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The reconstruction of mammal individual history: refining high-resolution isotope record in bovine tooth dentine

Antoine Zazzo [a,b,*], Marie Balasse [c], William P. Patterson [a]

[a] *Department of Geological Sciences, University of Saskatchewan, 114 Science Place, Saskatoon, SK, Canada S7N 5E2*
[b] *CNRS UPR 2147 ''Dynamique de l'Evolution humaine: Individus, Populations, Espèces'', 44 rue de l'Amiral Mouchez, 75014 Paris, France*
[c] *CNRS UMR 5197 ''Archéozoologie, Histoire des Sociétés humaines et des Peuplements animaux'', Muséum national d'Histoire naturelle, Département Ecologie et Gestion de la Biodiversité, 55 rue Buffon, 75231 Paris Cedex 05, France*

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Abstract

Longitudinal and transverse carbon isotope profiles were performed on tooth dentine from five steers (*Bos taurus*) initially fed C_3 and subsequently C_4-dominant food. Comparison of different protocols for bioapatite extraction revealed that the use of NaOCl considerably reduced the amplitude of variation of $\delta^{13}C$ within a tooth. Increasing contribution of C_4 food to the carbon isotope composition of bioapatite was found from the tip of the tooth crown to the neck and from the enamel–dentine junction toward the pulp cavity. These findings confirm that the model of dentine growth as a succession of stacked cones applies to bovines. Temporal resolution is estimated to be 4 months in transverse profiles, significantly better than in longitudinal dentine profiles (8–9 months) or even in profiles derived from enamel of the same individual (6–7 months). Temporal resolution could be improved by a factor of two by selecting a different sampling zone or refining our sampling protocol. This sampling strategy could also be applied to dentine collagen and has important ecological and archaeological implications including determination of the season of weaning, or the reconstruction of mobility strategies.
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Keywords: Dentine; Stable isotopes; Dietary reconstruction; Mammal; Intra-tooth profiles

1. Introduction

Research involving stable isotope analysis of vertebrate skeletal remains has lately focused on reconstruction of life history through sequential sampling of animal teeth. Investigating environmental variation and diet changes in wild and domestic animal life history at annual or subannual scale has served various applications in palaeoenvironmental and palaeoclimatological reconstruction (e.g. [17–19,25,26,29,40,41,45,47]), biological and physiological investigation (timing of tooth development: [17,28,41]; investigation of weaning: [8,22]) and anthropological research essentially related to zooarchaeological perspectives [5,6,9–12,34]. Most of these studies have focused on the mineral fraction (bioapatite) of tooth enamel for several reasons. First, because of its larger, more tightly packed and highly organized apatite crystals, enamel is less susceptible to post-depositional isotope exchange or alteration than other less densely mineralized tissues such as bone and dentine [31–33,48]. Secondly, because its deposition as a single layer that is not remodeled once fully mineralized made it a good candidate for the recording of isotope signals over the period of tooth growth, spanning a year to a few decades. Lastly, enamel has been preferentially used because it forms the exterior coating of teeth, and therefore it is more easily reached with minimal destruction of the specimen unlike dentine that forms the bulk of the tooth.

* Corresponding author. Present address: School of Biology and Environmental Sciences, UCD Agriculture and Food Science Center, University College Dublin, Belfield, Dublin 4, Ireland.
E-mail address: antoine.zazzo@usask.ca (A. Zazzo).

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However, some limitations also come with the use of enamel. First, the loss of most of the enamel organic matrix during maturation prevents routine stable isotope analyses of the organic fraction (N, C from dietary protein and S), eliminating the possibility of coupled isotope analyses of organic and inorganic tooth components. Moreover, interpretation of C and O isotope variations in tooth enamel are more problematic than initially thought, because of the multiple directions of mineralization waves, and the duration of the whole process [4,16,36,49]. Disentangling the isotope signal in enamel may be possible in some cases via inverse modeling but this procedure is rather complex and requires a detailed knowledge of the mineralization process for each species [37].

Contrary to enamel, mature dentine contains 20% organic matter by weight (30% of its volume, versus 0.4% by weight or 2% by volume in enamel: [20]), composed mainly of collagen (90%: [20]), allowing access to multiple isotope systems by the coupling of collagen and bioapatite analysis. Another advantageous property of dentine is that its matrix is heavily—although not fully—mineralized instantly after deposition [15], perhaps reducing the damping of the isotope signal measured in the mineral phase.

In spite of these properties, dentine has been used only in rare occasions in stable isotope studies involving sequential sampling in teeth. This is partly due to the geometry of dentine growth. Dentine is deposited by apposition of successive cone-shaped layers coating the pulp cavity, meaning that the whole dentine layer gains in width as it gains in length. Consequently the sequential sampling usually applied to enamel along the longitudinal axis of the tooth cannot be applied to dentine without considerable homogenization related to the mixing of histological features [8].

Koch et al. [25,26] and Hobson and Sease [22] produced time series from the dentine of proboscideans and marine mammals respectively, using microsampling techniques on transverse tooth sections following growth increments. Both demonstrated that dentine preserves a high-resolution isotopic record of behavioral and environmental changes throughout life. This sampling procedure has not been applied to other terrestrial mammals principally because of the small size of most mammal teeth and the difficulty of clearly identifying and sampling growth features on transverse sections.

Today, progress in micro-sampling procedures and analytical techniques permit recovery and analyses of more discrete samples and alternative sampling strategies. In the study presented herein, attempts were made to improve sampling strategies in dentine from the teeth of large herbivores commonly present in the archaeological record, by exploring the mineralization process through the thickness of the dentine layer, by comparing isotope analysis along the transversal and longitudinal axes of the tooth. The procedure was applied to modern bovines, and the mineralization process was traced using a change of diet (pure C_3 to $C_3 + C_4$) experienced by the individuals at the time of the second molar growth. Time resolutions were estimated both for the longitudinal and transverse sampling procedures. Comparison with isotope profiles derived from enamel of the same specimens [4,49] established the tissue/sampling strategy allowing more accurate recovery of original environmental variability. The implications for archaeological studies based on fossil bioapatites will be discussed in light of our experimental results.

