for reconstructing breastfeeding, weaning, and dietary ecology in living and fossil mammals. Future work linking macronutrient proportions, especially lipids, of breast milk with $\delta^{13}\text{C}_{\text{breath}}$ and/or $\delta^{13}\text{C}_{\text{enamel}}$ will further enhance the interpretive resolution of $\delta^{13}\text{C}$, particularly in

contexts where carbon isotopes are better preserved than nitrogen isotopes in the fossil record.

Limitations of this study

In the feeding experiment, we used a commercial chow diet to ensure reproducibility and long-term health for both mothers and their pups. Although the general dietary compositions of the commercial product are reported, the exact proportions are strictly confidential. Carbon isotope compositions of the bulk chow indicate ~30% of the carbon derives from C₄ sources, and fishmeal and beer yeast are also included to some extent. Thus, the diet is not isotopically homogeneous. We detected differential digestion in breath data ($\Delta C_{\text{breath-bulk diet}} = 2.4 \pm 0.6\text{‰}$), which led us to use the term “assimilated diet” rather than “bulk diet” or “diet” in this study. Likewise, breast milk can be synthesized differentially through a biological process that could not be controlled under our experimental conditions. We observed ¹³C depletion of up to 4 per mil in early-forming enamel, whereas we presume that the magnitude of ¹³C depletion would be lower with an exclusively C₃ diet. Despite these limitations, the offset between enamel and the progressively changing diet is consistently ~12‰ based on model-derived averages (11.8‰ measured from m3).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Yuri Kimura (ykimura.research@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This article does not report original code.
- All data reported in this paper are deposited in Mendeley Data (doi: <https://doi.org/10.17632/gd7nkrbzz8.1>).
- Any additional information required to reanalyze the data reported in this article is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.K., T.E.C., and L.L.J.; methodology, investigation, and formal analysis, Y.K., T.E.C., K.Y., and A.S.; writing – original draft, Y.K.; writing – review and editing, I.C.V., L.J.F., L.L.J., T.E.C., K.Y., and A.S.; and funding acquisition, Y.K., I.C.V., and L.J.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Charles River Formula-1	Oriental Yeast Co., Ltd. (Japan)	lot. 20180314
Charles River Formula-1	Oriental Yeast Co., Ltd. (Japan)	lot. 20190312
Charles River Formula-1	Oriental Yeast Co., Ltd. (Japan)	lot. 20190805
Deposited data		
Datas S1–S6	This study	DOI: https://doi.org/10.17632/gd7nkrbzz8.1
Experimental models: Organisms/strains		
Slc:SD brown rat	Japan SLC, Inc. (Japan)	–
Slc:Syrian golden hamster	Japan SLC, Inc. (Japan)	–
MON/Jms/GbsSlc Mongolian gerbil	Japan SLC, Inc. (Japan)	–
Slc:ddY house mouse	Japan SLC, Inc. (Japan)	–
Software and algorithms		
R 4.2.3	R Core Team ⁸²	https://www.r-project.org/
Rstudio	RStudio Team	https://www.rstudio.com/
R package stats 4.4.1	R Core Team ⁸²	https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html
R package multcomp 1.4.29	Hothorn et al. ⁸³	http://multcomp.r-forge.r-project.org/
R package rstan 2.32.7	Stan Development Team ⁸⁴	https://mc-stan.org/rstan/
R package ggmmcmc 1.5.1.2	Fernández-i-Marín ⁸⁵	https://cran.r-project.org/web/packages/ggmmcmc/index.html
R package ggplot2 4.0.1	Wickham ⁸⁶	https://ggplot2.tidyverse.org/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Experimental design

We conducted controlled feeding experiments on four species of muroid rodents—brown rat (*Rattus norvegicus*), golden hamster (*Mesocricetus auratus*), Mongolian gerbil (*Meriones unguiculatus*), and house mouse (*Mus musculus*)—fed an isotopically defined diet from infancy to adulthood (Data S6).

The experiment is designed to dynamically track enamel-diet isotopic offset and quantify the breastfeeding signal in carbon isotopes of tooth enamel across growth stages, by targeting a series of molars that form during the dietary transition from breastfeeding to adult diet and using breath $\delta^{13}\text{C}$ as a proxy for dietary input (Figure 1).

