



## Invited Research Article

## Calcium isotopic variability of cervid bioapatite and implications for mammalian physiology and diet

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## ABSTRACT

There are clues that calcium (Ca) isotope composition of vertebrate bioapatite is influenced by diet and trophic level. These clues however conflict with several cases of mammal species exhibiting Ca isotope compositions which are inconsistent with their trophic levels. These observations support that diet may not be the only factor driving the Ca isotope composition in mammalian enamel and bone. To investigate this question, we selected a modern *Cervus elaphus* population (Bauges Natural Regional Park, Alps, France) to serve as a model for Ca isotope physiology of cervids and other mammals. Subsequently, we reinvestigated the case of the fossil *Rangifer tarandus* population from Jaurens (Late Pleistocene locality, 32.6 to 29.7 kyr BP, France), a population for which abnormal <sup>44</sup>Ca-depleted isotope compositions (regarding the low trophic level of reindeers) have been previously documented. By combining bone samplings and serial enamel micro-samplings, we discuss the main potential sources of Ca isotopic variability in bioapatite of young and adult individuals. This includes the effects of gestation, lactation, antlerogenesis, browser-grazer ecologies, osteophagia and natural mineral licks. Our results suggest an important effect of lactation on bone Ca isotope composition ( $\delta^{44/42}\text{Ca} = +0.19 \pm 0.09\%$ ; 95% confidence interval), whereas other factors such as gestation or antlerogenesis seem more secondary. Our enamel micro sampling method allowed to detect when enamel Ca isotope composition could be affected by milk consumption or mineral supplementation. Thanks to these advances, we collected new data from the Pleistocene reindeers of Jaurens, which are now consistent with their known low trophic level and plant-based diet. This demonstrates that disentangling ecological and physiological signals within enamel Ca isotope compositions is possible by using serial micro-sampling. This approach allows retrieving accurate trophic information from this proxy and truly pushes forward the limits of Ca isotope applications regarding paleoecology, physiology and ethology.

## 1. Introduction

An increasing number of studies suggest that the stable Ca isotope composition of the hydroxylapatite of vertebrate bone and teeth, represents a record of diet within modern and fossil skeletal remains (Clementz et al., 2003; Chu et al., 2006; Reynard et al., 2010; Heuser et al., 2011; Clementz, 2012; Martin et al., 2015, 2017a, b, 2018; Hassler et al., 2018). Body Ca originates mainly from food and water for land animals, but the Ca isotope composition of animals diverges from their food. At the opposite of carbon and nitrogen patterns, heavy Ca isotopes

are discriminated against light Ca isotopes during their routing from food to tissues, notably in bone and enamel (Skulan and DePaolo, 1999; Chu et al., 2006; Hirata et al., 2008; Tacaïl et al., 2014; Heuser, 2016; Heuser et al., 2016). This results in a trophic level effect (hereafter TLE) with bones of herbivorous animals exhibiting more <sup>44</sup>Ca-depleted compositions compared to the plants they consume, and carnivorous predators following the same trend compared to their prey. This ultimately leads to an isotopic clustering of animal taxa as a function of their trophic level, with primary consumers exhibiting a heavier Ca isotope composition than tertiary consumers, and secondary consumers