2. Material and methods

2.1. Material

This study was conducted on five steers (*Bos taurus*) raised in an experimental farm between 1994–1996 (Ferme Expérimentale des Etablières, Vendée, France), where diet was controlled throughout life. Detailed records of diet management and composition have been previously published, together with results from previous investigations on the isotopic record of diet change in the jawbone and teeth of the individuals [4,7,8]. Calves were raised on a C_3-based milk diet during the first 9–10 months of life (lactating cows were fed C_3 grass $\delta^{13}C_{grass} = -26.5‰$ VPDB). At weaning, they were switched to a C_4-dominated diet (maize), and therefore the diet switch occurred during the formation of the second molar. Individuals labeled V3 and V5 were kept in stall 5, with maize providing 89% by weight of the total dietary carbon (estimated $\delta^{13}C_{diet} = -13.4‰$ VPDB). Individuals labeled V2, V4 and V6 were kept in stall 6, with maize providing 63% by weight of the total dietary carbon (estimated $\delta^{13}C_{diet} = -17.0‰$). The steers were slaughtered 8 months after the introduction of C_4 plants to their diet at age 17–18 months [7]. At time of death, the second molar was still erupting and the anterior lobe was entering into wear.

2.2. Sampling procedures

Dentine was sampled following (1) the longitudinal axis along the tooth height of the five steer using hand drilling, and (2) the transversal axis through the tooth thickness of individual V3 using micromilling technique. Sequential sampling of dentine from individuals 2–6 was performed along the tooth height from the apex to the cervix of the crown, and to the root following the conventional sampling strategy commonly used for enamel. Each sample consists of a 2-mm-high band perpendicular to the growth axis, spanning the width of half a lobe and crossing all of the dentine thickness. To facilitate direct comparison between enamel and dentine profiles from the same individual and among individuals we sampled underneath grooves drilled through enamel in Balasse [4]. Microsampling was performed on the posterior lobe from the lower second molar of individual V3 (V3M2). Six slices were cut perpendicular to the growth axis of the tooth using a diamond saw (Fig. 1). The thickness of each slice is 2.7–2.9 mm. Slices were glued to glass slides, with the portion of the tooth being sampled overlapping the edge of the slide. The glass side was then glued to a second glass slide that was fixed to the micromilling stage beneath a stationary diamond dental drill. Powder was recovered on weighing paper placed between the two glass slides during microsampling. Dentine from the mesostyle and from the parastyle areas was micromilled following the

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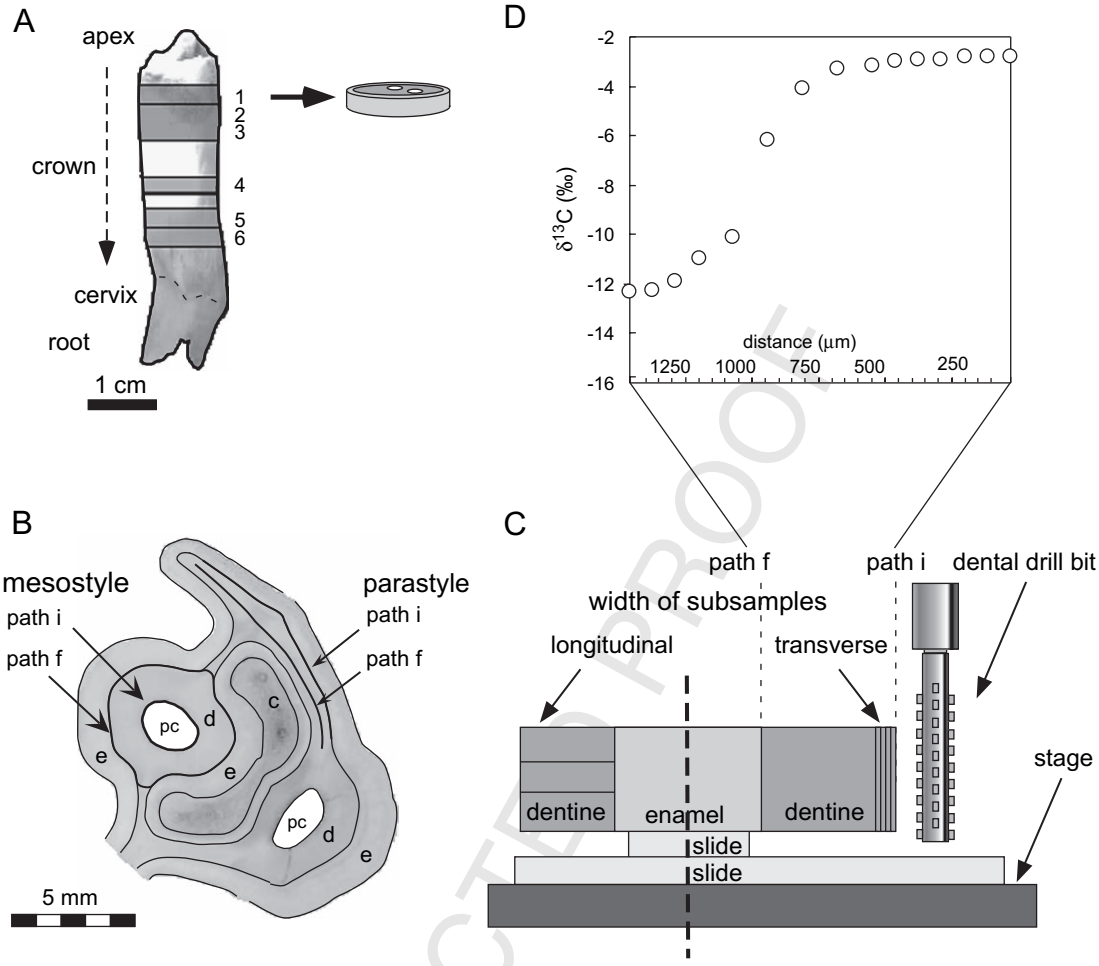

Fig. 1. Schematic representation of the microsampling procedure. (A) Preparation of the 3-mm-thick tooth slices. (B) Two-dimensional representation of tooth slice and manually selected three-dimensional coordinates for transverse sampling. Mesostyle dentine is sampled from the pulp cavity (path i) to the enamel–dentine junction (path f); Parastyle dentine is sampled from the enamel–dentine junction (path i) to the zone where the two dentine layers join (path f). e, enamel; d, dentine; pc, pulp cavity; c, cement. (C) Sampling device. Note that sampling width is much smaller than drill width. A schematic representation of longitudinal sampling is also given for comparison. (D) High-resolution $\delta^{13}C$ profile measured in tooth dentine (distance from pulp cavity).

methodology described in Wurster et al. [46]. For each zone, two paths (path i and path f) were digitized as a series of three-dimensional coordinates and ''smoothed'' intermediate coordinates were interpolated by cubic spline. Dentine from the mesostyle area (tooth slices 1–4), was micromilled from the innermost dentine layer in the pulp cavity to the enamel–dentine junction (EDJ). For the parastyle area (tooth slices 4–6), the enamel layer was first removed using the dental drill; path i is at the EDJ and path f is at mid-width of the dentine layer where the dentine layers join (cf. Fig. 1). Thirty to fifty intermediate sampling paths were calculated at every 20–50 µm between the two digitized curves, and were micromilled using the edge of a diamond dental drill. This methodology allows for subsamples to be taken contiguously at a spatial resolution that is only constrained by the precision of the step size (0.05 µm) rather than drill width as in conventional sampling. Powder from each micromilled dentine subsample was collected along the height of the tooth slice, and therefore represents a significant volume of about $10–20 \times 0.02–0.05 \times 3$ mm (l × w × h). The milled apatite powder (1–5 mg) was collected manually with a razor blade.