METHOD DETAILS

Animals, diet, and housing

Muroid rodents have reduced dentitions lacking premolars, but with a set of three molars (m1, m2, m3; lowercase for lower dentition) and a pair of ever-growing incisors. Rodent molars begin development prenatally and continue through the lactational stage and after weaning, which means that the first molars are formed during intake of breast milk and thus breast milk isotopically has greater influence on the first molars and lesser on the second and third molars (Table S1). Based on *in vivo* observation of molar dentition,⁸⁷ enamel formation of the first molar in the laboratory mouse is completed when nutrient intake is heavily dependent on maternal milk. The initiation timing of apposition is comparable between rats and hamsters, whereas the eruption timing (therefore, completion of enamel formation) of the first and second molars is earlier in hamsters than rats by nearly 10 days.⁶⁹ Hiatt et al.⁸⁸ observed that the apposition stage of m1 and m2 began on the 3rd and 6th postnatal days, respectively, in Mongolian gerbils, and m1 is expected to erupt by Day 30 in another gerbil species, *M. tristrami* (Yigit, personal communication, 2020).

A commercial chow diet, Charles River Formula-1 (CRF-1; Oriental Yeast Co., Ltd.), was selected for the experiment. During the course of experiments between 2018 and 2020, we used three different production lots. Carbon isotopic values of the whole diet are consistent among the lots (−22.3‰ for lot. 20180314, −22.4‰ for lot. 20190312, and −21.6‰ for lot. 20190805). Powdered samples were sent to Shoko Science Co., Ltd. (Kanagawa, Japan) for measurement of $\delta^{13}\text{C}$ values of the supplier diet.

Four males and four females of each species were obtained from Japan SLC, Inc. (Shizuoka, Japan). For isotopic acclimation, these animals were fed with an experimental diet and water of known isotopic composition for one month prior to 1:1 mating. This duration is slightly longer than a single half-life term of stable carbon isotope turnover of the whole blood in adult rats.⁵⁰ The mating pair was separated when pregnancy was confirmed, except for gerbils where the male was kept with a female and their pups until weaning because both genders provide parental care. Shredded paper bedding on the floor of the plastic cages was replaced regularly to keep the cage clean. Animals had free access to food and water in the environment on a repeated cycle of 12-hour light/12-hour dark at 24 °C with ambient humidity of 40 to 60%. They were euthanized with >5% Isoflurane at Day 60 or later. All tooth loci were fully erupted and functional in all individuals.

Breath sampling

Carbon isotope ratios of breath reflect those of diet and the amount of methane production.^{27,34} In previous studies, the isotope enrichment ϵ^* between breath and consumed diet was measured nearly 0‰ for small coprophagous hindgut fermenters with negligible methane production (0.04‰ averaged from the Ansell's mole-rat *Fukomys anselli* and European rabbit *Oryctolagus cuniculus*; Cerling et al., 2021). We approximated $\delta^{13}\text{C}$ of the assimilated portion of the commercial chow by $\delta^{13}\text{C}$ of breath CO_2 with a 0‰ correction for the studied rodents. Breath $\delta^{13}\text{C}$ (-19.66‰) was measured in adult females of the mouse (*Mus musculus*), whose values were monitored from the 3rd to the 30th days after giving birth (Figure S1). In our experiment, the isotope spacing between breath CO_2 and the supplier diet was consistently greater by a few permil in female adults (male adults were not measured). We presumed that this apparent isotope enrichment came from differential digestion of the nutrition-rich commercial chow (CRF-1), whose ingredients are strictly confidential by the manufacturer. Despite the differential digestion, we chose CRF-1 over a homogeneous diet to ensure well-balanced nutrition for both mothers and their pups.