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exhibiting an intermediate isotopic composition. This has been observed in land ecosystems (Chu et al., 2006; Reynard et al., 2010; Martin et al., 2017a, 2018; Hassler et al., 2018; Dodat et al., 2021) and marine ecosystems (Clementz et al., 2003; Clementz, 2012; Martin et al., 2015, 2017b), even though for marine animals the Ca originating from seawater likely buffers dietary Ca intakes. These studies are based on  $^{44}\text{Ca}/^{42}\text{Ca}$  or  $^{44}\text{Ca}/^{40}\text{Ca}$  analyses of the hydroxylapatite of bone and teeth, commonly expressed as  $\delta^{44/42}\text{Ca}$  and  $\delta^{44/40}\text{Ca}$ , respectively (equivalent to the variation in ‰ compared to the Ca isotope ratios of a reference material, further detailed in Section 2.6). Moreover, this technique allows to study the ecology of both modern and fossil specimens thanks to the good preservation potential of such mineralized tissues and their Ca (Heuser et al., 2011; Martin et al., 2017a). This body of studies supports that Ca isotopes are a promising tool for diet and trophic inferences in modern and paleontological contexts with a large temporal range of action, providing the fact that mineralized tissues are preserved. However, inferring a TLE within mammalian communities relies on first order observations of Ca isotopic variability among predators and their prey. As highlighted in previous work, many uncertainties remain concerning the mechanisms behind fractionation processes as related to physiology versus environmental sources. As such, not all taxa are strictly following the theoretical isotopic/trophic clustering in the faunas studied so far. For some specific faunas and trophic niches, diet seems hard to constrain with Ca isotopes only (Reynard et al., 2010; Melin et al., 2014). Moreover, bone or enamel Ca isotope compositions sometimes overlap between herbivores and predators, with herbivores occasionally exhibiting isotope compositions more  $^{44}\text{Ca}$ -depleted than predators from the same fauna (e.g. hippopotamidae, mammoths and cervidae; see Martin et al., 2017a, 2018; Dodat et al., 2021). This raises important questions about what can generate such issues, and highlights how critical it is for the accurate use of this proxy to reconcile the evidences of TLE with the occasional decoupling recorded between trophic level and Ca isotope compositions.

The isotopic offset between diet and bone which generates the TLE ( $\Delta^{44/42}\text{Ca}_{\text{diet-bone}}$ ) is relatively constant among mammal species with a value of  $-0.54 \pm 0.08\text{‰}$  (2 standard error, 20 individuals from 6 mammal species; reviewed in Tacail et al. (2017)), despite resulting from the combination of numerous body Ca fluxes associated with Ca isotope fractionation (Skulan and DePaolo, 1999; Chu et al., 2006; Hirata et al., 2008; Tacail et al., 2014; Heuser et al., 2016; Tacail, 2017). The most impactful fluxes identified so far are the kidney Ca reabsorption from primary urines (Skulan et al., 2007; Heuser and Eisenhauer, 2010; Morgan et al., 2012; Tacail et al., 2014; Channon et al., 2015; Heuser et al., 2016, 2019; Eisenhauer et al., 2019), the milk production and excretion (Chu et al., 2006; Reynard et al., 2010; Hassler et al., 2021) and the bone mineralization (Skulan and DePaolo, 1999; Skulan et al., 2007; Heuser and Eisenhauer, 2010; Reynard et al., 2010; Morgan et al., 2012; Channon et al., 2015), although the significance of this last parameter has been recently questioned (Tacail, 2017; Tacail et al., 2020; Hassler et al., 2021). Changing these fluxes, like during gestation, lactation (Ramberg Jr et al., 1970; Cross et al., 1995; Giesemann et al., 1998; Karlsson et al., 2001; Wysolmerski, 2002; Vanhouten and Wysolmerski, 2003; Gallego et al., 2006; Kovacs and Fuleihan, 2006; Tacail, 2017) or antlerogenesis (Mitchell et al., 1976; Muir et al., 1987a, b), likely modifies the Ca isotopic equilibrium of the organism, the resulting  $\Delta^{44/42}\text{Ca}_{\text{diet-bone}}$  offset, and could generate TLE discrepancies. Alternatively, the consumption of milk during nursing can also generate TLE discrepancies by changing the diet Ca isotope composition of non-weaned individuals compared to weaned individuals (Chu et al., 2006; Li et al., 2016, 2020; Tacail et al., 2017, 2019). Finally, Ca enriched mineral supplementation such as with mineral licks and osteophagia, as well as the Ca isotopic variability inherent to plants (Holmden and Bélanger, 2010; Gussone and Heuser, 2016; Schmitt, 2016; Moynier and Fujii, 2017; Martin et al., 2018; Griffith et al., 2020), are also able to blur the trophic clustering of Ca isotope compositions. The aim of this study is thus to investigate how these different factors

can affect bone and enamel Ca isotope compositions in modern animals, then to use this background to unravel a case study of TLE discrepancy previously documented.