2.3. Isotopic analyses

Thirty samples were split in half to test the effect of the NaOCl treatment on $\delta^{13}C$ values. Samples were reacted in 2.75% sodium hypochloride (NaOCl). The solution was changed every 6–12 h until no bubbling was visible (usually after 24 h maximum).

Between 12 and 17 untreated samples were analyzed for each individual following the longitudinal sampling. Between 13 and 32 untreated samples were analyzed per tooth slice using transverse sampling. About 500 µg of each sample was weighed and reacted in a Kiel III carbonate device interfaced with a Finnigan MAT 253 isotope ratio mass spectrometer in the Saskatchewan Isotope Laboratory, University of Saskatchewan. Carbon dioxide was generated by reaction of apatite carbonate with four drops of anhydrous phosphoric acid in individual reaction vessels at 70 °C for 5 min. Carbonate content was determined by coulometry during acid digestion with an analytical precision of ±0.3 wt% (1σ). Analytical precision for carbon isotopic analyses is ±0.03‰ (1σ), determined from replicate analyses of Miocene phosphorite NBS120c

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($\delta^{13}C = -6.32‰$, $n = 7$). Isotopic measurements were normalized to daily analyses of the international standards NBS-18 and NBS-19. $\delta^{13}C$ analyses are reported in permil notation relative to VPDB.

3. Results

Carbon isotope values and carbonate contents from unbleached and bleached dentine samples are presented in Table 1. Large differences in $\delta^{13}C$ values and were found between untreated ($\delta^{13}C$) and NaOCl-treated ($\delta^{13}C_{NaOCl}$) samples. This difference is expressed as $\Delta^{13}C_{NaOCl}$ values and varies from -2.5 to $+1.7‰$ as a function of $\delta^{13}C_{utr}$ values (Fig. 2). Average carbonate content is 7.9 ± 0.8 wt% for untreated samples and 4.1 ± 0.4 wt% for bleached samples.

Carbon isotope values from longitudinal profiles in individuals V2 to V6 are presented in Table 2 and Fig. 3. The sudden change in diet is reflected by a progressive increase in $\delta^{13}C$ values along the tooth length axis. The upper part of the crown has $\delta^{13}C$ values between $-10‰$ and $-14‰$, typical for a C_3 diet. Carbon isotope values of the tooth increase downward in the tooth to plateau in the lower third of the crown and root. The highest $\delta^{13}C$ values are proportional to the C_4 content of their diet. Individuals V2, V4, and V6, whose new diet contains 63% C_4 carbon ($\delta^{13}C_{diet1} = -17‰$) plateau at about $-5.5‰$, whereas V3 and V5 whose new diet had 89% C_4 carbon ($\delta^{13}C_{diet2} = -13.4‰$) plateau at a higher $\delta^{13}C$ value of approximately $-3‰$. These results indicate an $11–12‰$ ^{13}C-enrichment in the dentine apatite relative to diet values, close to previous estimates for bone apatite but $2‰$ lower than between enamel apatite and diet [14,30,33].

Table 1
Carbon isotope values and carbonate content before ($\delta^{13}C$, $[CO_3^{2-}]$) and after ($\delta^{13}C_{NaOCl}$, $[CO_3^{2-}]_{NaOCl}$) the NaOCl pretreatment

Sample	Position	$[CO_3^{2-}]$	$\delta^{13}C$	$[CO_3^{2-}]_{NaOCl}$	$\delta^{13}C_{NaOCl}$
Micro-milled series					
V3M2-2 (Z2)	120–350	8.2	−13.3	–	−11.9
V3M2-3 (Z1)	0–400	5.1	−2.9	–	−5.1
V3M2-3 (Z1)	800–850	8.5	−3.4	–	−5.0
V3M2-3 (Z1)	1450–1500	7.7	−11.9	–	−10.2
V3M2-3 (Z1)	1650–1700	8.2	−12.3	–	−10.6
V3M2-4 (Z1)	0–100	4.6	−2.8	–	−5.3
V3M2-4 (Z1)	400–500	8.6	−3.4	–	−5.2
V3M2-4 (Z1)	750–800	4.4	−3.5	–	−5.4
V3M2-4 (Z1)	1000–1050	8.9	−4.2	–	−5.6
V3M2-4 (Z1)	1400–1450	8.8	−8.4	–	−7.9
V3M2-4 (Z1)	1550–1600	8.1	−8.4	–	−8.2
V3M2-5 (Z2)	0–20	7.7	−3.3	–	−4.8
V3M2-5 (Z2)	20–40	7.2	−4.4	4.8	−5.8
V3M2-5 (Z2)	40–60	8.6	−3.8	4.6	−6.1
V3M2-5 (Z2)	620–640	7.8	−3.0	4.1	−5.1
V3M2-5 (Z2)	1100–1200	7.6	−2.6	4.1	−5.0
V3M2-6 (Z2)	940–1040	8.8	−2.8	4.2	−4.8
V3M2-6 (Z2)	1190–1290	8.4	−2.8	4.0	−5.0
V3M2-6 (Z2)	1440–1540	7.5	−2.6	3.9	−5.0
V3M2-6 (Z2)	1640–1740	6.2	−2.5	3.2	−5.0
V3M2-6 (Z2)	450–550	8.5	−3.5	4.0	−5.6
V3M2-6 (Z2)	700–800	8.6	−3.1	3.9	−5.2
Hand-drilled series					
V3M2	44.5–41.5	–	−10.1	–	−9.0
V3M2	37.5–34.0	–	−6.4	–	−6.6
V3M2	34.0–31.0	–	−5.9	–	−6.2
V3M2	27.0–22.5	–	−4.3	–	−5.1
V3M2	22.5–18.0	–	−3.5	–	−4.6
V3M2	18.0–14.0	–	−2.8	–	−3.9
V3M2	14.0–10.0	–	−2.6	–	−4.0
V3M2	10.0–06.0	–	−2.5	–	−3.9

Sample position is expressed as the distance from the enamel–dentine junction (in μm) and as the distance from the cervix of the crown (in mm) for micro-milled and hand-drilled samples, respectively.

