

RESEARCH ARTICLE

Intra-tooth isotopic analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of dentine collagen in high-crowned teeth: A new experimental study with modern sheep specimens

Celia Díez-Canseco^{1,2}  | Mònica Aguilera³  | Carlos Tornero^{2,4} 

¹Departament d'Història i Història de l'Art, Universitat Rovira i Virgili, Tarragona, Spain

²Institut Català de Paleoecologia Humana i Evolució Social (IPHES-CERCA), Tarragona, Spain

³Departament de Producció Vegetal i Ciència Forestal | AGROTECNIO Center, Universitat de Lleida, Lleida, Spain

⁴Department of Prehistory, Autonomous University of Barcelona (UAB), Bellaterra, Spain

Correspondence

Celia Díez-Canseco, Departament d'Història i Història de l'Art, Universitat Rovira i Virgili, Avinguda de Catalunya 35, Tarragona 43002, Spain.
Email: cdiezcanseco@iphes.cat

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Abstract

Hypsodont teeth molars develop in a short lapse of time. Both dentine and enamel dental tissues do not regenerate once formed. This fact allows the reconstruction of isotopic sequences covering short moments of the individual's life span, a useful approach in current zooarchaeological and paleontological studies. Sampling design usually follows a serial or sequential extraction of dental bands perpendicular to the tooth growth axis, from the apex to the root. However, dentine develops following a secretion of layers oblique to the tooth vertical axis, and this pattern makes hard to isolate isotopic signals. A new experimental study with modern sheep breeds was conducted, covering a whole year and where carbon and nitrogen isotopic signatures of consumed plants were known. Three diet periods with different isotopic compositions were alternated during sheep's life. In this contribution, we test the resolution of a dentine sampling procedure to obtain intra-tooth isotopic variability of $\delta^{13}\text{C}_{\text{collagen}}$ and $\delta^{15}\text{N}_{\text{collagen}}$ values tied to diet shifts in sheep. Dentine was sequentially sampled in previously demineralized second and third mandibular molars. Samples were treated with standard protocols of collagen purification and then subjected to stable carbon and nitrogen isotope analysis. In M2 and M3 isotopic sequences obtained, diet shifts are recorded along two progressive trends of increasing–decreasing values, but some affections of the sampling procedure are also present. We conclude that the dentine sequential sampling here implemented allows for obtaining reliable intra-tooth isotopic data tied to dietary conditions in high-crowned teeth, although dentine growth patterns should be considered in detail in final interpretations. The integration of this high-resolution analysis in archaeological studies offers a new scope of possibilities for studying dietary patterns, seasonal dietary changes, and animal husbandry practices in past populations.

KEYWORDS

carbon and nitrogen isotopes, modern reference data set, sequential analysis, serial sampling, tooth dentine

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1 | INTRODUCTION AND RESEARCH OBJECTIVES

During the last decades, biogeochemical analyses have shown a huge capability to investigate certain life conditions of past animal populations from archaeological faunal remains that were unreachable through traditional approaches. In zooarchaeological research, intra-tooth stable isotope analyses have become quite popular in hypsodont species because they allow for obtaining isotopic data with a high temporal resolution, in some cases with a seasonal extent, taking advantage from the growth dynamics of high-crowned teeth and the non-remodeling of the mineralized dental tissues. The possibility of obtaining biochemical data with an intra-annual resolution has turned these analyses into a powerful tool to investigate domestication processes and husbandry practices through the study of essential aspects of animal management, such as reproduction (Balasse et al., 2012, 2020, 2021; Blaise & Balasse, 2011; Tornero et al., 2013, 2020), feeding habits (Balasse, 2002; Balasse et al., 2009; Gillis et al., 2021; Makarewicz & Tuross, 2006; Navarrete et al., 2019), and mobility practices of domestic herds (Knockaert et al., 2018; Makarewicz et al., 2017; Tejedor-Rodríguez et al., 2021; Tornero et al., 2018), all three strongly tied to animal's seasonal cycles.

Nowadays, most intra-tooth or sequential stable isotope analyses performed on hypsodont species focus on inorganic compounds of teeth, commonly $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, or $^{87}\text{Sr}/^{86}\text{Sr}$ measurements in bioapatite enamel. However, dentine also offers possibilities for intra-tooth analysis. Neither enamel nor dentine undergoes remodeling once mineralized except for diseases (Bada et al., 1990; Nanci & Causa, 2013; Witzel et al., 2018), so they both record the isotopic signal from ingested sources during the whole time period of teeth development. In contrast with enamel where inorganic fraction forms $\sim 97\%$, organic fraction forms up to $\sim 20\%$ of dentine composition by weight, closer to bone composition (LeGeros, 1981; Skinner, 2005). This feature favors obtaining $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from dentine collagen through standard analytical techniques while obtaining high-temporal resolution data, an unfeasible goal in bone isotopic analysis considering bone remodeling rates. In addition, it is known that collagen isotopic composition reflects the protein part of diet (Ambrose & Norr, 1993; Tieszen & Fagre, 1993), so dentine may constitute a key tissue to complete the isotopic data spectrum in paleodietary studies developed through sequential analysis.

Despite the potential of implementing sequential analyses in dentine collagen, they have been scarcely performed in faunal remains regarding zooarchaeological research. Previous studies have been focused on defining weaning time in domestic cattle (Balasse et al., 2001; Balasse & Tresset, 2002; Gillis et al., 2013), feeding strategies on caprine herds (Makarewicz, 2014, 2017), or paleoenvironmental conditions in wild or free-range domestic specimens (Hopper et al., 2018; Kirsanow et al., 2008). The development of this analytical technique in high-crowned teeth has been limited by the shortage of methodological and experimental studies to support its archaeological implementation and to guarantee a reliable interpretation of the isotopic data. Sequential analysis on dentine is nowadays barely

understood because the technique needs to face an isotopic average resulting from mixing different dentine layers during sampling. In high-crowned teeth, dentine growth front is oblique to the vertical axis of molars, growing in width while the tooth is growing in length (Balasse et al., 2001; Kahle et al., 2018; Zazzo et al., 2006). This growth geometry complicates sampling following the natural deposition of dentine while obtaining samples with enough size to implement the standard collagen purification methods (Guiry et al., 2016).

Dentine's growth geometry makes difficult an alternative sampling: dentine thickness in one semi-lobule reaches around 1–1.5 mm wide (depending on the growth stage) and discriminating certain layers of dentine appositions becomes a complex challenge (e.g., Zazzo et al., 2006). Recent approaches implementing sequential dentine analysis in human molars develop the sampling by slicing sections of approximately 0.5–1 mm thickness following incremental layers of dentine (Czermak et al., 2018). However, this methodology is hardly suitable for hypsodont molars, where it is not possible to isolate incremental layers by hand (accumulated in less than 2 mm of dentine) and where it would become impossible to replicate a homogeneous sampling between different individuals, even using high-resolution transmission microscopy image (obtained prior to demineralization) as a reference. In addition, sample size obtained through these approaches implies omitting gelatinization, filtration, or NaOH steps during sample treatment (Czermak et al., 2018; Fernández-Crespo et al., 2018, 2020); all of them standardized steps accepted as beneficial and necessary to obtain reliable isotopic measurements. Drilling samples from teeth in a longitudinal axis would be more suitable for species with hypsodont teeth, but some problems turn up when implementing the demineralization of powder micro-samples by standard protocols in order to obtain reliable $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from dentine (Guiry et al., 2016).