We carried out the first part of this project by monitoring the bone and enamel Ca isotope compositions of a modern cervid population (red deer, *Cervus elaphus*) inhabiting the Bauges Natural Regional Park (NRP), Alps, (Savoie, France). This modern population then served as a model to discuss TLE discrepancies, and more precisely to discuss the case of the reindeers (*Rangifer tarandus*) from the Pleistocene locality of Jaurens (Corrèze, France). Martin et al. (2017a) showed that reindeers from this locality (dated between 32.6 and 29.7 kyr BP, Guérin et al., 1979) exhibit  $^{44}\text{Ca}$ -depleted compositions in tooth enamel down to a  $\delta^{44/42}\text{Ca}$  value of  $-1.75 \pm 0.09\text{‰}$  (2 standard deviation; 3 individuals). This is different from the Ca isotope compositions of the other herbivores of this locality, and closer to the one of lions and wolves (Martin et al., 2017a). In accordance with the documented effects of nursing on the body Ca isotope composition of the young (Chu et al., 2006; Li et al., 2016, 2020; Tacail et al., 2017, 2019), Martin et al. (2017a) proposed that these negative  $\delta^{44/42}\text{Ca}$  values could result from the consumption of maternal milk by reindeers at the time when their analyzed molars were mineralizing (second molars, M2). Although this hypothesis is tempting, no direct evidence was available to prove that this phenomenon was responsible for the  $^{44}\text{Ca}$ -depleted isotope composition reported among Jaurens fossil cervids, leading to this new investigation.

To ensure the completion of our study, it was necessary to assess at which degree the Ca isotope composition of bone and enamel are comparable. Previous studies found differences in  $\delta^{44/42}\text{Ca}$  values between bone and enamel or bone and enameloid (Heuser et al., 2011; Tacail et al., 2014; Martin et al., 2015, 2017a), but it was unclear whether this offset ( $\Delta^{44/42}\text{Ca}_{\text{bone-enamel}}$ ) was due to different Ca isotope fractionation coefficient during mineralization ( $\alpha_{\text{blood-bone}}$  and  $\alpha_{\text{blood-enamel}}$ ), to different mineralization timing, or to diagenesis. Our modern red deer population is free of diagenetic influence, and therefore, mineralization timings and differences between  $\alpha_{\text{blood-bone}}$  and  $\alpha_{\text{blood-enamel}}$  are the only factors affecting bone and enamel Ca isotopic differences. In other words, characterizing  $\alpha_{\text{blood-bone}}$  and  $\alpha_{\text{blood-enamel}}$  in red deer allow to accurately compare the different ontogenetic time periods recorded by these two tissues.

## 2. Material and methods

### 2.1. Modern specimens

We studied a total of 21 specimens of wild red deer of two age classes (subadult and adult) and sex (10 males, 11 females), coming from Bauges NRP, Alps, (Savoie, France, 45.69°N, 6.14°E) and slaughtered during the hunting season in 2015 (between October and November) as part of the local hunting activity. In order to identify lactation, gestation and antlerogenesis effects on the Ca isotope composition of mineralized tissues, we analyzed and compared the bone Ca isotope composition of females and males. Our two age classes allow to distinguish specimens free of strong lactation, gestation and antlerogenesis effects (two years old, referred as subadult) from the others (aged of three years or more, referred as adult). Ages of the individuals were estimated based on tooth eruption and attrition stages (Brown and Chapman, 1991a, 1991b; Table S1). The reproductive status of studied females was not directly monitored, but we know that most of females aged of two to three years already gave birth at least once (commonly more than 80% in comparable populations: (Gaillard et al., 2000; Bonenfant et al., 2002; Pellerin et al., 2014)). In addition, a large proportion of the females aged of three years or more are expected to have a fawn each year (about 80%). We can refine these estimations for the red deer population of Bauges NRP as we know the body mass of studied specimens and that fertility is strongly related to body mass in cervids. Females are usually fertile when attaining 80% of the adult body mass (Albon et al., 1986; Pellerin et al., 2014) and are around 30% heavier at the end of autumn if they did

not gave birth to a fawn earlier in the year (Mitchell et al., 1976; Clutton-Brock et al., 1982). These data allow to identify females which were too puny to be fertile, and females which were suspiciously too fat to have nursed a fawn recently. At the light of these data and knowing that the Bauges NRP deer population breed quite actively, we assume that adult females weighting between 65 and 105 kg have given birth to a fawn during the 2015 birth season (Table S1).