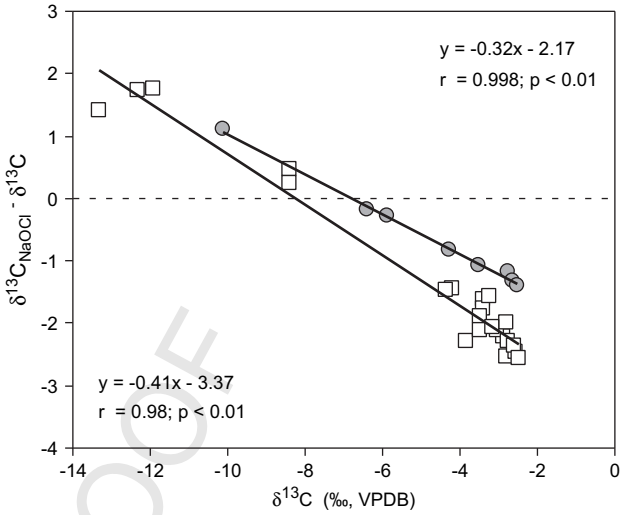

Fig. 2. Difference in $\delta^{13}C$ value between untreated and bleached dentine expressed as a function of dentine $\delta^{13}C$ values. Samples were processed in two different batches and data are given separately for micromilled (open squares) and hand-drilled (gray circles) samples.

Contrasting results were obtained in transverse profiles from the two sampling zones (Table 3, Fig. 4). In the mesostyle, large variations are recorded across the dentine thickness whereas stable isotope values are found in the parastyle. Profiles performed in the mesostyle exhibit increasing $\delta^{13}C$ values from the EDJ to inner dentine indicating that the introduction of C_4 plants into the steer's diet is recorded across the dentine thickness (Fig. 4A). The largest isotopic difference between inner and outer dentine is observed in the uppermost profile V3M2-1 where it equals that between the two diets ($13.0‰$), and decreases down the tooth axis. Outer dentine from the upper 30–35 mm of the crown exhibits $\delta^{13}C$ values between $-15.8‰$ and $-8.6‰$ suggesting that 50–100% of the apatite was formed in this zone before the diet switch. Inner dentine has the highest $\delta^{13}C$ values, ranging between $-2.6‰$ and $-3.5‰$, suggesting that this zone was mostly mineralized after the diet switch. On the contrary, transverse profiles in the parastyle exhibit remarkably stable carbon isotope values (Fig. 4B). Dentine in V3M2-2 was formed before the diet switch as suggested by low $\delta^{13}C$ values across the profile. Dentine in V3M2-5 and V3M2-6 was formed after the diet switch as suggested by high $\delta^{13}C$ values across the profile.

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Table 2
Carbon isotope values of bovine dentine apatite along longitudinal profiles

V2 ($h = 53$ mm)		V3 ($h = 48$ mm)		V4 (h = 51 mm)		V5 ($h = 48$ mm)		V6 ($h = 50$ mm)	
Position (mm)	$\delta^{13}C$ (V-PDB, ‰)	Position (mm)	$\delta^{13}C$ (V-PDB, ‰)	Position (mm)	$\delta^{13}C$ (V-PDB, ‰)	Position (mm)	$\delta^{13}C$ (V-PDB, ‰)	Position (mm)	$\delta^{13}C$ (V-PDB, ‰)
Crown									
53.0–50.5	−9.7	48.0–44.5	–	51.0–48.5	−13.9	48.0–45.5	–	50.0–48.0	−11.5
50.5–46.0	−8.6	44.5–41.5	−10.1	48.5–44.5	−12.8	45.5–41.0	−10.6	48.0–44.0	−11.2
46.0–41.5	−7.9	41.5–37.5	−8.2	44.5–40.5	−10.6	41.0–36.5	−8.6	44.0–40.0	−10.5
41.5–37.0	−7.0	37.5–34.0	−6.4	40.5–36.5	−9.9	36.5–32.0	−6.5	40.0–36.0	−9.9
37.0–32.5	−6.4	34.0–31.0	−5.9	36.5–32.5	−9.7	32.0–28.0	−4.7	36.0–32.0	–
32.5–28.5	−6.3	31.0–27.0	–	32.5–28.5	−9.0	28.0–23.5	–	32.0–28.0	−8.7
28.5–24.5	−5.7	27.0–22.5	−4.3	28.5–24.5	−6.2	23.5–19.0	–	28.0–25.0	–
24.5–20.5	−5.5	22.5–18.0	−3.5	24.5–20.5	−6.1	19.0–14.5	−2.7	25.0–21.0	−7.3
20.5–16.5	−5.4	18.0–14.0	−2.8	20.5–16.5	−6.2	14.5–10.0	−2.2	21.0–17.0	−6.4
16.5–12.5	−5.5	14.0–10.0	−2.6	16.5–12.5	−5.4	10.0–06.0	−2.0	17.0–13.0	−6.1
12.5–08.5	−5.5	10.0–06.0	−2.5	12.5–08.5	−4.9	06.0–02.5	−1.8	13.0–10.0	−5.5
08.5–04.5	−5.5	06.0–02.0	−2.4	08.5–04.5	−4.7			10.0–06.0	−5.3
				04.5–00.5	−4.9			06.0–02.0	−5.3
Root									
00.0–04.0	–	00.0–04.0	−2.8	00.0–04.0	−4.8	00.0–04.0	−2.3	00.0–04.0	−5.6
04.0–08.0	–	04.0–08.0	−2.6	04.0–08.0	−5.3	04.0–08.0	−2.1	04.0–08.0	–
08.0–12.0	−5.8	08.0–12.0	−2.4	08.0–12.0	−5.6	08.0–12.0	−1.9	08.0–12.0	−6.1
		12.0–16.0	−2.2	12.0–16.0	−5.6	12.0–16.0	−1.7		

Position is expressed as the distance (in mm) from the cervix of the crown. The molar crown height (h) was measured along the vestibular side of the posterior lobe.

