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The isotope record of short- and long-term dietary changes in sheep tooth enamel: Implications for quantitative reconstruction of paleodiets

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Abstract

Quantitative reconstruction of paleodiet by means of sequential sampling and carbon isotope analysis in hypsodont tooth enamel requires a precise knowledge of the isotopic enrichment between dietary carbon and carbon from enamel apatite (ε_{D-E}), as well as of the timing and duration of the enamel mineralization process (amelogenesis). To better constrain these parameters, we performed a series of controlled feeding experiments on sheep ranging in age from 6 to 24 months-old. Twenty-eight lambs and 14 ewes were fed isotopically distinct diets for different periods of time, and then slaughtered, allowing the timing and rate of molar growth to be determined. High resolution sampling and stable carbon isotope analysis of breath CO_2 performed on six individuals following a diet-switch showed that 70–90% of dietary carbon had turned over in less than 24 h. Sequential sampling and carbon isotopic analysis was performed on the first (M_1) and second (M_2) lower molars of four lambs as well as on the third lower molar (M_3) of 11 ewes. The changes in diet were recorded in all molars. We found that the length of enamel matrix apposition is approximately one-quarter of the final tooth length during crown extension, and that enamel maturation spans slightly less than 3 months in M_1, and 4 months in M_2 and M_3. Portions of enamel in equilibrium with dietary carbon were used to calculate ε_{D-E} values. Animals on grass silage diets had values similar to previous observations, whereas animal switched to pelleted corn diets had values ca. 4‰ lower, a pattern consistent with lower methane production observed for animals fed concentrate diets. The tooth enamel forward model of Passey and Cerling (2002) closely predicted the amplitude of isotope changes recorded in tooth enamel, but slightly underestimated the rate of isotope change, suggesting that the rate of accumulation of carbonate during maturation may not be constant over time. Although stable isotope profiles in tooth enamel represent underdetermined systems, our results demonstrate that they can provide useful information about dietary variability if the mineralization process is taken into account.
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1. INTRODUCTION

Because tooth enamel is not remodeled once fully mineralized, it holds a record of individual biochemical history over the time of tooth development. Its high resistance to diagenesis makes it a material of choice for paleodietary and palaeoenvironmental reconstruction using stable carbon and oxygen isotope analysis of carbonate or phosphate in biogenic apatite. In hypsodont (high-crowned) teeth, rapid (i.e., seasonal) changes in dietary preferences or climate can be documented by adopting a sampling strategy that

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follows the main direction of tooth growth. Koch et al. (1989) published the first attempt to investigate the record of seasonality in proboscidean tusk dentine by means of sequential sampling and stable isotope analysis, followed by Fricke and O'Neil (1996) who adapted the procedure to bison and sheep tooth enamel. The procedure, consisting of sampling along the crown to produce a series of samples perpendicular to the tooth growth axis, with each sample penetrating partly or completely through the enamel layer, was successful in detecting variations in the oxygen isotope composition of enamel phosphate, which was interpreted as reflecting seasonality. The procedure has since been widely applied, involving carbonate as well as phosphate oxygen isotope analyses (e.g., Stuart-Williams and Schwarcz, 1997; Fricke et al., 1998; Koch et al., 1998; Kohn et al., 1998; Sharp and Cerling, 1998; Fox and Fisher, 2001; Balasse et al., 2002, 2003, 2005, 2009; Zazzo et al., 2002). These reconstructions, however, are bound to remain essentially qualitative until two conditions are met.

First, a precise knowledge of the isotopic enrichment between diet and bioapatite is necessary to reconstruct the carbon isotope value of the food source based on enamel $\delta^{13}C$ values. Previous controlled feeding experiments have shown a rather large inter-species variability, with values usually ranging between 9‰ and 15‰ (DeNiro and Epstein, 1978; Ambrose and Norr, 1993; Tieszen and Fagre, 1993; Balasse, 2002; Howland et al., 2003; Jim et al., 2003; Passey et al., 2005a). These differences were interpreted as resulting from differences in the digestive physiology of these taxa, through methanogenesis (Metges et al., 1990; Cerling and Harris, 1999; Jim et al., 2003; Passey et al., 2005a). Among ruminants, a significant part of metabolic CO_2 is lost as methane, a by-product of microbial activity in the rumen (Van Soest, 1994). Because methane is depleted in ^{13}C by 30–40‰ relative to food (Rust, 1981), it leaves the remaining metabolic CO_2 enriched in ^{13}C by several permil (Schulze et al., 1997). This CO_2 then enters the blood stream in the form of bicarbonate which in turn is incorporated into bone and tooth apatite (Metges et al., 1990). This could explain why diet–apatite isotope discrimination is higher in ruminants than in rodents (Passey et al., 2005a). Moreover, intra-specific variability in diet–apatite isotope discrimination is not well documented and could play a role when animals switch between diets with different nutritional characteristics. This could lead to uncertainties when calculating the $\delta^{13}C$ value of an animal's diet based on carbon isotope analysis of tooth enamel.

Second, a detailed understanding of the mineralization process is required for meaningful interpretation of the isotope profiles performed along the tooth growth axis. Enamel formation is a two stage process, starting with the secretion of a mineral-poor matrix, followed by maturation into highly mineralized enamel (Weinmann et al., 1942; Suga et al., 1970, 1987; Suga, 1979, 1982; Sakae and Hirai, 1982; Moss-Salentijn et al., 1997). Because the entire process can take up to several months in large mammals, this can lead to underestimates in the magnitude and timing of short-term dietary or climatic changes recorded by tooth apatite. The issue of temporal resolution was first pointed out by Fisher and Fox (1998) in proboscidean molars, and the pattern of enamel mineralization was investigated through stable isotope analysis using controlled feeding experiments and modeling (Balasse, 2002, 2003; Passey and Cerling, 2002; Passey et al., 2005b; Zazzo et al., 2005). The models proposed by Passey and Cerling (2002) and Passey et al. (2005b) for mammals with continuously growing teeth and made a series of assumptions regarding the mineralization process, including unchanging maturation parameters, a linear increase in mineralization over time, and a constant growth rate. At this stage, it is still unclear whether these models are well-suited to ruminant teeth with definite growth.

In this paper we present the results of several controlled feeding experiments on sheep. The objectives of these experiments were threefold: (1) to refine our knowledge of the timing of tooth enamel development in sheep, (2) to determine the carbon isotopic enrichment between carbonate in enamel and different types of diets, and (3) to investigate the temporal and spatial dynamics of incorporation of carbonate in enamel. Sequential sampling and stable isotope analysis were performed on the first (M_1), second (M_2) and third (M_3) lower molars, because these teeth, together, provide a continuous record of individual history encompassing the first 2 years of life (Weinreb and Sharav, 1964; Milhaud and Nézit, 1991). Comparisons between measured isotope data and predictions of the forward model allowed us to quantify the time lag and attenuation of the isotope signal due to amelogenesis, increasing our ability to reconstruct the original environmental signal from an isotope signal measured in enamel.

2. MATERIALS AND METHODS

2.1. Experimental conditions

2.1.1. Lamb experiment: C_3 to C_4 switch

Twenty-eight Suffolk cross lambs (14 males and 14 females), born at the Teagasc Production Research Centre, Athenry, Co. Galway, Ireland between March and April 2006, were taken from their mothers at pasture 0 to 7 days after birth and raised on artificial milk for 6 weeks. During this period, the animals were slowly weaned from the artificial milk and introduced to a commercial diet (pre-experimental diet). The pre-experimental diet was offered *ad libitum* and was a mixture of cooked and flaked C_3 and C_4 plant material including barley flakes, maize flakes, maize gluten, cane, molasses and oats (for more details regarding the composition of the pre-experimental diet, see Zazzo et al., 2008). The animals were moved to the Teagasc Grange Beef Research Centre, Dunsany, Co. Meath, Ireland in June 2006 but maintained on the control diet until the start of the experiment in September 2006. At the beginning of the experiment, the animals were statistically blocked according to initial weight and sex, penned individually and assigned to an experimental diet for 0 (L1), 14 (L2), 28 (L3), 56 (L4), 98 (L5), 154 (L6), or 231 days (L7), followed by slaughter. The experimental diet consisted of 76% (wet weight basis) of pelleted maize concentrate produced in one batch at Teagasc Moorepark Dairy Production Research Centre, Fermoy, Co. Cork, Ireland, and

Table 1
Carbon isotope compositions and macronutrient contents of the feed used in this study.