These histological complications require some methodological efforts focused on untangling isotopic data resulting from the sampling protocols implemented in dentine. This study aims to develop a referential set of data for implementing sequential analysis of dentine collagen in hypsodont species, caprines in particular, to support its later archeological implementation. Our specific objective is to refine a main aspect related to the methodology: the relation between the isotopic input and the isotopic composition of the collagen serial samples obtained through the standard sampling and treatment protocol first developed by Balasse et al. (2001).

To achieve this proposal, we developed a new study with modern sheep specimens performing sequential stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) on tooth dentine collagen. This is the first study implementing intra-tooth analysis of dentine in experimental sheep raised under controlled life conditions and where the diet isotopic composition is known. This study focuses on post-weaning diet and covers a feeding schedule of 12 months where three fodder types with different isotopic compositions are alternated, introducing abrupt changes on sheep's diet in a 1-year time span. High-resolution isotopic data related to these diet changes are expected in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of dentine collagen.

2 | MATERIALS AND METHODS

2.1 | The SUMA modern reference herd

Between 2017 and 2019, an experimental animal science program with a modern reference set of *Ovis aries* (Linnaeus, 1758) specimens with known life conditions was developed at the Farm Services and Experimental Fields facilities of the Autonomous University of Barcelona (UAB), settled in Barcelona, Spain. This program was named SUMA and was designed by Dr. Carlos Tornero to implement several methodological studies dealing with sheep dietary reconstruction in zooarchaeological studies (e.g., Gallego-Valle et al., 2020). The different studies carried on were approved by the Animal Care and Use Committee from UAB. All investigated specimens belong to *ripollense* sheep breed, a local breed from NE Spain traditionally oriented to lamb production and meat exploitation (Caja et al., 2010; Esquivelzeta et al., 2011). The specimens used in this study come from a flock of 12 female sheep raised in a stall where diet conditions were controlled from October 2017 to November 2018.

The weaning age of the lambs was 2 months. Post-weaning diet consisted of three different monospecific diet periods, which alternate C₃ and C₄ plants. The sheep fed on alfalfa (*Medicago sativa*) (DIET1) during the first 10.5 months of life following weaning; C₄ diet period based on foxtail millet (*Setaria italica*) (DIET2) followed during the next 4 months; the last 4.5-month period the sheep fed on barley (*Hordeum vulgare*) (DIET3). The detailed diet schedule is shown in Figure 1. Fodder was provided by local agri-food companies using verified and numbered fodder bales. Fodder was daily supplied in general troughs where sheep ate ad libitum. During DIET1 and DIET2, fodder consisted of hay, thus including the whole plant. DIET3 was based on straw (mostly leaves and stems) but daily complemented with 1 kg of grain per specimen. Grain supplement was recommended to counteract the low nutritional contribution of barley straw to sheep's diet. Fodder origin was relatively stable during each diet period: each fodder type derived from the same supplier and same batch. Three specimens of this flock were selected to perform isotopic analyses: OV-08 (ID 711-170110), OV-07 (ID 704-170048), and OV-11 (ID 706-170080). They were born between March 1 and March 10, 2017, and they were slaughtered between November 7 and

November 21, 2018, at 20.3 (OV-08), 20.5 (OV-07), and 20.9 (OV-11) months of age.

2.2 | Fodder sampling and isotopic measurements

Fodder supplied to sheep during the three diet periods was sampled for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses. Fodder samples were collected from troughs from November 2017 until sheep's slaughtering. A total number of 40 fodder samples were analyzed, belonging to the four diet components: alfalfa, foxtail millet, barley straw, and barley grains. Each sample plot included at least five individuals and the representation of the whole plant in the case of alfalfa and foxtail millet, leaves and stems in barley-straw, and the whole grain in barley-grains. Samples were oven-dried, milled, weighted, and placed in tin capsules for subsequent analysis. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in fodder samples were measured using a Thermo Flash 1112 elemental analyzer (EA) coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer (IRMS) with a ConFlo III interface at the Institute of Environmental Science and Technology (ICTA-UAB) (Barcelona, Spain). The international standard laboratory IAEA 600 (caffeine) was used as control. The average analytical error was $<0.15\text{‰}$ (1σ) calculated for each isotopic measurement, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ separately. $\delta^{13}\text{C}$ values are expressed relative to Vienna PeeDee Belemnite (V-PDB) standard and $\delta^{15}\text{N}$ values relative to air N₂ (AIR): $\delta \chi^{1/4} (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000\text{‰}$, where χ stands for ¹³C or ¹⁵N and R stands for ¹³C/¹²C or ¹⁵N/¹⁴N.

M. sativa is a C₃ leguminous plant (N₂-fixing-plant), *H. vulgare* is a C₃ plant, and *S. italica* is a C₄ plant, the last two from *Poaceae* family. Our study foresaw important differences in $\delta^{13}\text{C}$ values between the three species according to their photosynthetic pathway (Farquhar et al., 1989; O'Leary, 1981). Further, low values in $\delta^{15}\text{N}$ values were expected in alfalfa according to N₂-fixing-plant condition (Kohl & Shearer, 1980; Virginia & Delwiche, 1982).

Some differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are also expected between both components of DIET3, barley grains and straw, according to the different isotopic composition reported between the different organs of plants (Codron et al., 2005; Heaton, 1999; Lightfoot et al., 2016). Taking into account that collagen isotopic

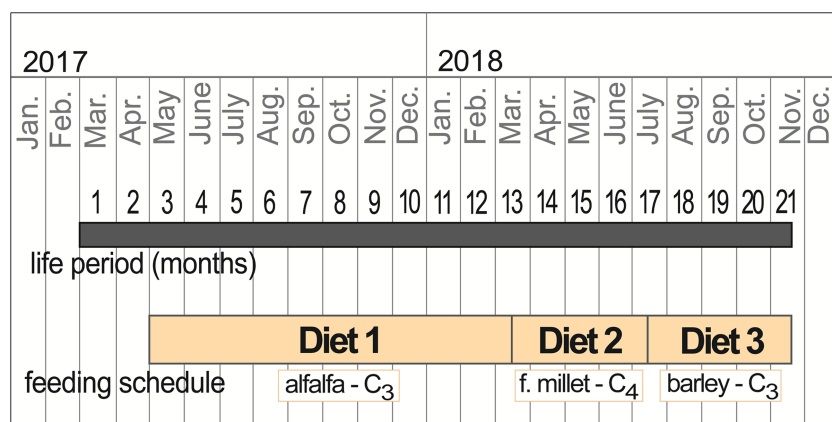


FIGURE 1 Life span period for the three sheep specimens analyzed in this study and duration of the three diet periods settled during the experimental program: DIET1, alfalfa; DIET2, foxtail millet; DIET3, barley [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

composition mainly reflects protein fraction of diet (Ambrose & Norr, 1993; Codron et al., 2018; Jim et al., 2004; Tieszen & Fagre, 1993), the estimation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the whole DIET3 was made considering the different protein contribution of straw and grain to the diet based on (1) their different protein content and (2) the different weight (g) contribution of both components. Protein contribution was estimated from the %N values (Balasse et al., 2001; Conklin-Brittain et al., 1999) and from dry matter (DM) content of barley forages (data extracted from National Research Council, 1985). Sheep daily ate the whole supplement of grain, that is, 1 kg (DM = 880 g). Because straw is supplied ad libitum, we estimated the daily DM voluntary intake of sheep following method from Minson (1990): $\text{DMI} = 60 \text{ g/kg } W^{0.75}$ (DMI, dry matter intake; W, bodyweight = ~60 kg).