4. Discussion

4.1. Pretreatment effects

Large differences in carbon isotope values between untreated and bleached dentine samples are found. This difference is not randomly distributed but is clearly dependant on $\delta^{13}C$ value of the sample. Whereas the effect of the acetic acid pretreatment on $\delta^{13}C$ and $\delta^{18}O$ values has been discussed in the past, potential bias due to the removal of organic matter using NaOCl had not been described thus far. Koch et al. [27] did an exhaustive study where they compared the $\delta^{13}C$ value of a bone sample before and after the NaOCl treatment but did not find any significant difference. This result however does not contradict our findings because their bone $\delta^{13}C$ value was close to −8‰ where minimal $\Delta^{13}C_{NaOCl}$ values are expected (Fig. 2). This result is important and must be confirmed because it has implications on the interpretation of isotope variability found in bone tissues in term of environment or ecology. Although the explanation for this result remains unclear we propose three hypotheses: (1) isotope fractionation due to incomplete reaction of carbonate with orthophosphoric acid in the presence of organic matter; (2) isotope exchange between carbonate apatite and bicarbonate dissolved in the NaOCl-solution during the pretreatment; (3) adsorption of dissolved bicarbonate on the surface of the apatite crystals. Initially, dissolved bicarbonate is not present in the solution but could originate from two different sources: dissolved atmospheric CO_2 and CO_2 derived from the oxidation of dentine organic matter. If hypothesis 1 is correct, then $\delta^{13}C_{NaOCl}$ values should be more reliable than $\delta^{13}C$ values. If hypothesis 2 or 3 is correct, then $\delta^{13}C$ values should be more reliable than $\delta^{13}C_{NaOCl}$ values. Reaction yields can be estimated by comparing carbonate contents measured before ($[CO_3^{2-}]$) and after ($[CO_3^{2-}]_{NaOCl}$) the NaOCl treatment. Because these values are expressed in wt%, the contribution of organic matter to untreated sample mass must be taken into account prior to comparing carbonate content estimates from untreated and bleached samples. Considering that dentine contains 25–30 wt% organic matter, assuming that bleaching removes all organic matter, and assuming a reaction yield with phosphoric acid of 100% for pretreated samples, average reaction yield is 69–74% ($n = 10$) for untreated dentine. Isotopic fractionation is likely to result from incomplete reaction. But what we observe is reduced variability, rather than offset $\delta^{13}C$ values.

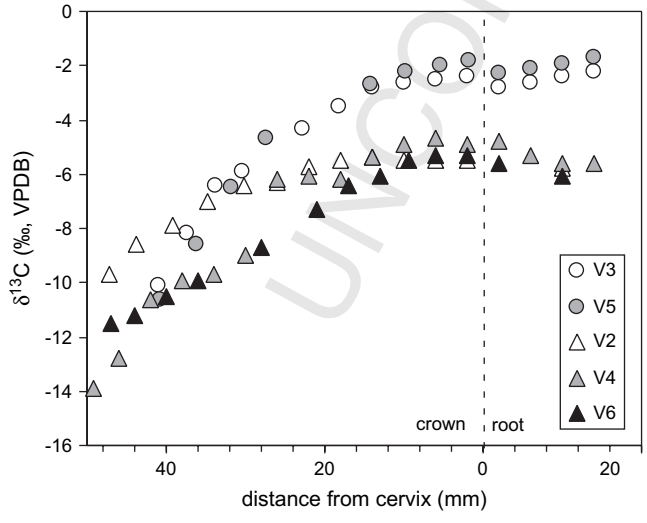

Fig. 3. Longitudinal intra-tooth carbon isotope profiles recorded in dentine apatite from the second lower molar of five steer. Series are plotted from the apex to the cervix of the crown, to the root.

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Table 3
Carbon isotope values ($\delta^{13}C$) across the dentine thickness of six horizontal tooth slices from individual V3M2

Mesostyle		Parastyle	
Position (μm)	$\delta^{13}C$ (‰)	Position (μm)	$\delta^{13}C$ (‰)
V3M2-1 (34.6–37.3 mm)		*V3M2-2 (30.9–33.7 mm)*	
100	−15.8	0	−13.7
150	−14.7	120	−13.3
200	−13.9	190	−13.6
350	−11.6	270	−13.3
400	−10.9	350	−12.9
450	−7.5	410	−13.5
500	−8.5	460	−12.4
550	−6.1	500	−11.9
600	−4.5	540	−12.2
650	−4.1	580	−12.3
700	−3.4	620	−12.2
850	−3.0	660	−12.3
950	−3.0	690	−12.6
1050	−2.8	740	−12.6
1150	−2.8	780	−12.5
1250	−2.8	820	−12.6
1350	−2.7	860	−12.6
1450	−2.7	900	−12.6
V3M2-2 (30.9–33.7 mm)		*V3M2-5 (10.3–12.9 mm)*	
0	−13.0	20	−3.8
50	−13.6	80	−3.9
250	−12.5	140	−3.9
550	−10.4	320	−3.9
700	−9.4	380	−4.0
800	−7.9	440	−3.6
850	−5.7	500	−3.4
900	−5.2	560	−3.3
950	−4.0	620	−3.0
1050	−3.9	680	−2.9
1300	−3.4	740	−2.8
1550	−3.0	800	−2.8
1800	−3.2	860	−2.8
2050	−3.5	920	−2.7
2200	−3.4	980	−2.7
		1040	−2.7
		1100	−2.7
		1160	−2.5
		1220	−2.5
		1280	−2.3
		1340	−2.3
		1400	−2.2
V3M2-3 (27.5–30.1 mm)		*V3M2-6 (07.1–09.5 mm)*	
0	−12.3	50	−2.8
100	−12.3	100	−3.4
200	−11.9	150	−3.5
300	−11.0	200	−3.3
450	−10.1	250	−3.5
600	−6.2	300	−3.6
750	−4.1	350	−3.5
900	−3.3	400	−3.1
1050	−3.2	450	−3.5
1150	−3.0	500	−3.5
1250	−2.9	550	−3.4
1350	−2.9	600	−3.4
1450	−2.8	750	−3.1
1550	−2.8	800	−3.1
1650	−2.8	850	−3.0
		900	−3.0
V3M2-4 (17.1–19.7 mm)		950	−2.8
		1000	−2.8
0	−8.6	1050	−2.9
100	−7.4	1100	−2.9
200	−6.1	1150	−2.9
300	−4.6	1200	−2.8
400	−3.8	1250	−2.7
500	−3.4	1300	−2.8
600	−3.5	1350	−2.8
750	−3.5	1400	−2.8
950	−3.3	1450	−2.7
1050	−3.0	1500	−2.5
1150	−2.8	1550	−2.5
1250	−3.0	1600	−2.5
1350	−2.6	1650	−2.5
		1700	−2.4

Positions of tooth slices are expressed as the distance from the cervix; positions of individual sub-samples are expressed as the distance from the enamel–dentine junction.

This feature can be produced by carbon isotope exchange between dentine carbonate and dissolved bicarbonate or adsorption of exogenous carbonate ions ($\delta^{13}C = -6$ to -8‰) onto apatite crystal surfaces. According to this scenario, $\delta^{13}C$ values of untreated samples are more reliable than $\delta^{13}C_{NaOCl}$ values. This is confirmed by examining V3M2-1 C-isotope variability. In untreated samples the difference between inner and outer dentine is 13‰, identical to the isotopic difference between the two diets. If we apply the $\Delta^{13}C_{NaOCl} - \delta^{13}C$ relationship, then we would underestimate the dietary shift by 4–5‰. For these reasons, we consider only $\delta^{13}C$ values for discussion. Further experiments will be required to fully understand potential biases resulting from the use of NaOCl treatment and how to avoid them.