		$\delta^{13}C$ (‰, VPDB)	Dry matter (DM, g/kg)	Crude protein (g/kg DM)	Ash (g/kg DM)	DM digestibility (g/kg)	Metabolisable energy (MJ/kg DM)
Grass silage (C_3)	Mean	−31.1	227	152	86	724	9.9
	±1 SE	0.4	2.4	6.6	6.2	15.4	0.23
	n	7	8	8	8	8	8
Maize silage (C_4)	Mean	−12.1	290	105	45	737	10.1
	±1 SE	0.3	2.8	7.3	6.5	21.9	0.32
	n	11	8	8	8	8	8
Hay (C_3)	Mean	—	823	104	57	631	8.5
	±1 SE	—	3.2	4.4	1.4	3.7	0.05
	n	—	4	4	4	4	4
Control diet (C_3/C_4)	Mean	−22.8	851	223	96	—	11.4
	±1 SE	0.4	1.3	9.9	15.4	—	0.3
	n	3	6	6	6	6	6
Maize concentrate (C_4)	Mean	−12.3	844	244	86	—	12.6
	±1 SE	0.4	1.4	4.5	2.2	—	0.14
	n	10	8	8	8	8	8

Chemical compositions were determined as described in Moloney and O'Kiely (1995).

24% (wet weight basis) of maize silage. The average carbon isotope value and macronutrient content of the control diet, pelleted maize concentrate and maize silage are given in Table 1. Each treatment group consisted of two male and two female animals which were further assigned to two groups of one male and one female animal at either a low energy allowance (LEA) or a high energy allowance (HEA) of the experimental diet. Feed allowances were adjusted regularly throughout the experiment to ensure a constant weight gain of 50 g d^{-1} for animals receiving the LEA and of 150 g d^{-1} for animal receiving the HEA. At the beginning of the experiment, the four lambs from group L7 weighed between 41 and 47 kg. Over the time of the experiment, the two LEA lambs (#9125 and #9169) gained ~10 kg, whereas the two HEA lambs (#9351 and #9646) gained ~25 kg (see Fig. 1 and Table 2 in Zazzo et al., 2008 for more detail). Food was offerered to each animal individually in one batch in the morning together with unlimited access to tap water.

2.1.2. Ewe experiment: C_3 to C_4 switch and C_3 to C_4 to C_3 switch

Fourteen Suffolk cross ewes born between March and April 2005 and raised in Co. Carlow, Ireland, were selected for the experiment. The ewes were raised outdoors on pasture from weaning. The diet $\delta^{13}C$ value was not directly measured, but we assume that it was close to the yearly average value of $-30.3 \pm 0.5‰$ ($n = 18$) for grass collected in various farms in Ireland (Zazzo et al., 2008), and that it varied seasonally between −32‰ (during winter) and −29‰ (during summer). Ewes were brought to the Teagasc Grange Beef Research Centre, Dunsany, Co. Meath, Ireland on the day prior to the switch to the experimental diet (August 28th, 2006) and penned individually. Five groups of two or three ewes were each offered the same experimental diet as the lambs for 0 (E1), 14 (E2), 28 (E3), 56 (E4), and 238 (E5) days. Animals from groups E1 and E5 were slaughtered after 0 and 246 days, respectively. Ewes from group E2 to E4 were switched back to a C_3 diet (grass silage for most of the time, then hay in the summer) and slaughtered 182–224 days later. The carbon isotope value and macronutrient content of grass silage and hay are given in Table 1. Food allowance was adjusted to ensure maintenance weight and was offered to each animal individually in one batch in the morning, together with unlimited access to tap water.

2.2. Breath sampling and analysis

Previous studies indicated that $\delta^{13}C$ values of expired CO_2 track the isotopic composition of blood dissolved inorganic carbon (DIC), which in turn is in isotopic equilibrium with carbonate in bioapatite (Tieszen and Fagre, 1993; Panteleev et al., 1999). Controlled experiments on horses also demonstrated that the isotopic composition of respired CO_2 closely tracks changes in the isotopic composition of food (Ayliffe et al., 2004) and can therefore be used as a tracer of the isotopic composition of blood DIC through time.

Breath CO_2 samples were collected from six sheep (two ewes from group E5 and four lambs from group L7) for up to 112 days following the diet-switch. Breath samples were collected intensively for the first 3 weeks to document short-term changes of breath $\delta^{13}C$ values following the diet-switch. Breath samples were collected by placing a flexible plastic cup over the animal's mouth. Attached to the base of the cup was a 60 ml syringe into which a mixture of respired and atmospheric gas was collected over a period of about 10 s. Gas was then injected through a rubber septum into a 10 cc headspace vial and stored in a cold room for up to 2–3 months. Samples were analysed by continuous flow–isotope ratio mass spectrometry on a Europa Scientific Hydra 20-20/ANCA-G instrument. Samples of IA-CO2-3 prepared in Exetainer tubes to 4% CO_2 were analysed as check samples during the analysis. Breath samples

collected typically contained ~1–6% CO_2 by volume (10,000–60,000 ppm). No correction was made for atmospheric CO_2 likely to be present in each sample, because atmospheric concentrations (ca. 360 ppm) are small compared to breath CO_2 concentrations, and the isotopic effect of atmospheric CO_2 contamination has been shown to be negligible (Passey et al., 2005a).

2.3. Animal selection, enamel sampling, and isotope analysis

Mandibles from all lambs and ewes were defleshed and cleaned by boiling. Molars were extracted from mandibular bone, enamel surfaces were cleaned by abrasion with a tungsten drill bit, and molar crown heights were measured. Measurements were taken on the buccal side of the anterior lobe of each molar. Teeth from four lambs (group L7) and 11 ewes (groups E2, E3, E4, and E5) were selected for stable isotope analysis. Selection of teeth for stable isotope analysis was guided by previous knowledge of the pattern of tooth development (Milhaud and Nézit, 1991). First (M_1) and second (M_2) lower molars were sampled from the lambs, whereas third (M_3) lower molars were sampled for the ewes. Sequential sampling of enamel was performed using a diamond drill bit. Each sample was a 1-mm-wide groove perpendicular to the tooth growth axis, taken on the anterior lobe from the buccal side through the thickness of the enamel layer. Enamel powder was treated with NaOCl 2–3% (24 h) to remove organic matter and rinsed three times with distilled water. It was then treated with 0.1 M acetic acid (4 h, 0.1 ml mg^{-1}), then rinsed five times and oven-dried at 50 °C. The acetic acid treatment was to remove exogenous carbonate and was carried out to ensure consistency with other studies involving archaeological material. Bioapatite samples weighing ~700 μg were reacted with 100% phosphoric acid at 70 °C for 4 min in a Kiel IV device, interfaced with a Delta V Advantage isotope ratio mass spectrometer. Analytical precision was ±0.03‰ for $\delta^{13}C$ (1σ) based on repeated analysis ($n = 106$) of our internal calcite standard (previously calibrated against NBS-19) over the period of analysis.

2.4. Modeling

We used the forward model of tooth enamel mineralization developed by Passey and Cerling (2002). A complete description of the model and examples are provided in Passey and Cerling (2002) and Podlesak et al. (2008). Mathematically, the model is very similar to a running average and assumes that the appositional and maturation fronts are laid down at the same angle. The main parameters of the mineralization model are the length of apposition (l_a), length of maturation (l_m), and mineral content during initial enamel deposition (f_{init}). Apposition length l_a is the length of crown over which the enamel matrix is accreted. This parameter was estimated post-mortem by visual examination of the developing molars: enamel matrix whose secondary mineralization has not started was identified by a change in colour and brightness. The initial fraction of mineralization (f_{init}) is deposited during this stage. Maturation length l_m is the length along the tooth over which secondary mineralization ($1 - f_{init}$) occurs. This parameter was estimated graphically, by measuring the distance between portions of enamel formed entirely before and after the diet-switch. The chronological framework was provided by our estimates of the timing and rate of molar growth and time of diet-switch. Input variables were determined by the isotope analysis of diets and breath CO_2. We did not directly estimate the amount of carbonate incorporated during the secretory stage (f_{init}) and set this parameter to 0.25 based on previous estimates of the mineral content of developing tooth enamel (Passey and Cerling, 2002).