2.3 | Dentine sampling and collagen isotopic analyses

Sheep mandibles were separated from the skull, partially defleshed, and boiled in distilled water for several hours. Second (M2) and third (M3) left mandibular molars were selected for analysis. At the time of death, M2s were partially worn, and enamel-root junction (ERJ) was recently formed. Roots were in an early stage of development at the time of death, barely exceeding the ERJ. M3 presents an early stage of eruption, the cuspids are slightly or not worn, and around 2/3 parts of the crown are formed (Figure 2).

The posterior lobe of M2 and anterior lobe of M3 were cut and separated from the tooth using a diamond disc. Teeth sampling and samples treatment were performed at the Biomolecular Laboratory of the Catalan Institute of Human Palaeoecology and Social Evolution (Tarragona, Spain). Dentine was sampled following Balasse et al. (2001). The selected lobes were demineralized in EDTA (0.5 M, pH 8) at room temperature (Tuross et al., 1988). The solution was renewed

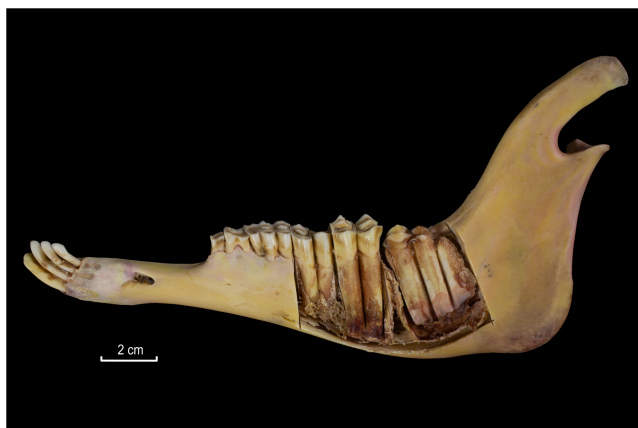


FIGURE 2 Stage of tooth development of one of the individuals analyzed (OV-08). All three individuals showed similar development of M2 and M3 molars [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

every 2–5 days. Complete demineralization took about 2 months for the M2 lobe and about 1 month for the M3 lobe. Once demineralized, the lobes were intensely rinsed with distilled water, including a soak in distilled water for a week, in order to remove EDTA completely. Sampling was then performed with a scalpel by cutting the buccal part of each lobe in 2-mm sections, following the vertical axis (i.e., from the top to the bottom of the tooth). A total of 18 to 20 samples were obtained in M2 molars and 14 samples in M3. In M2 lobes, the distance of the root base from ERJ was measured before demineralization. During sampling, the base of the sampled buccal lobe was considered as reference. Sample position was then expressed as distance from ERJ to allow for comparison between molars. Cuspid was used as reference for M3 sampling, because M3 molars were not worn or presented slight wear without compromising the tooth length. Each sample was soaked in 0.125M NaOH for 20 h at room temperature in order to remove some lipids (Ambrose, 1990; Ambrose & Norr, 1993) and then rinsed again with distilled water several times. Samples were homogenized by solubilization in a HCl 10^{-2} M solution (pH 2), at 100°C for 17 h (Bocherens et al., 1991), then filter and freeze-dried. A total of 80 samples were taken for isotopic analyses. Around 300 μg of collagen was weighed into 6×4 -mm tin capsules. Stable isotope measurements, notation, and standards used for δ calculation are described in previous Section 2.2. The average analytical error was also $<0.15\text{‰}$ (1σ) for each isotopic measurement, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ separately. The reliability of the isotopic signatures of the collagen extracts was addressed using carbon and nitrogen content values and the atomic C/N ratio, settled $3.1 < \text{C/N} > 3.5$. It was not possible to calculate the % of collagen yield because sampling was carried out once the whole tooth was demineralized.

3 | RESULTS

3.1 | Fodder $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values

The isotopic results from fodder samples are shown in Table 1. Alfalfa, foxtail millet and barley-straw samples provide $\delta^{13}\text{C}$ mean values of $-28.9 \pm 0.83\text{‰}$, $-13.1 \pm 0.30\text{‰}$, and $-28.2 \pm 0.41\text{‰}$, respectively, and $\delta^{15}\text{N}$ mean values of $+1.2 \pm 1.48\text{‰}$, $+11.7 \pm 0.74\text{‰}$, and $+3.6 \pm 1.78\text{‰}$, respectively. Barley-grain samples provide higher mean values than barley-straw samples, both in $\delta^{13}\text{C}$ ($-25.6 \pm 0.11\text{‰}$) and $\delta^{15}\text{N}$ ($+7.5 \pm 0.33\text{‰}$).

The two sampled C_3 plants species, alfalfa and barley, show large differences with C_4 foxtail millet, both in their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. The ^{13}C content is in accordance with their different photosynthetic pathways of C_3 – C_4 plants (Farquhar et al., 1989; Kohn, 2010). Both C_3 plants, alfalfa and barley, present similar $\delta^{13}\text{C}$ values around -28‰ (-25‰ in the case of grain). As for nitrogen isotope composition, DIET1-alfalfa shows the lowest $\delta^{15}\text{N}$ values, in accordance with N_2 -fixing-plants isotopic composition (Kohl & Shearer, 1980; Virginia & Delwiche, 1982). Differences in $\delta^{15}\text{N}$ values between DIET2-foxtail millet and DIET3-barley, both cereals and non- N_2 -fixing

TABLE 1 Carbon and nitrogen isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of sampled components supplied during DIET1, DIET2, and DIET3: Mean values and standard deviation (\pm), min. and max. values, and ranges of variation

		n	$\delta^{13}\text{C}$ (‰)				$\delta^{15}\text{N}$ (‰)			
			Mean \pm SD	Min.	Max.	Range	Mean \pm SD	Min.	Max.	Range
DIET 1	Alfalfa	3	-28.9 ± 0.8	-29.4	-27.9	1.5	$+1.2 \pm 1.5$	-0.5	+2.5	2.9
DIET 2	Foxtail millet	9	-13.1 ± 0.3	-13.5	-12.6	0.9	$+11.7 \pm 0.7$	+10.6	+12.8	2.2
DIET 3	Barley-straw	12	-28.2 ± 0.4	-28.7	-27.5	1.2	$+3.6 \pm 1.8$	+1.5	+7.0	5.4
	Barley-grain	16	-25.6 ± 0.1	-25.8	-25.4	0.4	$+7.5 \pm 0.3$	+7.1	+8.2	1.1