Our sample chamber is a screw-top container of an appropriate size (150 ml, 360 ml, 640 ml, 940 ml) with a silicone O-ring fitting. On the lid of the food container, a 1/4 in stainless steel ultra-torr vacuum fitting SS-4-UT-6-400 (Swagelok, USA) was attached as a sampling port, in which a 9.5mm (3/8 in) silicone septum Thermolite Plus Septa (Restek, USA) was placed (Figure S2). Two shut-off valves (PISCO, Japan) were installed for flushing air. After placing animals in the sample chamber with the bedding material, CO_2 -free air was flushed for 2 min to remove CO_2 derived from ambient air. We collected 5 mL exhaled breath samples by a gas-tight syringe A-2 series with a side port needle (VICI, USA). The sampling duration (the duration that an animals were placed in the chamber after flushing) was set between 1 and 5 min, depending on the size of the container and total body mass, to level the concentration of CO_2 in a vial as much as possible. For pups, four individuals were initially placed together in a chamber, with the number of individuals reduced as they grew. The sampled pups were separated by gender but otherwise selected randomly until each was raised in a separate cage. Each adult individual was analyzed separately. For mother-pup pairs, breath sampling was done first with a mother.

The sampled breath was stored in a 5-ml glass vial sealed with a freeze-drying butyl septum (stopper) and an open-top screw cap. To inject collected breath without piercing the butyl septum, the vial was held with the bottom up inside a plastic bag and flushed with nitrogen by placing a stainless-steel capillary tube between the legs of the butyl septum so that the glass vial and plastic bag were filled with nitrogen in a closed condition as much as possible. The breath sample was injected between the legs immediately after taking out the capillary tube. Before injection, the gas-tight syringe was set at 4ml or less with the on-off valve closed, and the excess of the sample gas was released with a quick on-off motion to flush out CO_2 from ambient air inside the syringe needle. The glass vial was then stored in a vacuum-sealed bag until stable carbon isotope analysis.

Tooth collection

These pups were euthanized with >5% Isoflurane at Day 60 or later. All tooth loci were fully erupted and functional in all individuals. Animal bodies were cleaned by flesh-eating beetles (*Dermestes* sp.). The skulls were soaked with 2% papain enzyme solution at 38 °C over 24 hours to break down connective tissues attached to teeth. Teeth were then pulled off the softened bones in a wet condition. For molars with complex tooth roots, a sewing needle was inserted between tooth roots and lifted by lever action to pull out molars without damaging the skulls and mandibles. The iron-pigmented orange-brown labial enamel of incisors typical of rodents was completely abraded using a low-speed drill with a dental diamond bur. After mechanically cleaning with a brush and an insect pin, teeth were soaked in 2% NaOCl overnight at room temperature to remove organic matter, rinsed with deionized water several times, and treated with 0.1M buffered acetic acid for 1 hr to remove calcified residuals, if present, on the tooth surface, followed by rinsing with deionized water again.

Stable carbon isotope analysis

Carbon isotope compositions of breath CO_2 samples were analyzed by a gas chromatography–isotope ratio mass spectrometry (GC-IRMS) equipped with an on-column injection port and cryo-focusing trap at the Institute of Science Tokyo, Japan. High purity helium (99.9999%) was used as the system carrier gas. Breath samples were injected with a gas-tight syringe through the on-column injection port, cryogenically concentrated in the cryo-focusing trap for 1 min, passed through a GC column (HP-PLOT/Q; 30 m×0.32 mm, 20 μm) kept at 40 °C at a He flow rate of 1.5 mL/min, and then transported to the Finnigan Delta Plus XL mass spectrometer via a GC Combustion III interface (Thermo Fisher Scientific). Any residual gas was baked at 250 °C for 5 min after every run. As a daily blank check, helium carrier gas was collected in the cryo-focusing trap for 1 min and measured, but the signals were negligible and undetected by the instrument during all analyses. For every session, CO_2 gas ($\delta^{13}\text{C} = -10.40\text{‰}$) from OZTECH (OZTECH

Trading Corporation, USA), adjusted to a CO₂ concentration of approximately 0.5% in the same vial as the breath sample, was measured for isotopic standardization to the VPDB scale. The overall precision (1 standard deviation, SD) of the subsampled standard gas was $\pm 0.4\%$.