3. RESULTS

3.1. Timing and rate of tooth growth

Results obtained are shown in Fig. 1. In 6 months-old lambs, the M_1 crown had already reached its full length, whereas half of the M_2 crown was formed. M_3 crown growth was complete when the animals were ~11–12 months old, a time when ~1/3 of the crown of M_3 was formed. Development of the third molar started before the lamb reached 11 months and was completed between 18 and 25 months in ewes. These results are in good agreement with results previously published by Milhaud and Nézit (1991) on a different breed of sheep (Préalpes du Sud). Based on radiography and visual inspection of 48 individuals, Milhaud and Nézit (1991) found that tooth crown development starts *in utero* and ends at 6–7 months for M_1 starts at one month and ends at 11–12 months for M_2 and starts at 9–10 months and ends at 20–22 months for M_3. Witter and Misek (1999) observed that in sheep, the first molar starts growing two months before birth. Based on these data, as well as average crown heights measured in unworn teeth in the present study, an average growth rate of 132 ± 15 μm d^{-1} was

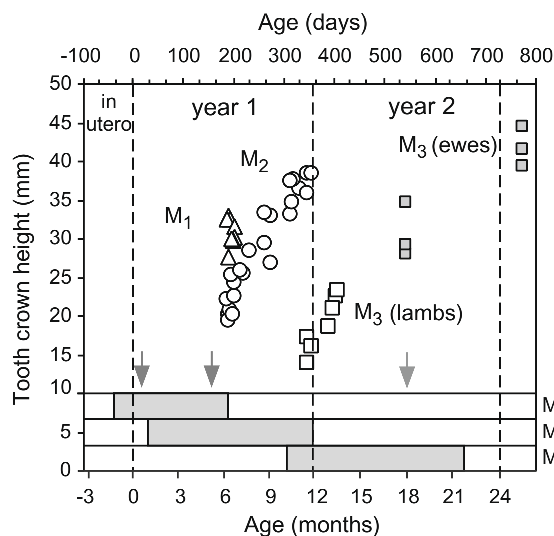

Fig. 1. Timing of tooth crown growth in the first (open triangle), second (open circle) and third molars (open squares for lambs and grey squares for ewes) of sheep based on our data and Milhaud and Nézit (1991). Arrows indicate average tooth crown height at the time of the initial diet-switch.

calculated for M_1 assuming that this 32 mm-long tooth takes 8 ± 1 months to grow. Average growth rates of $115 \pm 10\ \mu m\ d^{-1}$ were calculated for the second and third molars.

3.2. Carbon isotope record of diet-switch

3.2.1. Breath CO_2

Breath $\delta^{13}C$ values increased very rapidly following the diet-switch and approached equilibrium with the experimental diet very quickly (Fig. 2). Between 70% and 90% of the isotope difference between the pre-experimental and the experimental diet were found in sheep breath sampled less than 24 h following the diet-change. However, almost all breath $\delta^{13}C$ values measured 3 weeks or more after the diet-switch were still 0–4‰ more negative than the diet $\delta^{13}C$ value. This is in contradiction with Passey et al. (2005a) who found that ruminant (cattle) had breath $\delta^{13}C$ values that were 3‰ more positive than diet. We also discovered that animals that were sampled more than 10 h after feeding showed $\delta^{13}C$ values that were consistently (up to 5‰) lower than when breath was sampled 3–10 h after feeding. Similar results were observed by Passey et al. (2005a) on voles. Because most of the samples between weeks 2–16 were taken immediately before or following the animal feeding, this diurnal variation could potentially have affected to some extent all the breath isotope values during that period.

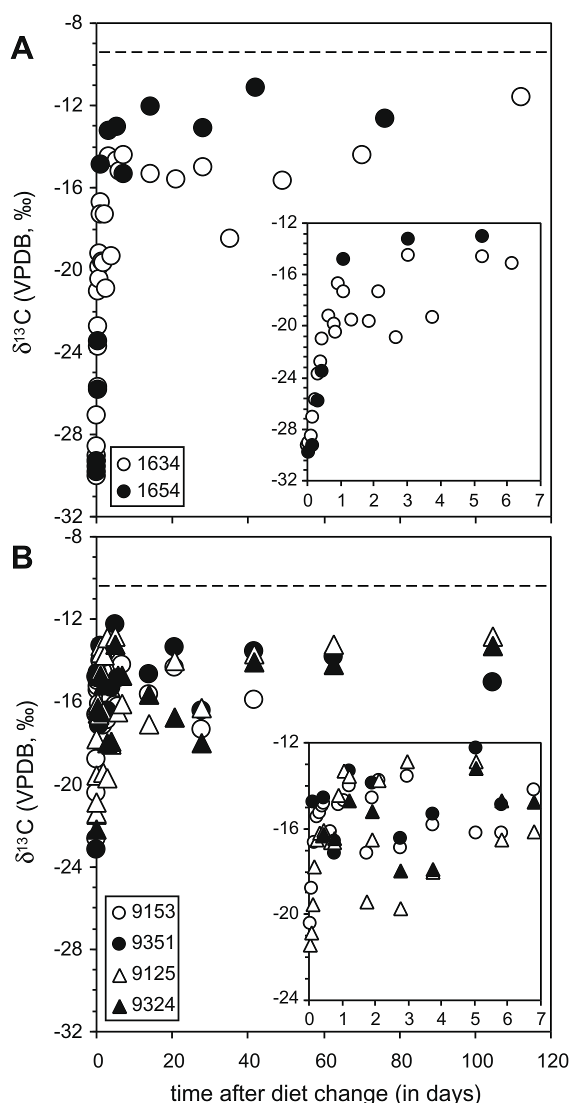

Fig. 2. Carbon isotope ratios ($\delta^{13}C$) of breath CO_2 collected from two ewes (A) and four lambs (B) following a change in diet. Inset is the plot of the $\delta^{13}C$ values during the first week following the diet-switch. The dotted lines correspond to the $\delta^{13}C$ value expected for breath in equilibrium with the experimental diet, assuming a breath–diet enrichment of 3‰ for ruminants (Passey et al., 2005a).

3.2.2. Tooth enamel carbonate

Carbon isotope values of tooth enamel are summarized in Fig. 3 and reported in the Appendix. Lamb M_1 recorded a two-step increase in $\delta^{13}C$ values, separated by a plateau (Fig. 3A). The lowest $\delta^{13}C$ values are measured in the uppermost part of the crown and were likely influenced by the mother's C_3 diet during *in utero* life. Carbon isotope values then increased, and stabilized around −9‰. It is likely that this 5–10 mm-long portion of enamel was formed when the animal had the pre-experimental (mixed C_3/C_4) diet. Finally, the lowest part of the M_1 recorded a 5–7‰ increase in $\delta^{13}C$ values, corresponding to mineralization while the lambs were on the experimental C_4 diet. Overall intra-tooth amplitude of change ranged between 7.0‰ and 10.5‰.

Isotope values recorded in the upper part of lamb M_2 matched the values measured in lower part of the M_1 (Fig. 3B). Increasing values were measured in the upper third of the M_2, followed by a plateau at around −0.7‰ suggesting that the lower 2/3 of M_2 were formed entirely when the animal received a C_4 diet. This is independently confirmed by measurements taken on M_2 from lambs slaughtered 2 weeks after the beginning of the experiment showing that the crown was less than 20 mm long at the time of diet-switch. Intra-tooth amplitude of change ranged between 3.8‰ and 4.4‰.

The two consecutive diet-switches C_3–C_4–C_3 were recorded in the third molars, demonstrating that our estimate of the timing of M_3 growth was essentially correct (Fig. 3C–E). Low isotope values (between −16‰ and −17‰) were measured in the uppermost sample of all but two (E4) individuals, suggesting that mineralization of the third molar had started several months before the beginning of the experiment, when the ewes were grazing outdoors. In the first three groups of animals (E2–E4) isotope values increased in the upper third of the crown as a result of the introduction of C_4 plants in the diet, stabilized for about 10 mm, then decreased again as a result of reintroduction of C_3 plants in the diet (Fig. 3C–E). Animals from group E5 recorded a progressive $\delta^{13}C$ increase in the upper two third of the crown (Fig. 3F). The slope of this isotope change became steeper ~15 mm from the top of the tooth. This increase in the isotope values was followed by a plateau in the lower third of the crown around −0.5‰. Intra-tooth variation of enamel $\delta^{13}C$ values ranged between 16.0‰ and 16.3‰ for these three ewes.