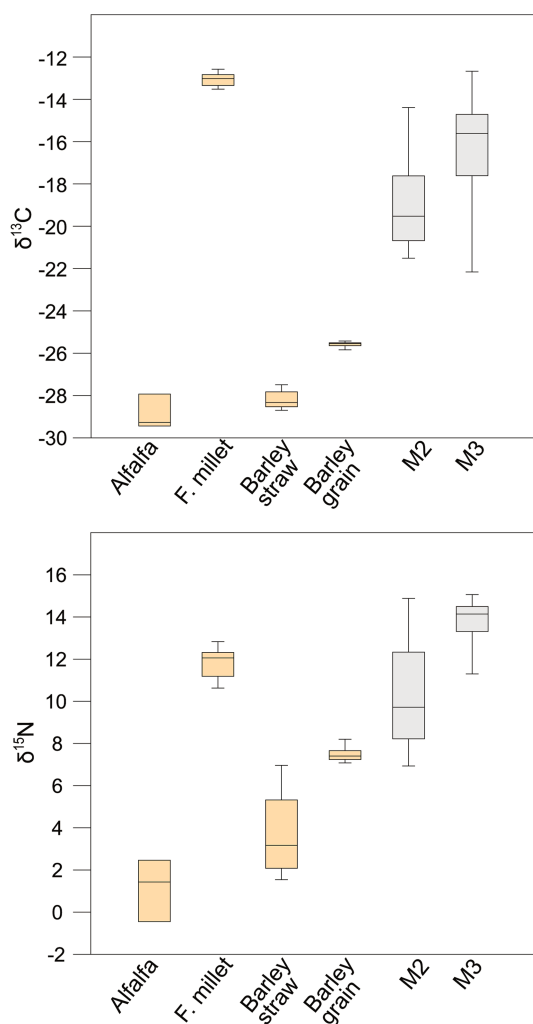


FIGURE 3 Box plot showing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for the four fodder types analyzed and for the collagen samples of the three sheep specimens plotted by sampled molar [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

plants, probably refer to different field origin and soil conditions of both crops types (manure intensity or natural abundance of ^{15}N in soil) (Bogaard et al., 2007; Handley et al., 1999; Handley & Scrimgeour, 1997; Treasure et al., 2016), more than referring differences in the plant physiology (Terra et al., 2000). The same explanation regarding differences in soil conditions applies to the

intra-specific variability existing in $\delta^{15}\text{N}$ values (Figure 3), because several bales of hay and straw were employed for fodder supplies during each period.

The two barley components—grain and straw—involved in DIET3 present large differences in their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. In this sense, it is important to underline that most protein contribution to sheep's diet will come from grain, considering two main aspects. (1) Based on the different nitrogen content (%N) of the two components, we can assume a larger crude protein content present in grain (CP of 12.3%) than in straw (CP of 2.5%) (following Kjeldahl method, Conklin-Brittain et al., 1999). (2) Considering a daily voluntary intake of 1.3 kg of DM for each sheep specimen (Minson, 1990), a larger daily intake of grain in relation to straw should be expected. The isotopic composition estimated for the whole DIET3 according to the different protein contribution of the two components is shown in Table 2. We are aware that some differences could exist between $\delta^{13}\text{C}_{\text{whole-plant}}$ (bulk sample) and $\delta^{13}\text{C}_{\text{protein}}$ (Fagre et al., 1991), so plant carbon isotopic results are not fully representative of the real isotopic contribution coming from protein.

3.2 | Dentine collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values

Carbon and nitrogen isotopic composition, carbon and nitrogen content values, and C:N ratios from dentine collagen samples are presented in Table 3. All samples present carbon content values, nitrogen content values, and C:N ratios according to modern collagen data (Ambrose, 1990; Van Klinken, 1999). All samples yield C:N ratios between 3.1 and 3.4. Carbon content (in wt% C) ranges from 44.8% to 24.9% (mean $36.8 \pm 3.45\%$), and nitrogen content (in wt% N) ranges from 16.2% to 9.3% (mean $13.5 \pm 1.21\%$).

Collagen samples from M2 molars present mean values of $-19.0 \pm 2.04\%$ in $\delta^{13}\text{C}$ and $+10.3 \pm 2.46\%$ in $\delta^{15}\text{N}$ (Table 4). Collagen samples from M3 are slightly enriched in ^{13}C , showing mean values of $-16.4 \pm 2.42\%$ in $\delta^{13}\text{C}$ and $+13.8 \pm 0.92\%$ in $\delta^{15}\text{N}$. Serial samples yield high intra-tooth isotopic variation in both second and third molars (Figure 3) as a consequence of the different feeding periods involved in sheep's diet: in M2 samples, $\delta^{13}\text{C}$ values range from -21.5% to -14.4% and $\delta^{15}\text{N}$ values range from $+6.9\%$ to $+14.9\%$; in M3 samples, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values range from -22.2% to -12.7% and from $+11.3\%$ to $+15.1\%$, respectively.

TABLE 2 Mean isotopic composition of whole DIET3 based on the different protein contribution of the two components supplied to sheep (straw, grain)

		Weight (g)	Dry matter ^a intake (g)	%N	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	Protein contribution
Barley	Straw	Ad lib.	420	0.4	-28.2	+3.6	8%
	Grain	1000	880	2.0	-25.6	+7.5	92%
Isotopic composition from protein part of DIET3					$\delta^{13}\text{C}_{\text{protein}}$	$\delta^{15}\text{N}_{\text{protein}}$	
					-25.8‰	+7.2‰	

Note: Dry matter intake of straw (supplied ad libitum) has been estimated considering a daily voluntary intake of 1.3 kg for each sheep specimen (following Minson, 1990). Mean carbon isotopic composition for the protein part of DIET3 ($\delta^{13}\text{C}_{\text{protein}}$) is an estimation (see text).

^aDry matter content of barley forages from NRC (1985).