Due to small sample size of the studied rodents, the in-situ thermal laser ablation GC-IRMS method, designed by Sharp and Cerling⁴⁸ and enhanced by Passey and Cerling,⁸⁹ was performed to analyze carbon isotope composition in tooth enamel of molars (m1, m2, m3) and incisors at the University of Utah. Tooth samples were mounted with Blu-Tack (Bostik) on a plate of the laser chamber. For lower molars, the lingual side was preferably used for analysis because its surface is flatter than that of the labial side, but the labial side was analyzed when data results from the lingual side were not reliable due to low CO₂ yield or organic contamination derived from the dentine (visible as charring) or Blu-Tack (visible as heavy smoke). Targeting on the previous laser pits was avoided. The CO₂ laser (wavelength of $\sim 10.6\ \mu\text{m}$) was set to range from 5.5 to 17 W with 8.5 ms pulse duration. Laser-generated CO₂ from multiple ablation pits was cryogenically trapped for 270 sec, inlet to a gas chromatographic column at 60 °C, and sent to a Finnigan Delta Plus XL mass spectrometer via a GC/CP interface. Leaking between the ends of the glass chamber and metal holder plates is not negligible even with tight sealing by O-rings and N-grease. Blank correction was made by placing liquid nitrogen in the cold finger trap for the same duration as the samples. Internal standard CO₂ was analyzed for additional normalization (1SD = $\pm 0.4\%$). To correct for the offset of carbon isotope values obtained by the laser method from those with the conventional H₃PO₄ method, a piece of molar enamel of an extant African elephant—whose carbon and oxygen isotope values were determined by a H₃PO₄ method—was measured in each laser session. If multiple analytical runs were performed for a single specimen, the average value of the measurements was used for further analyses. *Mus* (house mouse) teeth were excluded from the laser-based isotope analysis because m3 is too small to analyze with the instrument.

Carbon isotope enrichment

Apparent carbon isotope enrichment, $\varepsilon^*_{\text{enamel-}^{13}\text{C}_2} = [(1000 + \delta^{13}\text{C}_{\text{enamel}})/(1000 + \delta^{13}\text{C}_{\text{assimilated portion of adult diet}}) - 1] \times 1000$, was calculated,⁹⁰ approximating $\delta^{13}\text{C}$ of assimilated portion of adult diet by $\delta^{13}\text{C}$ of breath CO₂ in non-pregnant adult mice ($\delta^{13}\text{C} = -19.66\%$) with a 0‰ offset. The superscript * indicates that the biochemical process involving digestion, diffusion in body fluid, and precipitation of carbon as bioapatite in tooth enamel is a non-reversible reaction.⁹⁰ We approximated $\delta^{13}\text{C}$ of the assimilated portion of the adult diet by average $\delta^{13}\text{C}$ of breath CO₂ ($\delta^{13}\text{C} = -19.66\%$) in non-pregnant adult mice ('CO₂') with a 0‰ offset,³⁴ instead of directly measuring $\delta^{13}\text{C}$ of the commercial chow, because we confirmed that the commercial chow was not homogeneously assimilated in the analyzed animals.

This assumption is reasonable as exhaled breath CO₂ rapidly reaches isotope equilibrium with $\delta^{13}\text{C}$ of diet in small mammals and birds.^{51,78,79} Regarding metabolic rates at different growth stages, similar isotopic behavior was observed between juveniles and adults of the Eurasian shrew (*Sorex araneus*), in $\delta^{13}\text{C}$ of breath CO₂ during a short-term dietary-switch experiment.⁵¹ We take advantage of a previous study showing the isotope enrichment ε^* between breath and diet is known to be nearly 0‰ – specifically 0.04‰ averaged from the Ansell's mole-rat *Fukomys anselli* and European rabbit *Oryctolagus cuniculus* – for small mammals with minimum methane production.³⁴ The breath CO₂-based approximation for $\delta^{13}\text{C}$ of assimilated diet (= 'CO₂') is necessary because we need to monitor a progressive isotopic change of assimilated nutrients from exclusive breast milk through varying mixing degrees to the fully adult diet.

To clarify which values are being compared in our experimental data, $\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}$ describes ¹³C enrichment in tooth enamel relative to breath CO₂-approximated assimilated portion of adult diet, whereas $\varepsilon^*_{\text{enamel-progressive diet}}$ expresses the enrichment relative to progressively changing diet (also breath CO₂-based approximation) during a transition from milk to adult diet.