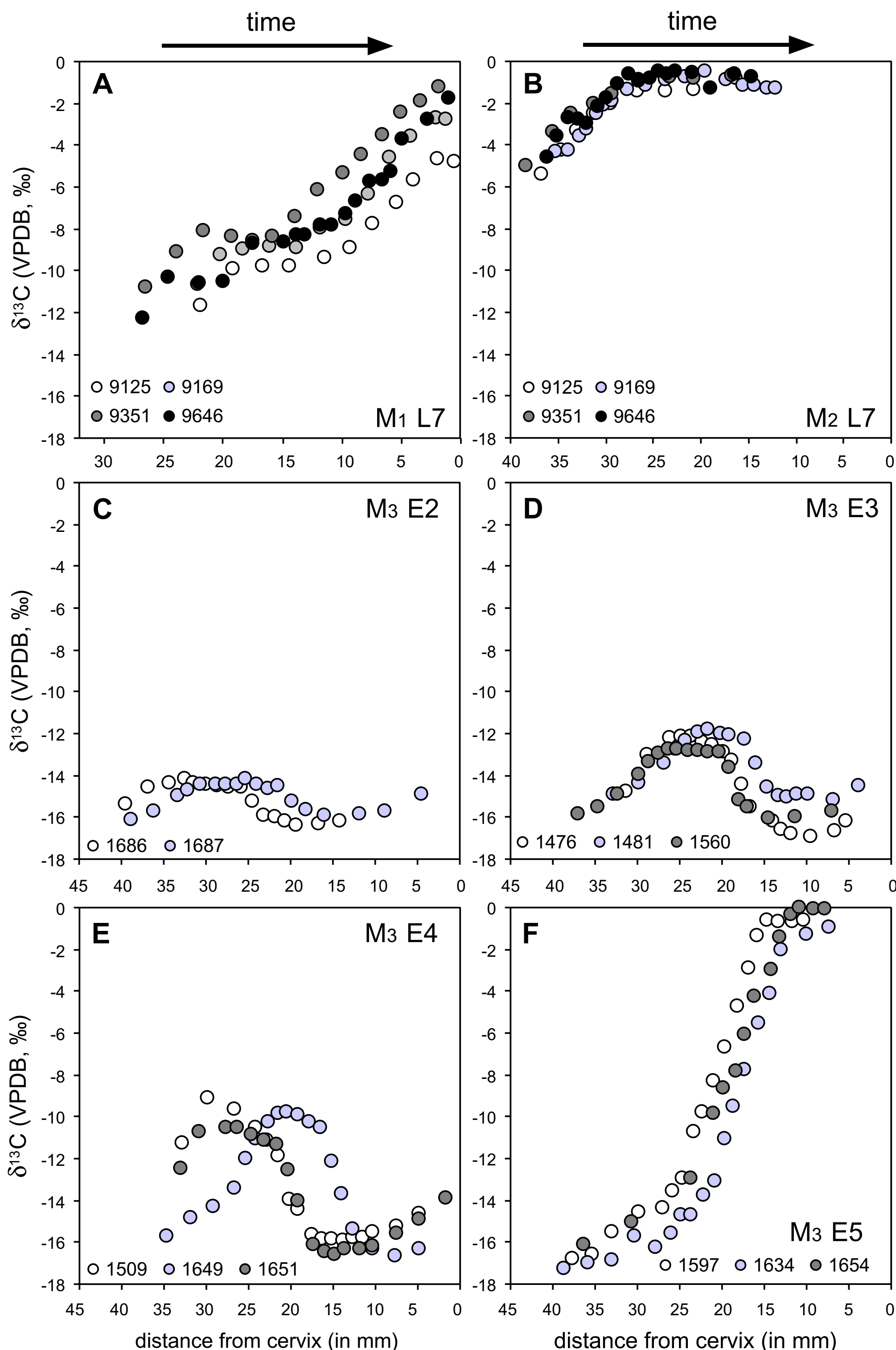

Fig. 3. Variation in carbon isotope ratios (δ^{13}C) measured along the tooth crown of the first (A) and second (B) molars of four lambs from group L7, and along the tooth crown of the third molar of 11 ewes (C–F) from groups E2 to E5. Information regarding the dietary history for each study group is reported in the Section 2. The cervix represents the youngest enamel, and the temporal record is oriented as earlier to the left and later to the right as indicated by the arrow.

Finally, we notice a large inter-individual variability in the part of the crown where the diet-change is recorded. In sheep #1686 (Fig. 3C), the increase in δ^{13}C values begins in the uppermost part of the crown whereas it starts only at mid-height for other individuals (see #1649 in Fig. 3E, or #1634 in Fig. 3F). This ~15 mm difference suggests the

existence of a temporal variability of approximately 130 d in the timing of mineralization of M_3 within this population of sheep. This result is in agreement with the 6 month range of variation estimated for the time of eruption of that tooth in improved sheep breeds (Weinreb and Sharav, 1964; Silver, 1970; Milhaud and Nézit, 1991).

3.3. Diet–apatite isotope enrichment

The fractionation factor α and the isotope enrichment ε between dietary carbon (D) and carbon from enamel apatite (E) were calculated using the following formulae:

$$\alpha_{D\text{–}E} = [1000 + \delta_D]/[1000 + \delta_B] \quad (1)$$

$$\varepsilon_{D\text{–}E} = (\alpha_{D\text{–}E} - 1)/1000 \quad (2)$$

Here, we use the notation ε*, which indicates a fractionation not associated with chemical equilibrium (Cerling and Harris, 1999). Calculation of $\varepsilon_{D\text{–}E}$ values requires that 100% of enamel carbonate is formed during exposure to that diet. Only portions of enamel with stabilized $\delta^{13}C$ values fulfil this condition. For M_1, we assume that the plateau at mid-height in L7 lambs ($-9.4 \pm 0.8‰$) reflects formation during pre-experimental feed and the average enamel value was used to calculate a $\varepsilon_{D\text{–}E}$ of $13.7 \pm 0.8‰$. High and stabilized isotope values in the lower third of the M_2 ($-0.9 \pm 0.3‰$) and M_3 ($-0.5 \pm 0.4‰$) from group E5 were used for calculation of $\varepsilon_{D\text{–}E}$ between maize diet and tooth enamel of $11.5 \pm 0.3‰$ and $11.8 \pm 0.4‰$, respectively. Finally, low values in the lower third of the M_3 in E2–E4 ($-16.2 \pm 0.5‰$) were used to calculate $\varepsilon_{D\text{–}E}$ between C_3 silage and tooth enamel of $15.4 \pm 0.5‰$.

3.4. Estimation of l_a and l_m

Visual inspection of the surface of developing molars led to the identification of a translucent and fragile zone at the bottom of the tooth, which we interpreted as the appositional zone. Average lengths of apposition l_a of 9.8 ± 0.7 mm ($n = 13$) and 10.0 ± 0.7 mm ($n = 7$) were calculated for M_2 and M_3, using 6–9 months-old lambs (for M_2) and 11–14 months-old lambs (for M_3). We used young animals only to calculate l_a because the length of apposition becomes shorter and vanishes to zero at the conclusion of tooth crown formation.

Length of maturation l_m can be estimated using the value found for l_a. In his histological study of developing enamel in sheep molars, Suga (1982) estimated that the time required for the completion of the maturation was about twice as long as that required for the matrix formation. This estimation includes the last wave of mineralization involving an increase in the mineralization gradient only in the thin subsurface layer of enamel (15–20 μm in width), which might not be detected here because of the small amount of carbonate involved. This final stage of maturation takes about 1/3 of the whole maturation time (Suga, 1982). Taking this into account, a length of apposition l_a of 10 mm, as it was estimated in this study, would correspond to a length of maturation l_m (excluding the final phase) of 13.3 mm. This corresponds approximately to 120 d using the average growth rate of 0.111 mm d^{-1} calculated for M_3.

Because M_1 formation was almost complete at the beginning of the experiment, the average length of apposition could not be determined. We assumed that l_a and l_m represent a constant fraction of an animal crown length for a given species and calculated values of 7.5 and 10 mm for l_a and l_m, based on the observation that M_1 is approximately 25% shorter than M_2 and M_3.

4. DISCUSSION

4.1. Variability of $\varepsilon^*_{bioapatite-diet}$ values

Inference of $\delta^{13}C$ value of animal diets based on enamel $\delta^{13}C$ value requires a precise knowledge of the isotopic enrichment between diet and bioapatite. The sheep results are plotted on Fig. 4 and compared with values estimated from previous controlled feeding experiments on different mammals. There is a rather large inter-species variability, with ε values usually ranging between 9‰ and 15‰. Sheep ε values cover about two-thirds of the variability documented for other mammals. Sheep fed pre-experimental (mixed C_3/C_4) diet and C_3 silage show the highest ε values, similar to values calculated for cattle raised under controlled conditions (Balasse, 2002; Passey et al., 2005a) and for wild ruminants ($14.1 \pm 0.5‰$, Cerling and Harris, 1999). These high ε* values are consistent with the hypothesis that methanogenesis, i.e. production of methane by microorganisms within the rumen is responsible for elevated $\delta^{13}C$ values in bone and tooth apatite of ruminants (Metges et al., 1990; Cerling and Harris, 1999; Jim et al., 2003; Passey et al., 2005a).