TABLE 3 Carbon and nitrogen isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), carbon and nitrogen content values (%C and %N), and C:N ratio of collagen samples

	M2						M3					
	Dist. from ERJ (mm)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	C:N	Dist. from cuspid (mm)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	C:N
OV-07	38.0	-20.2	+7.0	38.3	14.0	3.2	1.5	-15.8	+11.7	38.6	14.0	3.2
	34.0	-20.5	+7.8	40.2	14.6	3.2	4.3	-15.4	+13.3	32.7	11.9	3.2
	30.3	-20.7	+8.0	39.1	14.3	3.2	6.5	-14.6	+13.9	36.3	13.3	3.2
	26.5	-20.9	+8.3	38.9	14.2	3.2	8.3	-14.5	+14.3	39.8	14.6	3.2
	23.0	-20.5	+9.1	39.9	14.5	3.2	10.3	-15.0	+14.5	30.2	11.2	3.1
	19.3	-19.6	+9.8	44.8	16.2	3.2	12.5	-15.2	+14.6	40.4	14.7	3.2
	17.0	-19.1	+10.4	40.0	14.5	3.2	14.8	-15.5	+14.5	37.2	13.8	3.1
	15.0	-18.4	+11.1	41.1	14.9	3.2	17.0	-16.0	+14.6	26.7	9.7	3.2
	13.0	-18.1	+11.8	42.1	15.1	3.3	20.8	-17.8	+14.1	37.6	13.7	3.2
	11.0	-18.0	+11.9	39.5	14.3	3.2	25.0	-20.4	+13.0	28.3	10.5	3.1
	8.8	-17.4	+12.3	40.2	14.4	3.2	29.8	-20.9	+13.4	24.9	9.3	3.1
	6.3	-16.2	+13.7	37.9	13.8	3.2						
	4.0	-15.0	+14.7	37.8	13.7	3.2						
1.5	-17.9	+14.2	41.9	15.2	3.2							
OV-08	35.1	-20.4	+8.0	36.7	13.6	3.1	1.3	-15.6	+11.3	33.6	12.4	3.2
	31.6	-20.6	+8.5	39.0	14.3	3.2	3.2	-14.7	+13.0	36.4	13.5	3.2
	28.4	-20.7	+8.6	39.9	14.5	3.2	4.4	-14.4	+13.6	38.3	14.2	3.1
	24.3	-20.7	+8.6	35.7	13.2	3.2	6.0	-14.0	+14.2	36.8	13.7	3.1
	20.8	-20.4	+8.9	36.9	13.6	3.2	8.5	-14.0	+14.7	38.0	14.0	3.2
	17.8	-19.8	+9.2	38.4	14.1	3.2	10.6	-14.7	+14.9	41.0	15.1	3.2
	15.8	-19.4	+9.4	36.9	13.7	3.1	12.3	-14.9	+14.9	35.7	13.3	3.1
	14.0	-18.7	+10.2	34.3	12.8	3.1	13.7	-15.7	+15.1	37.8	14.0	3.2
	12.3	-18.2	+10.6	30.6	11.3	3.1	17.0	-16.7	+14.9	35.2	13.1	3.1
	11.1	-17.6	+11.3	34.9	13.0	3.1	21.4	-18.9	+14.2	39.2	14.4	3.2
	9.7	-16.7	+12.3	34.5	12.7	3.2	27.0	-20.4	+14.1	31.3	11.7	3.1
	7.6	-15.6	+13.4	37.0	13.7	3.2						
	5.5	-14.4	+14.5	34.6	12.8	3.2						
3.5	-15.6	+14.9	32.4	12.0	3.1							
1.3	-19.1	+14.0	34.7	12.8	3.2							
OV-11	36.0	-21.4	+6.9	37.2	13.6	3.2	1.5	-12.7	+13.4	40.8	15.0	3.2
	33.3	-21.4	+7.3	35.6	13.0	3.2	4.0	-14.2	+14.0	39.5	14.5	3.2
	31.5	-21.5	+7.5	36.7	13.4	3.2	6.0	-14.9	+13.9	38.1	14.2	3.1

(Continues)

TABLE 3 (Continued)

M2						M3					
Dist. from ERJ (mm)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	C: N	Dist. from cuspid (mm)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	C: N
29.6	-21.3	+7.5	37.8	13.8	3.2	7.7	-15.2	+14.2	38.0	14.1	
27.4	-21.5	+7.6	36.7	13.4	3.2	9.7	-15.7	+14.2	37.0	13.6	3.2
25.4	-21.4	+7.9	35.9	13.2	3.2	12.0	-16.0	+14.2	38.6	14.3	3.1
22.9	-21.1	+8.1	37.3	13.7	3.2	13.5	-16.3	+14.4	39.9	14.7	3.2
20.3	-20.8	+8.4	38.1	14.1	3.2	15.0	-17.4	+13.9	41.6	15.1	3.2
18.3	-20.7	+8.5	36.2	13.4	3.2	19.5	-20.1	+12.7	38.0	14.1	3.1
16.2	-20.2	+8.9	36.6	13.5	3.2	24.0	-20.7	+12.5	35.6	13.2	3.1
14.1	-19.2	+9.6	37.5	13.9	3.1	28.5	-22.2	+12.4	30.1	10.4	3.4
11.9	-18.2	+10.6	36.2	13.3	3.2						
9.8	-17.6	+11.7	35.4	13.1	3.2						
8.3	-16.4	+12.6	36.9	13.7	3.1						
6.1	-15.1	+14.0	37.3	13.7	3.2						
3.5	-16.4	+14.1	35.6	13.1	3.2						
1.2	-20.3	+12.0	31.4	11.9	3.1						

Note: Minimum and maximum isotopic values are shown in bold. Distance (dist.) from ERJ in M2 and from cuspid in M3 represents the intermediate point between sections.

TABLE 4 Summary table showing mean collagen isotopic values, standard deviation, and range for the three individuals and teeth analyzed

		n	$\delta^{13}\text{C}$ (‰)			$\delta^{15}\text{N}$ (‰)		
			Mean	SD	Range	Mean	SD	Range
OV-07	M2	14	-18.7	1.8	5.9	+10.7	2.5	7.6
	M3	11	-16.5	2.3	6.4	+13.8	0.9	2.9
OV-08	M2	15	-18.5	2.1	6.3	+10.8	2.4	6.9
	M3	11	-15.8	2.1	6.4	+14.1	1.1	3.8
OV-11	M2	17	-19.7	2.1	6.4	+9.6	2.4	7.1
	M3	11	-16.9	3.0	9.5	+13.6	0.7	1.9