Dynamic monitoring of isotope enrichment

We expect that isotopic dietary shift from breast milk to the adult diet is described as one-compartment modelling,⁸⁰ if not strictly analogous to isotopic turnover in tissues by incorporating a new diet into a growing body. We modelled the dietary transition from breast milk to adult diet as a one-compartment process, using standard equations.^{50,90–93}

For enamel,

$$\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(t) = \varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(eq) + [\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(0) - \varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(eq)] e^{-(k+m+b)t} \quad (\text{Equation 1})$$

where $\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(t)$ is isotope enrichment between enamel and breath CO₂-approximated assimilated portion of adult diet (-19.66‰, Figure S1) at timing (postnatal t days) of tooth eruption into oral cavity ($\approx$ completion of enamel precipitation) (Figure 1B and Table S1). $\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(0)$ is the initial enrichment of breath at time 0 (day of birth), $\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}(eq)$ is asymptotic enrichment value at equilibrium, k is the growth rate, and m is the metabolic constant. A new term b is the dietary behavioral constant (turnover due to progressive dietary switch from breast milk). That is, $k+m+b (= \lambda)$ represents the turnover rate of isotope ratios resulting from growth, metabolic processes, and dietary behavioral change.

The vertical spacing of two fitted curves for enamel ($\varepsilon^*_{\text{enamel-}^{13}\text{C}_2}$) and breath CO₂ ($\varepsilon^*_{\text{breath at day } t\text{-}^{13}\text{C}_2}$) yield age-specific ε^* between enamel and the progressively changing diet. We fit exponential curves to ε^* for breath CO₂ and enamel using the *nls* function of the R package *stats*.⁸² A self-starting function (the *SSasympt* function) was used to evaluate initial estimates of the parameters, $\varepsilon^*(0)$, $\varepsilon^*(eq)$, and λ for our datasets. Furthermore, we estimated Bayesian credible intervals to quantify the uncertainty of unobserved parameters,

using the R package *rstan*⁸⁴ with the *ggmcmc* package.⁸⁵ The lower and upper bounds of uninformative prior distributions of the model parameters sufficiently cover the initially estimated values. A noise term was assumed to be normally distributed. In the *stan* function, Markov chain Monte Carlo (MCMC) sampling was set as follows: chains=4, iterations=5000, warmup=2500, thin=1 for 500 samples.

Reaction progress models

We used the reaction progress model to determine turnover rates of C in breath CO₂ and molar enamel.^{90,94} The model expresses a turnover curve as a fraction of completion of a switch from one isotopically-distinct source to a different source. The reaction progress variable begins with transposing Equation 1 as follows.

$$\frac{\delta^{13}\text{C}(t) - \delta^{13}\text{C}(eq)}{\delta^{13}\text{C}(0) - \delta^{13}\text{C}(eq)} = e^{-\lambda t} \quad (\text{Equation 2})$$

Turnover rates of carbon in breath CO₂ and molar enamel were determined by the reaction progress model.^{90,94} The reaction progress variable (F) is scaled between 0 and 1 as a fraction of change at time t and can be written as

$$\frac{\delta^{13}\text{C}(t) - \delta^{13}\text{C}(eq)}{\delta^{13}\text{C}(0) - \delta^{13}\text{C}(eq)} = 1 - F \quad (\text{Equation 3})$$

Equations 2 and 3 were then combined as

$$\ln(1 - F) = -\lambda t$$

which is a straight line with a slope of turnover rate λ determined by plotting $\ln(1-F)$ vs. time. The half-life $t_{1/2}$ was given by

$$t_{1/2} = \ln(2)/\lambda$$

where λ was calculated based on data from Day 0 to Day 40.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses and calculations described above were performed using R 4.2.3.⁸² In the calculations of ϵ^* and subsequent statistical analyses, the values were rounded to one decimal place only for the final descriptive summaries. The significance level was set to 0.05 (*=0.01, **=0.001, ***<0.001) for ANOVA and Tukey post-hoc tests. The *glht* function in the R package *multcomp*⁸³ was used for the post-hoc tests. The data were visualized with the *ggplot2* package⁸⁶ in R.

Permission

Experiments were conducted with approvals of the Institutional Animal Care and Use Committee at Hamri (18-068 and 19-H099) and the National Museum of Nature and Science, Japan (22-002) in accordance with the Guidelines for the Care and Use of Laboratory Animals (AAALAC International).