The low ε values found for lambs ($11.5 \pm 0.3‰$) and ewes ($11.8 \pm 0.4‰$) on the C_4 diet are somewhat surprising.

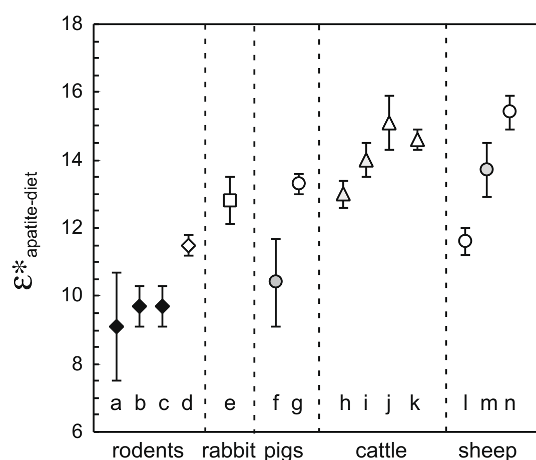

Fig. 4. Single axis plot of epsilon values (mean ± standard deviation) calculated for sheep (circles) compared with published values taken from the literature for rodents (diamonds), rabbit (square), pigs (circles), and cattle (triangles). Open symbols correspond to animals given pure diets; closed symbols correspond to animals given mixed diets (grey), or to cases where bone was used instead of tooth enamel (black). Data are from Tieszen and Fagre (1993) (a); Jim et al. (2003) (b); Ambrose and Norr (1993) (c); Passey et al. (2005a) (d, e, g, k), Howland et al. (2003) (f); Balasse (2002) (h–j); this study (k–m).

A large intra-specific variability in ε^* values was also found for steers and pigs fed different diets (Balasse, 2002; Howland et al., 2003). In the case of pigs, animals were fed mixed C_3/C_4 diets, thus preferential digestion of isotopically distinct components almost certainly influenced ε^* values. Intra-specific variability decreases to 1‰ if only animals with the purest C_3 or C_4 diet are considered. In the case of steers, animals fed a pure C_3 diet, followed by an 89% pure C_4 diet displayed a 2‰ difference in ε^* values (Balasse, 2002). Here, we show that animals raised on pure C_3 and C_4 diets can display 3–4‰ differences in ε^* values. Like in steers, these differences are recorded for the same individual fed different diets in sequence. Two mechanisms can be invoked to explain this result: a long lasting contribution of old carbon to blood DIC, and a diet-related variation in methane production.

One possible mechanism for low epsilon values is old carbon contributing to blood DIC. Different pools are contributing to blood DIC, and differ by their kinetic properties (Ayliffe et al., 2004; Cerling et al., 2007; Podlesak et al., 2008). Metabolic carbon is thought to have a rapid turnover rate, whereas body reserves which are exclusively composed of old carbon at the time of diet-switch have a lower turnover rate. Virtually all the breath $\delta^{13}C$ measured values after the diet-switch were more negative than the diet $\delta^{13}C$ value, whereas the opposite is expected for ruminants (Passey et al., 2005a). The decrease in breath values observed for animals which have not eaten for more than 10 h is also consistent with this hypothesis (Hatch et al., 2002). Hatch et al. (2002) showed that while metabolic carbon dominates breath $\delta^{13}C$ value following dietary intake, the contribution of endogenous lipids becomes more noticeable following a fast. However, given the amount of weight gain (about 10 and 25 kg, respectively for lambs on the LEA and the HEA diets) and time lapsed on the experimental diet for the lambs (231 d) and the ewes (246 d), this explanation is essentially impossible. In addition, there is no difference in ε values between the ewes on a maintenance weight (11.4–12.4‰), the lambs on a LEA (11.0‰ and 11.6‰), and on a HEA (11.6‰ and 11.7‰), further suggesting the old carbon is not the cause for the low ε values. Therefore we rule out the old carbon mechanism, and the real mechanism probably relates to digestive physiology.

Intra-individual differences in ε values for sheep fed different diets in sequence could also be the result of a diet-related variation in methane production. In vivo and in vitro experiments have demonstrated that methane emissions in ruminants are influenced by many factors including type of carbohydrate in the diet, level of feed intake, and food processing (Johnson and Johnson, 1995; Moss et al., 2000). The fermentation of carbohydrates in the rumen gives rise to the formation of large amounts of lower volatile fatty acids (formic, acetic, propionic, and butyric acids) which are absorbed from the rumen into the blood stream. Diets rich in starch favour propionate production, a competitive pathway for hydrogen recycling and will therefore decrease methane production in the rumen. Conversely, a roughage-based diet will increase methane production. A decrease in residence time (through consumption of ground and pelleted diet) and increase in intake induce lower methane losses as a percentage of daily energy intake (Moss et al., 1995; Johnson and Johnson, 1995). Although we did not measure CH_4 production during our experiment, one could reasonably expect to find high amounts of methane production for animals fed silage at a maintenance level, and lower amounts of methane production for animals fed concentrates at high intake. Likewise, ε^* values were 2‰ lower when steers were fed mostly with maize grain and soy bean than when they were fed C_3 grass and milk (Balasse, 2002).

These differences in ε^* values are significant when compared to differences in the $\delta^{13}C$ values of plants that utilise C_3 and C_4 photosynthetic pathways and could potentially lead to large uncertainties when estimating $\%C_3$ or C_4 browse in the ruminant diet. Future studies will have to verify if this observation also applies to animals switching between C_3 and C_4 in the wild.

4.2. Modeling the mineralization process

4.2.1. Blood DIC input signal

In order to model the expected tooth enamel signal, we first had to determine the time-transgressive pattern of change in the $\delta^{13}C$ of dissolved inorganic carbon in blood (blood DIC), because this is the pool from which enamel carbonate is derived and is in equilibrium with. This forms the input signal (*sensu* Passey and Cerling, 2002) that drives the change in tooth enamel isotopic composition. Using the ewe breath data as a proxy for sheep blood DIC and the 3-pool exponential turnover model of Ayliffe et al. (2004) (Eq. (3)), we determined the turnover rate constants for sheep. This approach was described in much detail elsewhere (Ayliffe et al., 2004; Cerling et al., 2007). In short, the model offers several advantages over the traditional approach of fitting a single exponential function to data, including the relatively simple recognition of multiple pools and an improved fit (higher correlation coefficient, r^2). Even if this approach is graphical and does not allow to assign names to pools, it is probably valid to assume that the pool with the most rapid turnover rate corresponds to exogenous (dietary) carbon whereas the pools with slower turnover rates correspond to principally endogenous (tissue turnover) sources. The multi-component model gave half-lives of 0.32, 1.99, and 35 days, with fractional contributions of 0.42, 0.50, and 0.08, respectively for the three pools. This is close to values found for adult horses by Ayliffe et al. (2004). The lamb dataset was too noisy to allow an accurate determination of turnover rate constants and we assumed similar values for lambs. These constants were then used to calculate the evolution of blood DIC $\delta^{13}C$ values following a diet-change (Fig. 5).

4.2.2. Mineralization pattern of sheep molars

Using the reconstructed blood DIC input signal and the $\varepsilon^*_{\text{bioapatite–diet}}$ values corresponding to each diet, we modeled the expected pattern of tooth enamel isotopic variation in the first, second and third molars and searched for the best fit between modeled and measured data. Part of the pre-experimental dietary history of our animals was missing and had to be estimated. To model the isotope composition