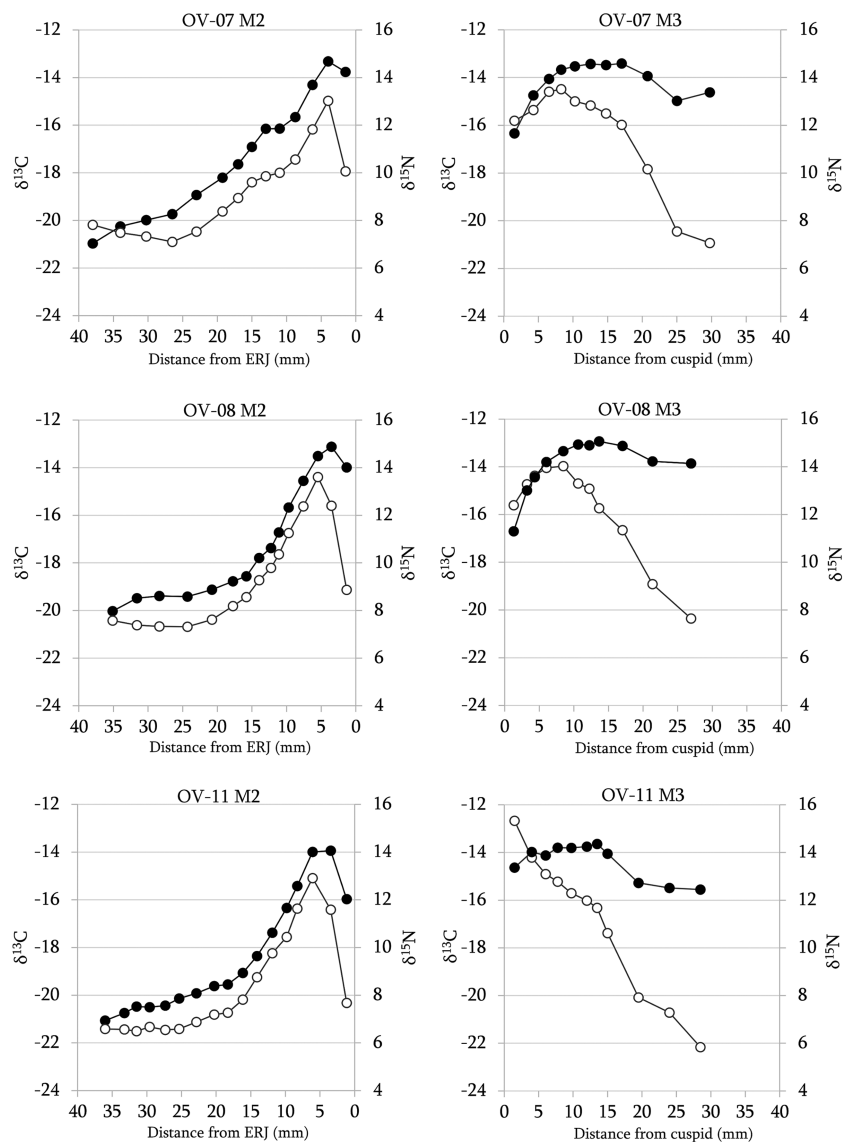
The three sheep individuals show similar intra-tooth isotopic variation as expected from animals raised and fixing isotopic signatures under identical conditions: (1) the range of variation for the three individuals present mean values of 6.2 ± 0.26 and 7.4 ± 1.77 in $\delta^{13}\text{C}$ and 7.2 ± 0.38 and 2.9 ± 0.93 in $\delta^{15}\text{N}$, M2 and M3, respectively; (2) according to maximum values, inter-individual variability is $\leq 0.7\%$ and $\leq 1.8\%$ in $\delta^{13}\text{C}$ and $\leq 0.8\%$ and $\leq 0.7\%$ in $\delta^{15}\text{N}$, M2 and M3, respectively; according to minimum values, inter-individual variability is $\leq 0.8\%$ and $\leq 1.8\%$ in $\delta^{13}\text{C}$ and $\leq 1.0\%$ and $\leq 1.1\%$ in $\delta^{15}\text{N}$, M2 and M3, respectively.

Intra-tooth isotopic sequences for each individual are presented in Figure 4. In all sampled specimens, isotopic variation along the M2 molars follows a progressive increase in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values along most part of the crown, followed by an abrupt decrease represented in the last 2–4 mm. In the M3 crowns, sequences show a short ascendant trend in $\delta^{13}\text{C}$ values during the first 8 mm (except for OV-11) then interrupted by a sudden deviation into a trend of

decreasing values. In $\delta^{15}\text{N}$ values, the M3 sequences show a short increase until forming a “plateau” in the middle of the crown, followed by a less accentuated decrease –both in amplitude and extension– than in $\delta^{13}\text{C}$ values.

Minimum $\delta^{13}\text{C}$ values recorded in sheep dentine samples from the first part of the M2 sequences show a mean of $-21.0 \pm 0.43\%$, consistent with a C_3 diet. Mean maximum $\delta^{13}\text{C}$ values in M2s ($-14.8 \pm 0.38\%$) and M3s ($-13.7 \pm 0.93\%$) are consistent with an important intake of C_4 plants in sheep's diet. Last samples of M2 and M3 crowns show again low $\delta^{13}\text{C}$ values closer to a consumption of C_3 plants ($-19.1 \pm 1.19\%$ and $-21.1 \pm 0.92\%$, respectively). As for $\delta^{15}\text{N}$, minimum values are recorded in the first part of M2 crowns and show a mean of $+7.3 \pm 0.58\%$, while the maximum values recorded in the last mm of M2s show a mean of $+14.5 \pm 0.43\%$. In M3 crowns, maximum values of $\delta^{15}\text{N}$ provide a mean of $+14.7 \pm 0.36\%$, whereas last samples yield $\delta^{15}\text{N}$ mean values of $+13.3 \pm 0.84\%$.

FIGURE 4 Sequential $\delta^{13}\text{C}$ (white) and $\delta^{15}\text{N}$ (black) values measured in collagen along the crown of the second (M2) and third (M3) molars of the three sheep specimens analyzed (OV-07, OV-08, OV-11)



4 | DISCUSSION

4.1 | Intra-tooth isotopic variation related to diet shifts

The pattern of intra-tooth variation of $\delta^{13}\text{C}_{\text{collagen}}$ and $\delta^{15}\text{N}_{\text{collagen}}$ obtained through dentine sequential sampling fits well with the differences observed between fodder types in their carbon and nitrogen isotopic composition. Diet shifts—succession of DIET1, DIET2, and DIET3—are recorded in the M2 and M3 isotopic sequences along two progressive trends of increasing-decreasing values in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Collagen highest isotopic values recorded in both molars necessarily correspond to the DIET2 signature entirely based on a C_4 plant enriched in ^{15}N (foxtail millet). DIET1 period based on alfalfa is mainly recorded in the first half of M2 crown by low values, whereas DIET2 and DIET3 (barley) are mainly recorded through the last part of the M2 and along the whole crown of the M3. In third molars samples, the smaller range of variation of nitrogen isotopic values compared

with carbon values is also consistent with plant data, where the isotopic variation between DIET2-millet and DIET3-barley is much larger in $\delta^{13}\text{C}$ values than in $\delta^{15}\text{N}$ values.

The oscillation pattern of carbon and nitrogen values found along the M2 and M3 sequences is highly uniform for all three sampled individuals. In addition to the identical dietary conditions existing for all sheep, this aspect suggests that sampled animals followed similar patterns of crown formation. Inter-individual variability in maximum and minimum values is $\leq 1.0\text{‰}$ for M2s and $\leq 1.8\text{‰}$ for M3s, in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$; however, it is worth noting that excluding OV-11, inter-individual variability in M3s is reduced to $\leq 0.6\text{‰}$ in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Similar small inter-individual isotopic differences between same species specimens raised under the same dietary conditions have already been noticed in collagen composition (for small mammals with monoisotopic diets: Ambrose & Norr, 1993; Tieszen & Fagre, 1993; for medium-large mammals with non-monoisotopic diet: Balasse et al., 2001; Doherty et al., 2022; Von Holstein et al., 2013; Webb et al., 2016) or other proteinaceous tissues (Sponheimer, Robinson,

Ayliffe, Passey, et al., 2003; Sponheimer, Robinson, Ayliffe, Roeder, et al., 2003), as a result of individual metabolic and physiological factors affecting the final tissue isotopic composition. Moreover, the dissimilarities seen in the M3 of OV-11 against the others M3s of OV-07 and OV-08—both in the oscillation pattern and in the max. and min. isotopic values—are probably related to a slightly-later development of this third molar in OV-11, a scenario where the first diet isotopic signature previous to the 13th month would be absent. Previous studies have shown a larger variability in the M3 growth timing than in M2 within sheep populations (Milhaud & Nezit, 1991; Weinreb & Sharav, 1964; Zazzo et al., 2010), a feature also observed through isotopic approaches (Blaise & Balasse, 2011; Tornero et al., 2013).