4.2. Geometry and timing of dentine mineralization

Preliminary histological observations on thin sections [3] suggested that bovine dentine grows by apposition of stacked cones as suggested in other terrestrial [21,28] and marine mammals [39]. Growth layers appear sporadically in thin sections produced along the longitudinal axis. They show a very low angle with the EDJ and are almost vertical except in the uppermost part of the crown at the top of the cone. The coupling of longitudinal and transverse sampling confirms this view of the geometry of dentine growth and gives additional information concerning the timing of dentine growth. The sudden change from C_3 diet to the $C_3 + C_4$ mixed diet is reflected in longitudinal profiles by a gradual ^{13}C-enrichment starting in the uppermost part of the crown and ending in the lower third of the crown (Fig. 3). In transverse profiles, $\delta^{13}C$ values increase from the EDJ to the inner dentine. Pre-diet switch $\delta^{13}C$ values are found in the uppermost part of the crown close to the EDJ, whereas post-diet switch $\delta^{13}C$ values are recovered from the lower part of the crown, near the pulp cavity. This result demonstrates that the mineralization gradient is oblique

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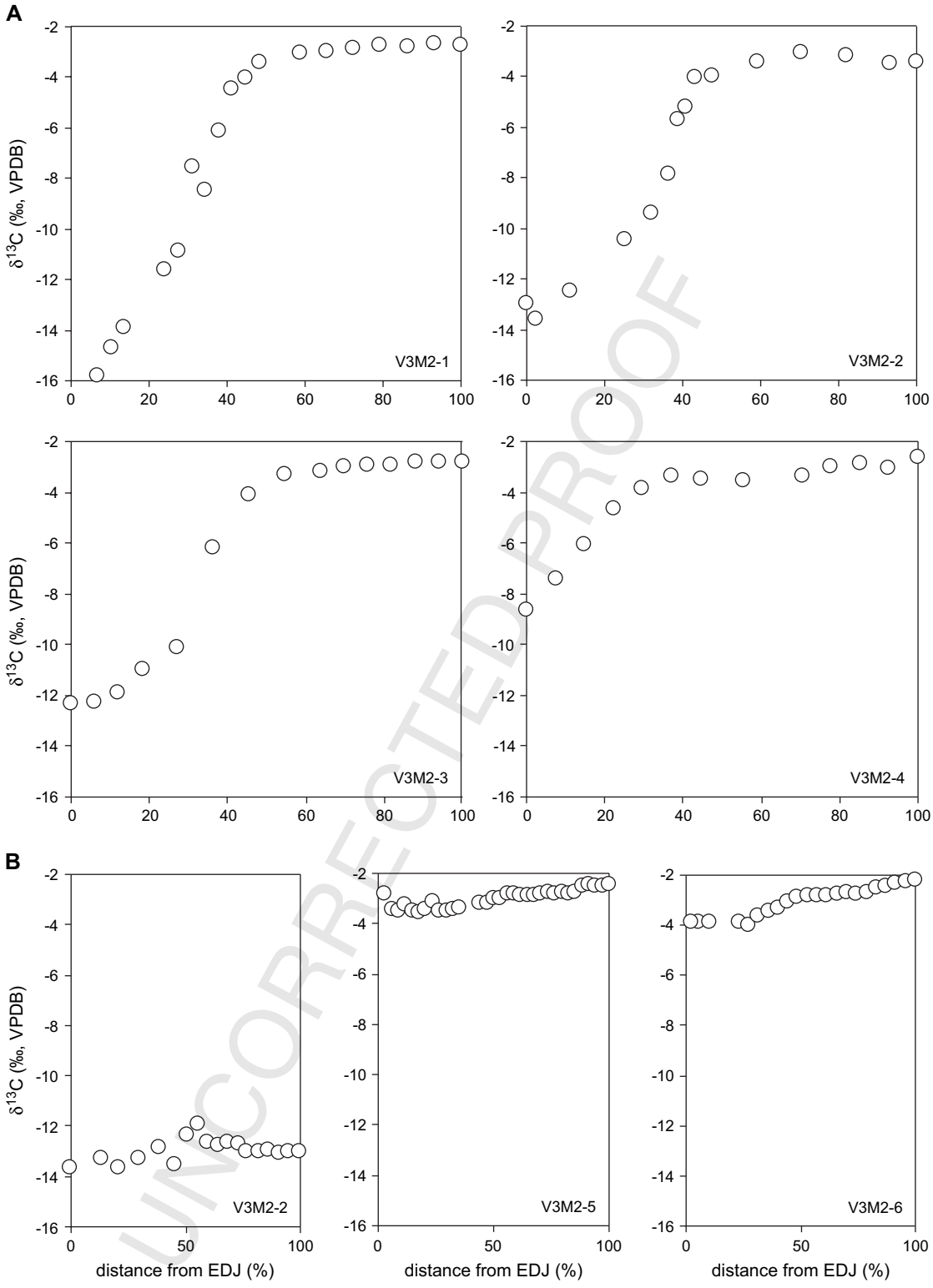

Fig. 4. Transverse intra-tooth carbon isotope profiles recorded across the dentine thickness of the second molar of individual V3. Series are plotted from enamel–dentine junction to pulp cavity. (A) Mesostyle profiles. (B) Parastyle profiles. Profiles are plotted from apex (V3M2-1) to cervix (V3M2-6).

and that the mineralization front runs diagonally through the dentine thickness extending inwards from the EDJ at a low angle. Thus, our data agree with the model of dentine growth by addition of stacked cones along the surface of a roughly conical pulp cavity at the proximal end of the growing tooth. A diagram showing this growth pattern can be found in Balasse et al. (Fig. 6, [8]). Furthermore, the examination of three successive profiles performed at mid-height of the crown through the tooth dentine show that they can be superimposed for the most part (Fig. 4, profiles 1–3). Only the portions of the