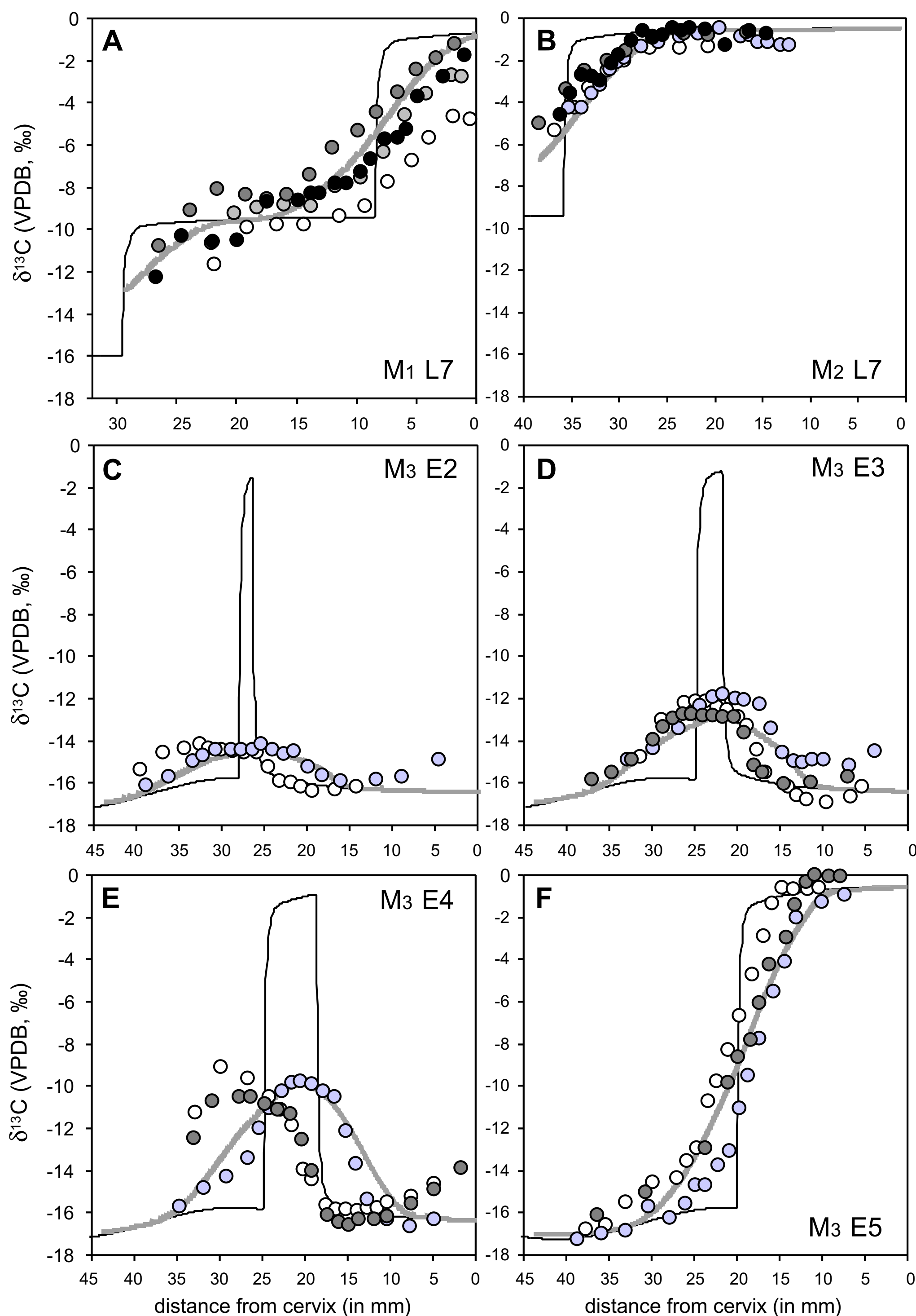

Fig. 5. $\delta^{13}C$ of the modeled dissolved inorganic carbon corrected for isotope fractionation between DIC and enamel in apatite (black line), of the measured carbonate tooth enamel (circles) and of the modeled enamel (grey line) for the first (A) and second (B) molars of lambs from group L7 and for the third molars of ewes from groups E2 to E5 (C–F). Information regarding the dietary history for each study group is reported in the Section 2. Symbols used correspond to results graphed for the same individuals in Fig. 3. Model parameters (l_a, l_m) were 7.5 and 10 mm for M_1, and 10 and 13.3 mm for M_2 and M_3 and $f_{init} = 0.25$ for all teeth. The apparent temporal shift between the modeled input (black line) and modeled tooth enamel (grey line) is an artefact of plotting a temporal signal (modeled input) on a spatial scale (grey line, distance along tooth). See Section 2.2, and Passey and Cerling (2002), for additional discussion of this phenomenon.

of lambs' M_1 we needed to determine pre-birth conditions as this tooth starts forming *in utero*. The mothers were raised on a C_3 diet and we assumed a $\delta^{13}C$ value of $-16‰$ for enamel in equilibrium with this diet. Also, we

did not measure the isotope composition of the grass ingested by the ewes during the 6–9 months preceding the diet-switch. Our pre-experimental input signal is a sinusoidal function varying between −17.2‰ and −15.7‰, based on average values calculated for "winter" and "summer" conditions recorded in modern sheep enamel from Scotland fed pure grass (Balasse et al., 2009).

Knowledge of the average crown deposition rate of the teeth allowed us to plot a time-transgressive signal like the blood DIC input signal on a tooth enamel length axis. We chose to reconcile the time and length scales with reference to the outer enamel surface (OES), as opposed to the enamel-dentine junction (EDJ), because this corresponds to the convention utilised in Passey and Cerling (2002). Regardless of the convention used, there will always be an apparent temporal offset between diet and modeled tooth enamel in graphical displays such as Fig. 5, for example as illustrated for simple dietary input scenarios in Fig. 4 of Passey and Cerling (2002). Because of the temporal variability in the age of eruption of the M_3, the position of the modeled series was adjusted to fit one tooth only per treatment group.

Assuming that M_1 starts to form 2 months prior to birth (Witter and Misek, 1999), the model was successful in reproducing the shape of the isotope profile, including the ~6 mm-long plateau at mid-height (Fig. 5A). The model did not predict this plateau when values of l_a and l_m similar to those measured for M_2 and M_3 were used. A good agreement between model and data was also found for M_2, assuming that this tooth starts to form when the lamb is 1 month-old (Fig. 5B).

The different dietary histories recorded by the third molars in groups E2–E5 provide a stronger test of the coherence of the forward model because the same combination of parameters l_a, l_m, and f_{init} must predict equally well isotopic variation measured in each group of animals. The results of the tooth enamel forward modeling for M_3 are shown in Fig. 5C–F, along with the measured data for each group. The model suggests that the change from C_3 to C_4 diet (indicated by the thin line) occurred when the third molar was 22 (for #1686) to 36 mm (for #1634) long. This estimate is independently confirmed by tooth lengths of 27–35 mm measured on three animals slaughtered at the time of the first diet-switch (E1, Fig. 1). Overall, the model closely predicted the amplitude and shape of the measured tooth enamel signal.

4.2.3. Model-data discrepancies

Small but important discrepancies were observed between the prediction of the model and the measured isotope profiles. For example, the model did not predict the plateau visible in the two individuals from group E2 and individual #1560 from group E3 (Figs. 3 and 5). Also, the predicted rate of isotope change was lower than the measured rate of change in many of the teeth. This trend is opposite to what was found by Podlesak et al. (2008) for rats after a water switch. These discrepancies were exaggerated by the large difference in isotope value between the diets (10–20‰, depending on the treatments) which exceeds by far what is usually recorded in animal teeth. In this respect, the relatively good fit between model and data suggest that the model will adequately describe most of the situations experienced by animals in natural environments.

Several reasons can be invoked to explain these discrepancies. The forward model used in this study is simplistic, assuming a constant growth rate and constant dimensions of maturation parameters. In non-continuously growing teeth like sheep teeth, parameters such as l_a and l_m may change as crown formation proceeds. For example, the cusp may mature rapidly, the main part of the crown may have a characteristic maturation length, and the enamel near the root may mature at a yet-different rate. Changes in tooth growth rates or in the angle between the apositional layer and the enamel-dentine junction could constrict or expand certain portions of the isotope profile.

Second, the model assumes a linear increase in the degree of mineralization during maturation. This is inconsistent with the description of different maturation dynamics within the enamel layer (Suga, 1979, 1982; Suga et al., 1970, 1987). For example, the innermost enamel layer was shown to mature rapidly following deposition whereas full mineralization of the uppermost layer was the slowest (Suga, 1982; Tafforeau et al., 2007). Although these two layers have different maturation lengths than the enamel between them, they represent only a small (~10%) fraction of the total enamel and should therefore not affect the isotope results too much as long as each sample represents a significant fraction of tooth enamel thickness.

Finally, discrepancies between model and data could be due to an incorrect characterisation of the pattern of accumulation of carbonate during mineralization. The rationale for choosing $f_{init} = 0.25$ was guided by the measure of the phosphorus content of a hippo tooth during the secretory stage (Passey and Cerling, 2002). Phosphorus can be used as an indicator of hydroxyapatite content because the rates and pattern of accumulation of calcium and phosphorus during the process of enamel development seem to co-vary (Suga et al., 1970; Robinson et al., 1978, 1987, 1988; Engel and Hilding, 1984; Sydney-Zax et al., 1991). On the contrary, the ratio of carbonate content to phosphorus is not constant, and decreases constantly from forming to maturing stages (Robinson et al., 1978; Sydney-Zax et al., 1991). For instance, the carbonate content per weight of mineral was found to decrease by 30–40% during maturation of human and bovine enamel (Sydney-Zax et al., 1991). However, this decrease is insufficient to be explained by simple dilution during crystal growth, and therefore, carbonate ions must be incorporated during the maturation stage as well.