DIET2 and DIET3 periods are represented in the isotopic sequences over a different length (in mm) between second and third molars, despite representing the same time span in both molars (4 months span each period). This fact is well consistent with the variable tooth growth rates existing along the crown, where the deposition rate of dentine is higher during the first months of tooth development than towards the latest growth stages when the growth rate decreases (Kahle et al., 2018; Witzel et al., 2018). This histological feature (i.e., attenuation of the crown growth rate) results in a more prolonged representation of a certain isotopic signature in the upper part of the crown, while the same time span would be cramped in the last part of the tooth. This trend has also been highlighted in previous works implementing enamel sequential analysis in hypsodont species, due to similar differential growth rates of this tissue along the tooth crown (Balasse, 2003; Bendrey et al., 2015; Zazzo et al., 2010). Our study found that, as for enamel, when implementing dentine sequential analysis in sheep, the dental growth stage when diet variations occur determines the representation of the isotopic signatures in the sequences, not only in their position in the crown but also in their length. Hence, dentine growth rates must be considered in final interpretations.

4.2 | Effects of the sampling approach on intra-tooth isotopic variation

In both M2 and M3, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values obtained from collagen samples show a quite reduced range of variation in contrast with the large isotopic differences existing between each diet period. Results from fodder analysis show that diet changes entailed drastic shifts in the sheep's isotopic input. For the first and second diet change, respectively, an input variation of around $\sim 15.8\text{‰}$ and $\sim 12.7\text{‰}$ in $\delta^{13}\text{C}$ and $\sim 10.5\text{‰}$ and $\sim 4.5\text{‰}$ in $\delta^{15}\text{N}$ could be expected. However, dentine samples obtained through sequential sampling hardly reach such ranges of variation in their isotopic values. Collagen shows, in M2 and M3, respectively, a maximum range of 7.1‰ and 9.5‰ in $\delta^{13}\text{C}$ values and a maximum range of 7.9‰ and 3.8‰ in $\delta^{15}\text{N}$ values.

This observation is mainly related to the effect that the sampling procedure here implemented is having on the isotopic intra-tooth variation. Dentine is deposited by apposition of layers oblique to pulp cavity and therefore oblique to the tooth vertical axis, a growth model

previously demonstrated through histological observations (Kahle et al., 2018; Weinreb & Sharav, 1964; Witzel et al., 2018) and through dentine isotopic data (Balasse et al., 2001; Balasse & Tresset, 2002; Zazzo et al., 2006). As a result, when a longitudinally sampling procedure cross-cuts the whole dentine thickness, it causes the admixture of dentine increments synthesized in different moments of life. Therefore, it is expectable that in our study—performed during 1 year—dentine samples from M2 and M3 may yield isotopic values from more than one feeding period, depending on the position of the samples in the tooth crown. When this dentine admixture affects samples yielding maximum and minimum isotopic values, it results in shorter ranges of intra-tooth variation than expected based on diet input.

This fact is most evident in the highest $\delta^{13}\text{C}_{\text{collagen}}$ values, too depleted to represent the C₄ diet period exclusively (DIET2), both in M2s samples (mean observed diet-collagen enrichment: $\Delta^{13}\text{C}_{\text{diet2-collagen}} = -1.7 \pm 0.38\text{‰}$) and M3s samples (mean observed diet-collagen enrichment: $\Delta^{13}\text{C}_{\text{diet2-collagen}} = -0.6 \pm 0.93\text{‰}$). These values seem quite depleted taking into account that a diet-collagen enrichment of 3–5‰ should be expected in $\delta^{13}\text{C}$ in primary consumers (Ambrose & Norr, 1993; Bocherens & Mariotti, 2002; Froehle et al., 2010; Lee-Thorp et al., 1989; Tieszen & Fagre, 1993). Considering dentine growth geometry, in these samples, the DIET2 isotopic signature is probably attenuated because of some incorporation of later increments representing DIET3, more depleted in ^{13}C . As a sampling-related issue, similar attenuation may exist in maximum values of $\delta^{15}\text{N}_{\text{collagen}}$, although in this case, the diet-collagen differences are larger (attending max. Values: $\Delta^{15}\text{N}_{\text{diet2-collagen}} = 1.4 \pm 0.43\text{‰}$ in M2s and $\Delta^{15}\text{N}_{\text{diet2-collagen}} = 1.6 \pm 0.36\text{‰}$ in M3s) and somewhat more coherent with usual $\delta^{15}\text{N}$ enrichment values. The interference of DIET3 signature in maximum values of $\delta^{15}\text{N}$ is here less evident, probably because DIET2 and DIET3 present more similar isotopic values in $\delta^{15}\text{N}$ than in $\delta^{13}\text{C}$.

A similar sampling effect could also be expected in both minimum $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of M2s, according to the dentine growth pattern and the position of these samples in the first part of M2 crowns. Here, they may include some dentine increments developed during the following diet periods, therefore yielding higher isotopic values than expected from DIET1 when isolated. For the same histological reason, samples from the last part of the crown should, in contrast, include less dentine admixture, because dentine is here thinner and represents only the final growth development (for illustrating dentine growth geometry, cf. Kahle et al., 2018, and Witzel et al., 2018).

Finally, it is worth noting that in the 20-month-old sheep specimens analyzed here, dentine does not exceed ERJ either in second or third molars at death time. However, dentine continues developing along the whole life of the individuals, conforming the roots and narrowing the pulp chambers and the root canal (Hillson, 2005; Milhaud & Nezit, 1991). Therefore, when applying this sampling procedure, isotopic variations obtained from dentine will be progressively attenuated as the tooth continues developing and growing in thickness with the apposition of new dentine. Our study suggests that tooth development stages should be considered in order to implement

dentine isotopic sequential analysis in sheep, and younger specimens should be preferably selected when possible.

4.3 | Temporal resolution

The diet changes induced on sheep were abrupt, produced in 1 day. However, diet changes are apparently represented in intra-tooth isotopic sequences along two progressive trends of increasing-decreasing values. Around 1.5 and 2 months of diet-tissue isotopic equilibration is expected (Jones et al., 1981; Sponheimer, Robinson, Ayliffe, Roeder, et al., 2003), but effects of the longitudinal sampling procedure should also be considered to explain this observation. Considering the dentine growth geometry of hypsodont molars, it is reasonable to assess that the isotopic variation observed in the intra-tooth sequences starts with the incorporation of inner and later-formed dentine to the sample, thus representing the progressive admixture of subsequent increments instead of the actual collagen variation through time (i.e., equilibration period). Although the equilibration process is indeed recorded in dentine collagen, the admixture of dentine derived from the longitudinal sampling necessarily overstates this 2-month process, resulting in an earlier and longer representation of the isotopic variation. This aspect had been previously noticed in cattle molars by M. Balasse et al., where diet shifts were represented in dentine intra-tooth sequences earlier than expected

according to crown development timings known for cattle (Balasse et al., 2001).