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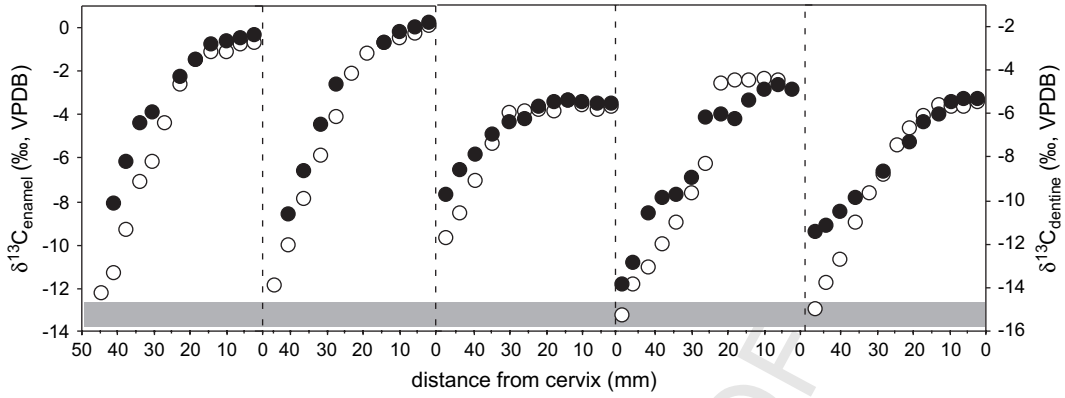

Fig. 5. Comparison of the pattern of carbon isotope in enamel (open circles) and dentine (black circles) apatite measured along the second molar of five steer. Gray zone represents the pre-diet switch endmember. The *y*-axis is shifted by 2‰ to account for the difference in diet-apatite fractionation factor in enamel and dentine.

curves located close to the EDJ differ slightly because of the decrease in the volume of the whole dentine layer synthesized before the diet change from the top to the bottom of the tooth. Our results suggest that apatite is rapidly mineralized as soon as the newly formed dentine layer is deposited. This is a major difference with enamel where two successive profiles could exhibit very different shape (concave or convex) and absolute $\delta^{13}C$ values shifted by several permil [49].

4.3. Time averaging

4.3.1. Longitudinal profiles

In the case of longitudinal profiles, time averaging essentially corresponds to the time required for the tooth dentine to gain its full thickness. The time represented in each subsample from the profile can be estimated by measuring the time elapsed between the mineralization of pre-diet switch dentine and post-diet switch dentine. Dentine mineralized exclusively after the diet switch is clearly visible as indicated by high invariant $\delta^{13}C$ values in the lower third of the crown. In the upper part of the crown, there is no pre-diet switch plateau but the $\delta^{13}C$ value corresponding to the C_3 diet can be predicted assuming that the C_3 and C_4 components of the diet are metabolized similarly. If we subtract the isotope difference between the two diets from the post diet-switch dentine value, we predict that the pre diet-switch dentine should be around −15‰. In the five steer, $\delta^{13}C$ values in the upper part of the crown range between −9.7‰ and −13.9‰ suggesting that dentine was still accreting when the diet switch occurred and was influenced by the C_4 signal. In bovines, the crown from the second molar starts growing when the animal is one month old [13]. The diet-switch in our experiment occurred at age 9–10 months. Therefore, the time required to complete dentine growth in thickness can be estimated to be at least 8–9 months for the highest part of the tooth at this stage of tooth development, and is likely to become even greater while the tooth completes its formation and as the pulp cavity fills in throughout life. Each sub-sample collected by drilling segments perpendicular to the tooth length axis and crossing all of the dentine thickness will represent a signal time-averaged by this amount of time, although time averaging should be slightly less towards the lower part of the tooth where dentine thickness is smaller. Fig. 5 presents a comparison between enamel and dentine longitudinal profiles from the five steers. Direct comparison is straightforward because the sampling protocol is similar for enamel and dentine, each sample crossing all of the enamel and dentine thickness, and dentine samples were recovered from beneath the grooves drilled for enamel in Balasse [4]. Intra-individual amplitudes of variation are reduced by 1 to 4‰ in dentine compared to enamel due to greater time-averaging in dentine (8–9 months) than in enamel (6–7 months, [4]). Therefore we conclude that (1) longitudinal sampling in mesostyle dentine is not accurate enough to assess the rate or the amplitude of a change in the diet or

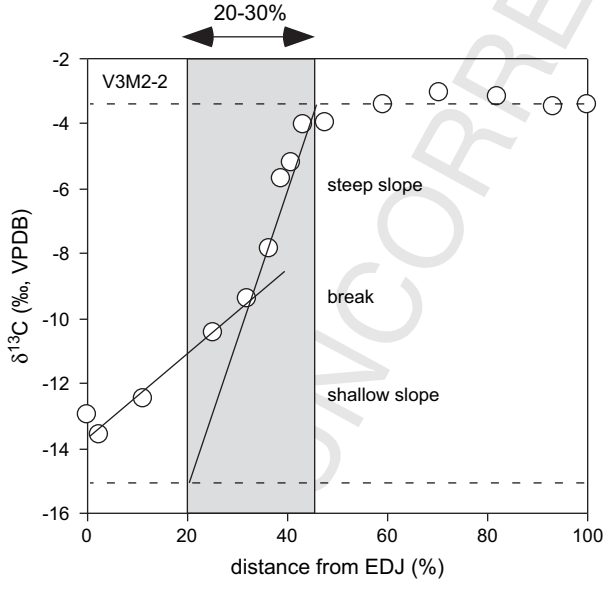

Fig. 6. Schematic representation of the effect of sampling on time-averaging. Slope break is interpreted as reflecting a change in the match between sampling path and the geometry of dentine apposition. Assuming that the steep portion of the curve represents the best match, the transition from pre- to post-diet switch dentine carbon isotope values (dotted lines) could represent only 20–30% of the dentine thickness (shaded area), an equivalent of 7–11 weeks of growth.

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environment of an animal and (2) in the absence of micromilling device, conventional sampling should be restricted to enamel rather than dentine.

4.3.2. Transverse profiles

Considering transverse profiles, it appears that the full difference in isotope values between the two diets (13‰ in specimen V3) can be recorded within one single tooth slice (Fig. 4). In V3M2-1, the carbon isotope value of the sample close to the EDJ is not influenced by the C_4 plant signal, and $\delta^{13}C$ values plateau at mid-width of the dentine layer. Because the time required to complete dentine growth in thickness was estimated to be 8–9 months for individual V3, we conclude that each subsample from the transverse profiles is time-averaged by about 4 months. This represents a 50% increase in time resolution compared to enamel.

4.4. Can we do a better job?

The quality of our high-resolution investigation is limited by two parameters: (1) gradual turnover of the metabolic nutrient pool (equilibration time between dietary carbon and blood bicarbonate); (2) correspondence of the micro-sampling paths to the geometry of dentine growth.