Fig. 6 shows the prediction of the model for different values of f_{init}. The first scenario assumes that the carbonate incorporated during the secretory stage is absent from mature enamel, or is completely replaced during subsequent mineralization ($f_{init} = 0$). The agreement between model and data is reasonably good in E2 but the rate of isotope change is underestimated in all the other groups. Setting the amount of carbonate incorporated during secretory stage at 50% ($f_{init} = 0.5$) gave better results: it reproduced the amplitude of the isotope excursion (E2–E4) and predicted detailed features of the isotope profiles such as the change in slope in the upper part of the tooth (E2–E5),

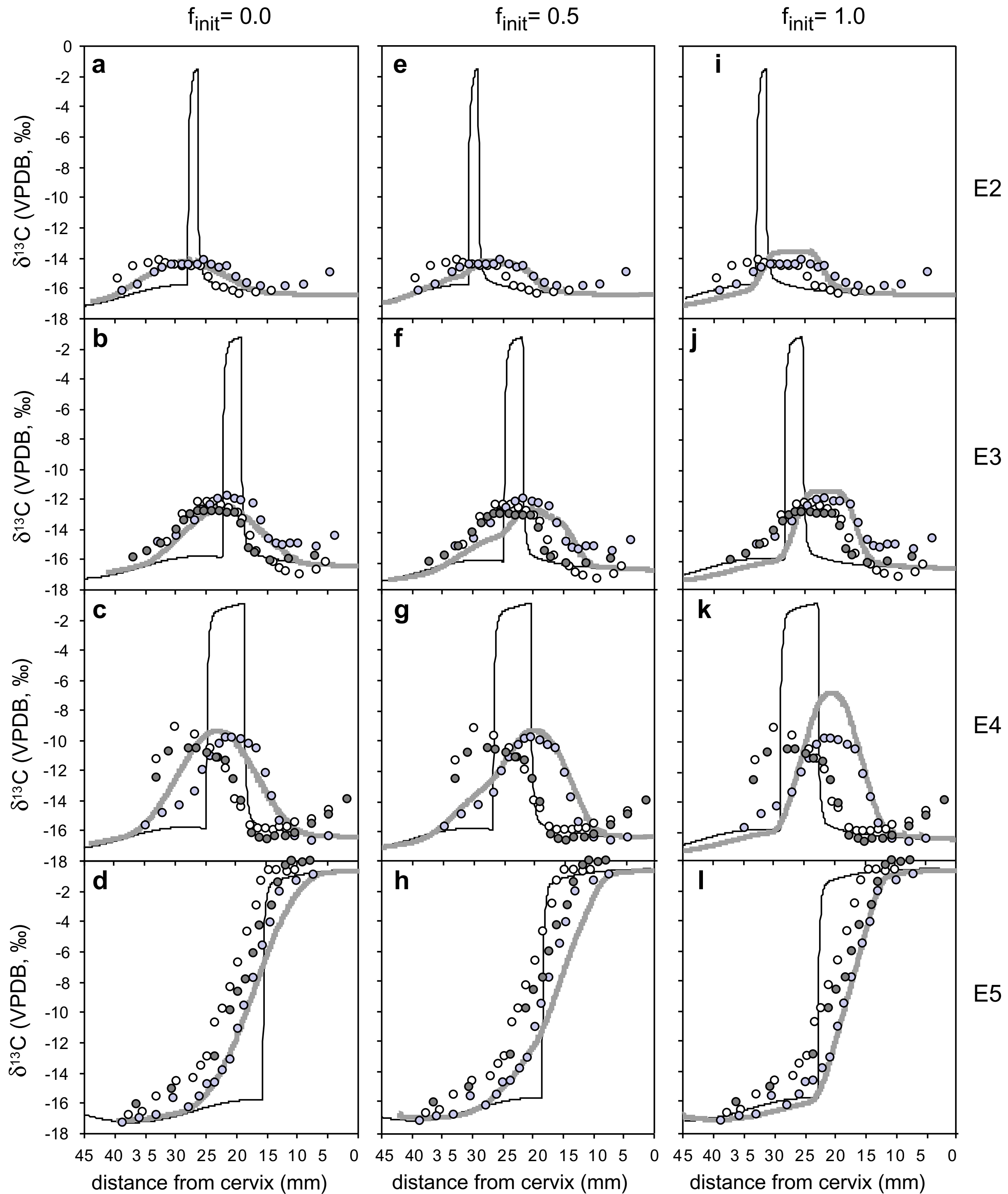

Fig. 6. Comparison between model predictions and isotope profiles measured along M_3 crown height for $f_{init} = 0$ (a–d), $f_{init} = 0.5$ (e–h) and $f_{init} = 1$ (i–l).

and the abrupt decrease in $\delta^{13}C$ value following the return to C3 diet (E3–E4). The last scenario ($f_{init} = 1$) predicts the plateau (E2–E3) but overestimates the rate and the amplitude of the isotope change (E2–E4). In conclusion, even if no combination of model maturation parameters perfectly predicted the shape and amplitude of isotope change in all groups, our results suggest that carbonate is incorporated during both the secretion and the maturation stages. The pattern of carbonate accumulation is probably more complex than previously thought. More work is needed to determine more precisely the exact timing and rate of incorporation of carbonate in developing tooth enamel, thereby enabling a more meaningful interpretation of intra-tooth isotope profiles.

5. CONCLUSIONS

Sequential sampling and stable isotope analysis of tooth enamel performed on animals fed controlled diets proved successful in addressing the questions of the systematics and kinetics of carbonate uptake during amelogenesis. Controlled feeding experiments performed on a large number of sheep allowed us to determine the timing of the first, second and third molar development, the fractionation between diet and apatite, and the duration of amelogenesis in each of these teeth. Carbon isotope analysis of breath CO_2 showed that the turnover of dietary carbon was rapid and that blood DIC had nearly reached equilibrium with the new diet less than a week after diet-switch. The changes in diet were also recorded in the sheep molars and allowed us to calculate maturation parameters as well as the isotopic enrichment between diet and apatite. Length of apposition l_a and length of maturation l_m were either determined graphically (for M_2 and M_3) or estimated (for M_1) and represented ~1/4 and ~1/3 of total crown length, respectively. Sequential sampling and isotopic analysis of tooth enamel from sheep fed C_3–C_4–C_3 diets in sequence showed that the attenuation of the diet isotope signal recorded in enamel as a result of delayed mineralization was inversely proportional to the time of exposure to the C_4 diet.

The tooth enamel forward model of Passey and Cerling (2002) closely predicted the lag and attenuation of isotope changes recorded in tooth enamel but slightly underestimated the rate of isotope change, suggesting that the rate of tooth growth and/or the pattern of accumulation of carbonate during maturation are more complex than assumed by the model. Despite this misfit, it is encouraging that a model developed for a very specific, simplified case—ever-growing teeth with constant growth rate and unchanging maturation parameters—is successful at predicting the general form and amplitude of isotopic changes in high-crowned ungulate teeth. This may in part relate to the idea that growth of the prismatic portion of high-crowned teeth

Table A1
Carbon stable isotope ratios of carbonate enamel sampled along the tooth crown of the first and second lower molars of four lambs from group L7. Sample position is expressed as the distance from crown neck, in mm.