In the sheep specimens analyzed in this study, these effects are well evident in the representation of the first change of diet—occurring at the 13th month of life—along the M2 crown. The ascending trend in isotopic values starts around 25 mm from ERJ, while it would be expectable that this moment of life would be represented much onwards in the crown, in the last 1/5 part according to crown growth rates reported in sheep in previous studies (Kahle et al., 2018; Witzel et al., 2018). When considering these dentine growth timings reported for M2 and M3 crowns, in contrast, the moments on sheep's life when diet shifts occurred seem to fit better with the final position of ascending/descending trends instead of the starting point of variation (Figure 5). This fact is coherent with the dentine growth geometry and the sampling implemented: final points of the variation trends should correspond to the crown's position where the previous diet signature is no longer sampled, that is, the part of the crown first developing—in length—under the new isotopic background.

Taking this into account, we suggest that final points of the ascending/descending trends reached in intra-tooth sequences obtained through this sampling may coincide with the part of the crown growing in length when collagen is being synthesized under the new period isotopic composition, thus representing that particular period of life after the equilibration period. This is an important aspect because it entails that through sequential dentine analysis, it could be possible to situate at a temporal level different diet periods existing in

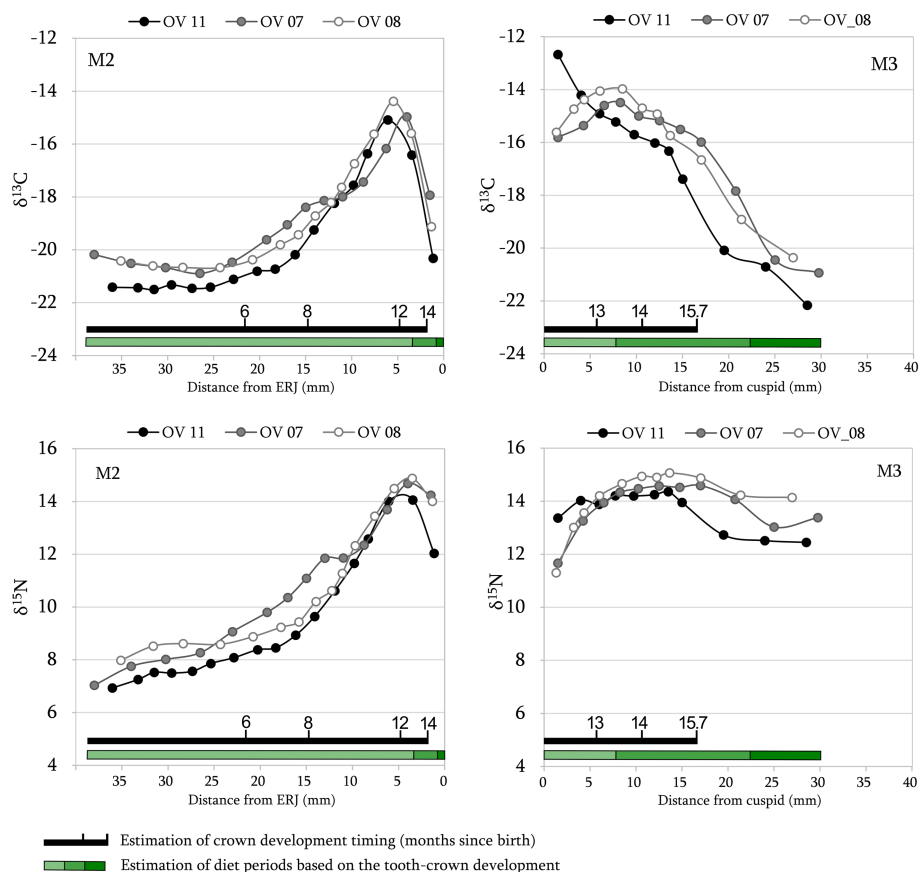


FIGURE 5 Sequential $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values measured in dentine-collagen of second (M2) and third (M3) molars. The horizontal black bars represent an estimation of the crown development timing in the tooth vertical axis (months since birth), according to dentine growth rates reported by Witzel et al. (2018) for sheep's M2 and M3 molars. The horizontal green bars indicate an estimation of the three diet periods. Note that isotopic variations are represented earlier than expected [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

the animal's life, provided that dental growth timings are known in detail. Moreover, it is worth noting that because dentine collagen is synthesized during the first phase of pre-dentine deposition (Carlson, 1991; Nanci & Causa, 2013), it is expectable that the later mineralization process does not distort the isotopic signature of the organic fraction. More specific knowledge of dentine development would allow for determining in a precise way the temporal resolution of the sampling (i.e., when diet changes occurred in the animal's life period).

5 | CONCLUSIONS

The sampling procedure implemented in this study enables obtaining isotopic data based on the longitudinal growth dynamics of high-crowned molars, a key feature in performing high-resolution analysis on tissues developing progressively during annual time spans. Results from this study show that this procedure is suitable for representing a minimum of three different scenarios of dietary conditions in sheep's life along second and third molars, even when they present different tooth growth stages. Furthermore, it allows identifying intra-tooth isotopic variation patterns within populations, because inter-individual differences are not related to sampling and rely on uncontrollable factors like metabolism or growth timing differences. Although the admixture of dentine increments is also represented in the isotopic sequences, it is possible to assign temporal information to the intra-tooth isotopic data obtained. Methodological efforts are needed in this direction.

Regardless of the growth-geometry handicap, the main strength of implementing a longitudinal post-demineralization sampling is obtaining dentine samples susceptible to be treated with the standard protocols of collagen purification, supporting its applicability to the archaeological record where some collagen preservation problems may exist and where it becomes essential to guarantee reliable $\delta^{13}\text{C}_{\text{collagen}}$ and $\delta^{15}\text{N}_{\text{collagen}}$ values.

Animal management is an essential aspect in archaeological research concerning the socioeconomic organization and complexity of prehistoric communities. Animal's dietary and nutritional conditions are often constraint to seasonal cycles, and their control by human communities is a key factor for the survival, optimal growth, and reproduction of domestic animals. The development of intra-tooth analysis of $\delta^{13}\text{C}_{\text{collagen}}$ and $\delta^{15}\text{N}_{\text{collagen}}$ offers new possibilities for studying fluctuations of the protein part of diet during the animal's cycle, unapproachable through the serial analysis of inorganic components that reflect the whole part of diet. Exploring the wide scope of possibilities for sequential stable isotope analysis of dentine-collagen, in particular $\delta^{15}\text{N}_{\text{collagen}}$ analysis, may become a new pathway for zooarchaeological research.

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CONFLICT OF INTEREST

The authors declare no competing interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Celia Díez-Canseco  <https://orcid.org/0000-0003-0221-745X>

Mònica Aguilera  <https://orcid.org/0000-0002-4502-735X>

Carlos Tornero  <https://orcid.org/0000-0002-2870-8989>

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