However sudden a dietary change may be, it is unlikely reflected by an abrupt change in the isotope values of the synthesized tissues, because of the equilibration period during which turnover of the metabolic pool occurs. Dietary switch experiments in dairy cows show a very rapid turnover of labile carbon (completed in about 3 weeks), with ~80% of the new diet recorded in breath CO_2 within a week [35]. Similarly steer feces achieve equilibrium with the new diet after about a week [23]. Based on individuals from the same experiment, Jones et al. [24] demonstrated that the time required to incorporate the new diet isotope composition into blood bicarbonate, then into a newly synthesized tissue, is much longer. In these bovines, isotopic equilibrium with the new diet was progressively reached in hair 50–70 days after the dietary change depending on the steer. Considering the time lag for the new portion of the hair to reach the skin surface, Balasse et al. [8] estimated the equilibration time to be a little shorter, between 1.5 and 2 months. This physiological constraint gives us a theoretical limit of the temporal resolution attainable in steer.

Based on our estimates, time averaging is about 4 months in transverse profiles. It means that the remaining 2–2.5 months are probably the result of laboratory sampling, bias that could be minimized by refining our sampling strategy. Our sampling strategy, consisting of successive concentric shavings from the pulp cavity to the EDJ (in the mesostyle) or vice versa (in the parastyle), can only be a rough approximation of the true incremental structure and is likely to mix portions of dentine mineralized at different times. In the case of profiles performed in the mesostyle, sampling bias is expected to increase from the first to the last micromilled sample because the pulp cavity serves as a physical guide for the geometry of deposition of the dentine layer. This view is confirmed by looking more into detail at the shape of the upper three isotope profiles which share a similar pattern of isotope variation (Fig. 4A). Following the sequence of sampling, this pattern is characterized by (1) high and stable $\delta^{13}C$ values in the inner half of the profile; (2) rapid decrease followed by (3) a gradual decrease toward low $\delta^{13}C$ values. The slope break occurs at about 500 μm from the EDJ and could be due to a change in the geometry of deposition of the dentine layers. Assuming that the steep slope represents the best match between sampling path and the geometry of dentine apposition, extrapolation of the steep portion to a theoretical pre-diet switch $\delta^{13}C$ values at around −15‰ suggest that the C_3 plateau should be recorded much earlier in the profile (Fig. 6). According to our estimates, the switch from C_3 to C_4 dentine would represent no more than 20–30% of the dentine thickness, an equivalent of 7–11 weeks of growth. This time lag would then become very close to the limit imposed by the turnover of the nutrient pool (6–8 weeks).

Lowering the time averaging in steer dentine to 2–2.5 month will require greater concordance between sample paths and the growth bands, or selection of another zone where the risk of mixing different layers is more limited. The technique developed to examine growth increments in marine mammal teeth could potentially be applied to bovines [44]. But this method requires the preparation of transverse thin sections examined with transmitted polarized light, and would probably not be ideally suited for micromilling mainly because thin section will not allow the collection of sufficient powder for isotope analyses. An alternative solution would be to select a different zone where dentine deposition occurs only for a short period of time. Dentine from the parastyle could reveal a good candidate. In tooth slice V3M2-2 profiles were performed in the parastyle and the mesostyle areas. The profile performed in the parastyle shows low and invariant $\delta^{13}C$ values averaging −14‰ and indicating very little C_4 contribution to dentine apatite (Fig. 4B). Similar values are only found in the first three samples from the mesostyle profile, close to the EDJ (Fig. 4A, profile 2). This result suggests that dentine formation started at the same time in both regions but ceased much earlier in the parastyle probably because there was no room left in this zone for accretion of additional material. It is also evidence for allometric growth of dentine. During the same period of time 900 μm of dentine were deposited in the parastyle whereas only 250 μm where deposited in the mesostyle. More detailed surveys of dentine apposition in the parastyle will be required to precisely define the amount of time represented in this area. Sampling this zone might offer the advantage of significantly increasing the temporal resolution of isotope series while eliminating the problem of accurately sampling the growth layers.

4.5. Implication for high resolution studies

If longitudinal sampling in the parastyle of a molar is demonstrated to reduce time damping due to overlapping of histological features, this procedure might facilitate the drilling of larger samples than in transverse sections, allowing extraction

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of both collagen and bioapatite from the same sample and simultaneous analysis of multiple isotopes. This would considerably extend the perspectives, essentially by introducing the possibility to analyze changes in the N stable isotopes from collagen while the seasonal cycle is traced in the O and C stable isotopes from bioapatite. Nitrogen stable isotopes have been used to trace numerous dietary, physiological and environmental events [1]. Simultaneous analysis of $\delta^{15}N$ in collagen and $\delta^{18}O$ in bioapatite would allow, for example, investigation of the season of weaning of domestic animals, that for the herders implies the season of the year when lactation and milk production drops; seasonal foddering with leguminous cultivates; seasonal physiological stress due to drought or malnutrition.

An accurate correspondence between the bioapatite and the collagen isotope signals will be difficult if time damping applies differentially to bioapatite due to a possible short mineralization delay in dentine. A solution might be to correct this delay by comparing the bioapatite and in collagen $\delta^{13}C$ signal. This will be possible when the $\delta^{13}C$ of the diet protein, from which most of the collagen C derives, does not differ significantly from the $\delta^{13}C$ of the whole diet, from which bioapatite is synthesized [2,30,33,42,43]. This might be true most of the time in large herbivores.

5. Conclusion

For the first time, high-resolution carbon isotope profiles have been generated within the dentine thickness using micromilling techniques. Our sampling strategy has been demonstrated capable of identifying large variations in $\delta^{13}C$ values (up to 13‰) within a single tooth slice, corresponding to a diet-switch experienced by the steers during tooth growth. The amplitude measured in the same tooth applying the longitudinal sampling in dentine was considerably lower (7.7‰) due to cross-sampling of histological features. These results confirm the model of dentine growth as the apposition of stacked cones and allow us to estimate time resolutions for both sampling procedures. Time averaging was estimated to 8–9 months when the longitudinal sampling procedure is applied, compared to 6–7 months when the same procedure is applied to enamel. However, contrary to enamel, the factor responsible for time averaging in dentine is not duration of mineralization, but mixing of dentine layers deposited over a period of time, and therefore the averaging might get even more pronounced as the animal gets older and dentine accumulates within the pulp cavity. For this reason, the transverse sampling performed in the mesostyle, which better matches the geometrical structure of dentine growth more accurately mimics growth banding, thereby decreasing time averaging by half.

Improvement of the transverse sampling procedure necessitates a better understanding of the timing of deposition of growth layers (seasonal changes in growth rates). At a larger scale, contrasting results obtained in the mesostyle one the one hand, and in the parastyle on the other hand suggest allometric growth of bovine dentine, which would necessitate further investigations. Fast growing zones might be of great potential for isotope studies involving sequential sampling.

Sequential sampling in dentine permits the use of additional stable isotope information present in collagen and bioapatite, thereby increasing the potentials for archaeological applications. Application to archaeological specimens will require careful examination of the preservation state of this tissue that is known to be more susceptible to diagenetic alteration than enamel.

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[38]

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