9125			9169			9351			9646		
#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)
1	21.8	−11.7	1	22.0	−10.7	1	26.4	−10.8	1	26.7	−12.3
2	19.1	−9.9	2	20.2	−9.2	2	23.9	−9.1	2	24.6	−10.3
3	16.6	−9.8	3	18.2	−9.0	3	21.6	−8.1	3	21.9	−10.6
4	14.4	−9.8	4	16	−8.8	4	19.2	−8.3	4	19.9	−10.5
5	11.4	−9.3	5	13.8	−8.9	5	17.4	−8.6	5	17.4	−8.7
6	9.3	−8.9	6	11.7	−8.0	6	15.7	−8.3	6	14.8	−8.6
7	7.3	−7.7	7	9.6	−7.5	7	13.8	−7.4	7	13.8	−8.3
8	5.3	−6.8	8	7.7	−6.4	8	11.9	−6.1	8	13.0	−8.3
9	3.9	−5.6	9	5.9	−4.6	9	9.8	−5.4	9	11.8	−7.8
10	1.9	−4.7	10	4.1	−3.6	10	8.2	−4.5	10	10.8	−7.8
11	0.5	−4.8	11	2.0	−2.7	11	6.5	−3.5	11	9.6	−7.3
12	1.2	−2.8	12	5.0	−2.4	12	8.7	−6.7			
						13	3.3	−1.9	13	7.6	−5.7
						14	1.8	−1.2	14	6.6	−5.7
									15	5.9	−5.3
									16	4.9	−3.7
									17	2.8	−2.8
									18	0.9	−1.7
1	34.7	−5.4	1	33.3	−4.3	1	36.3	−5.0	1	34.1	−4.6
2	32.7	−4.3	2	31.9	−4.3	2	33.6	−3.4	2	33.1	−3.6
3	31.1	−3.3	3	30.8	−3.6	3	31.6	−2.5	3	31.9	−2.7
4	29.2	−2.5	4	30.0	−3.2	4	29.3	−2.0	4	30.9	−2.8
5	27.5	−2.0	5	29.0	−2.5	5	27.3	−1.6	5	30.0	−3.0
6	24.7	−1.4	6	28.2	−2.1	6	24.5	−0.8	6	28.8	−2.2
7	21.7	−1.4	7	27.4	−1.9	7	21.2	−0.7	7	28.0	−1.8
8	18.7	−1.3	8	25.7	−1.4	8	18.7	−0.8	8	26.7	−1.1
9	14.8	−1.1	9	23.8	−1.1	9	14.7	−0.7	9	25.5	−0.6
			10	21.7	−0.9				10	24.5	−0.9
			11	19.7	−0.8				11	23.4	−0.8
			12	17.5	−0.5				12	22.4	−0.5
			13	15.3	−0.9				13	21.5	−0.6
			14	14.3	−0.8				14	20.6	−0.5
			15	13.5	−1.1				15	18.9	−0.5
			16	12.4	−1.2				16	16.9	−1.3
			17	11.1	−1.3				17	14.4	−0.6
			18	10.2	−1.3				18	12.6	−0.8

Table A2
Carbon stable isotope ratios of carbonate enamel sampled along the tooth crown of the third lower molars of 11 ewes from groups E2 to E5. Sample position is expressed as the distance from crown neck, in mm.

1686 (E2)			1687 (E2)			1481 (E3)			1476 (E3)			1560 (E3)		
#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)
1	38.5	−15.4	1	37.8	−16.143	1	31.8	−14.9	1	30.4	−14.8	1	36.0	−15.9
2	35.9	−14.6	2	35.1	−15.738	2	28.8	−14.4	2	27.8	−13.0	2	33.6	−15.5
3	33.3	−14.3	3	32.4	−14.977	3	25.9	−13.4	3	25.2	−12.2	3	31.4	−14.9
4	31.5	−14.2	4	31.1	−14.682	4	24.5	−12.7	4	23.8	−12.2	4	28.9	−14.0
5	30.5	−14.3	5	29.6	−14.452	5	23.3	−12.4	5	22.7	−12.1	5	27.6	−13.3
6	29.0	−14.4	6	27.9	−14.428	6	21.9	−11.9	6	21.4	−12.4	6	26.5	−13.0
7	27.7	−14.5	7	26.7	−14.406	7	20.6	−11.8	7	20.2	−12.6	7	25.4	−12.8
8	26.4	−14.6	8	25.4	−14.422	8	19.2	−12.0	8	18.8	−12.8	8	24.3	−12.8
9	24.9	−14.5	9	24.4	−14.138	9	18.1	−12.0	9	17.9	−13.3	9	23.0	−12.8
10	23.5	−15.2	10	23	−14.412	10	16.3	−12.3	10	16.6	−14.4	10	21.8	−12.8
11	22.2	−15.9	11	21.7	−14.657	11	15	−13.4	11	15.6	−15.5	11	20.6	−12.9
12	20.9	−16.0	12	20.5	−14.51	12	13.6	−14.6	12	13	−16.2	12	19.3	−12.9
13	19.6	−16.2	13	18.9	−15.243	13	12.4	−14.9	13	12	−16.6	13	18.1	−13.7
14	18.4	−16.4	14	17.1	−15.651	14	11.4	−15.0	14	10.8	−16.8	14	17.0	−15.2
15	15.6	−16.3	15	15	−15.887	15	10.2	−14.9	15	8.5	−17.0	15	16.0	−15.5
16	13.1	−16.2	16	10.8	−15.85	16	8.9	−14.9	16	5.7	−16.6	16	13.5	−16.0
17	10.0	−15.8	17	7.8	−15.718	17	5.9	−15.2	17	4.3	−16.2	17	10.3	−16.0
18	6.0	−15.4	18	3.5	−14.932	18	2.8	−14.5				18	6.0	−15.7

1509 (E4)			1649 (E4)			1651 (E4)			1597 (E5)			1634 (E5)			1654 (E5)		
#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)	#	Position (mm)	$\delta^{13}C$ (‰, VPDB)
1	31.9	−11.3	1	33.7	−15.7	1	32.0	−12.5	1	36.6	−16.8	1	37.7	−17.3	1	35.3	−16.1
2	28.8	−9.1	2	30.9	−14.8	2	29.8	−10.7	2	34.4	−16.6	2	34.8	−17.0	2	29.6	−15.0
3	25.7	−9.6	3	28.2	−14.3	3	26.6	−10.5	3	32	−15.5	3	32	−16.8	3	22.6	−12.9
4	23.1	−10.5	4	25.7	−13.4	4	25.4	−10.5	4	28.9	−14.6	4	29.3	−15.7	4	20	−9.8
5	21.8	−11.1	5	24.3	−12.0	5	23.6	−10.8	5	26	−14.3	5	26.8	−16.3	5	18.8	−8.6
6	20.5	−11.9	6	23.1	−11.1	6	22.2	−11.1	6	24.8	−13.5	6	25	−15.6	6	17.4	−7.8
7	19.1	−14.0	7	21.7	−10.2	7	20.7	−11.3	7	23.7	−13.0	7	23.9	−14.7	7	16.3	−6.1
8	18.1	−14.4	8	20.5	−9.8	8	19.4	−12.5	8	22.4	−10.7	8	22.6	−14.7	8	15.1	−4.3
9	16.5	−15.6	9	19.5	−9.8	9	18.1	−14.1	9	21.3	−9.8	9	21.2	−13.8	9	13.2	−3.0
10	15.3	−15.8	10	18.2	−9.9	10	16.4	−16.1	10	20	−8.3	10	19.9	−13.1	10	12.1	−1.4
11	14.2	−15.9	11	16.8	−10.2	11	15.0	−16.4	11	18.6	−6.7	11	18.7	−11.1	11	10.8	−0.4
12	12.9	−15.9	12	15.5	−10.5	12	13.8	−16.6	12	17.2	−4.7	12	17.6	−9.5	12	9.8	0.0
13	11.7	−15.8	13	14.2	−12.1	13	12.6	−16.3	13	15.8	−2.9	13	16.3	−7.8	13	8.2	−0.1
14	10.5	−15.7	14	13	−13.7	14	10.9	−16.3	14	14.9	−1.3	14	14.6	−5.6	14	6.9	0.0
15	9.4	−15.5	15	11.7	−15.4	15	9.4	−16.2	15	13.7	−0.6	15	13.4	−4.1			
16	6.5	−15.2	16	9.3	−16.3	16	6.5	−15.6	16	12.3	−0.7	16	12	−2.0			
17	3.9	−14.6	17	6.6	−16.6	17	3.8	−14.9	17	10.7	−0.7	17	9	−1.3			
18	1.1	−13.8	18	3.9	−16.3	18	0.7	−13.9	18	9.3	−0.6	18	6.3	−1.0			

is analogous to the growth of evergrowing teeth. As such, the model evaluated here may be broadly applicable to high-crowned teeth such as those found in many species within the equid, bovid and rhinocerotid families, as well as species within the lagomorph, and rodent orders.

Finally, our study also has implications for the investigation of seasonality of past climates, the timing of birth in fossil mammals, and pastoralism in an archaeological context (Fricke et al., 1998; Zazzo et al., 2002; Balasse et al., 2003; Balasse and Tresset, 2007). These studies may rely on the assumption that the timing of tooth development is fixed within a species, and that individuals born in the same season will record the same part of the seasonal cycle in the same part of the tooth. The inter-individual variability of ~4–5 months found here in the timing of M_3 development suggests that this tooth may not be well-suited to infer timing and duration of seasonal events. However, this range of variation might be exacerbated in the improved sheep breeds used in our study. Indeed, a much smaller range of variation was observed for the timing and growth of the M_3 in a limited number of sheep belonging to the unimproved North Ronaldsay breed (Balasse et al., 2005: Fig. 3). More work is needed to establish whether this conclusion applies to ancient breeds and wild animals.

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APPENDIX A

(See Tables A1 and A2)

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