Bone samples were collected from mandibles, previously manually cleaned and boiled prior to be integrated to the collection of the PALEVOPRIM laboratory (UMR CNRS 7262 - University of Poitiers, France). For each mandible, bone sample powder was collected few centimeters under the second molar, with the help of a handheld drill (8200 Dremel equipped with a tungsten steel solid carbide bit). In order to remove any trace of dirt or other environmental contaminants, the first layer of bone surface was removed with the drill then cleaned with pure ethanol prior to sampling. Between 0.4 and 0.7 mg of bone powder were sampled and weighted prior to chemical preparation.

Along with bone sampling and in order to investigate intra-tissue and inter-tissue  $\delta^{44/42}\text{Ca}$  variability, we selected two specimens for serial enamel micro-sampling. The first is an adult male with premolars P2, P3, P4, and molars M1, M2, M3 fully emerged and moderately worn (specimen ID number: UP-15CE5672, lab name: AB). The second is a juvenile female with deciduous premolar DP2, DP3, DP4, and molars M1, M2 fully emerged and with an erupting M3 in ongoing mineralization (specimen ID number: UP-15CE3734, lab name: JVB). The serial micro-samplings performed on these specimens provide snapshots of the enamel Ca isotope composition at different ages, depending on when each enamel zone mineralized. This ultimately allows to identify isotopic anomalies produced by milk consumption, osteophagia, mineral licks or occasional consumption of  $^{44}\text{Ca}$ -depleted plants. These deer specimens (AB and JVB), also had some plant leftovers trapped between their teeth. We collected these rests for elemental concentration analyses.

To assess the effect of antlerogenesis and antler consumption we selected an adult male red deer preserved with its skull, mandible and antlers in the collections of the Confluence Museum (Lyon, France). This specimen (ID number: MHNL-50002207, lab name: SPB) is of unknown European origin and has been sampled following the same bone sampling procedure than specimens from Bauges NRP. One sample has been retrieved from the mandible, the other three were retrieved on the main beam of the right antler, respectively at the base, the middle and the top of the antler.

## 2.2. Fossil specimens

In order to study the trophic position of reindeer from the Late Pleistocene Jaurens fauna and their peculiar Ca isotope composition, we selected four specimens to carry out serial enamel micro-sampling. We focused our efforts on enamel sampling instead of bones in order to limit diagenetic influence. All specimens are part of the paleontological collections of the Laboratory of Geology of Lyon (LGL-TPE, France). This includes two mandibles (one adult and one juvenile) with their associated teeth, and two isolated teeth (lower M1 and a lower DP4) from two different individuals. One of the two mandibles comes from an adult specimen with the teeth P2, P3, P4, M1, M2, M3 fully emerged and with significant tooth wear on the M1 (specimen ID number: UCBL-FSL 451.409, lab name: AJ). The second mandible comes from a juvenile specimen with the teeth DP2, DP3, DP4, M1 fully emerged and with the M2 erupting (specimen ID number: UCBL-FSL 451.389, lab name: JVJ). The isolated teeth are a lower left DP4 (specimen ID number: UCBL-FSL 451.398, lab name: ISO DP4) and lower right M1 (specimen ID number: UCBL-FSL 451.384, lab name: ISO M1), both with a minimal wear level. The lessons learned from modern specimens allow to critically discuss our new and previous (Martin et al., 2017a) enamel Ca isotope data collected on fossil reindeers from Jaurens.

## 2.3. Micro-sampling

We designed two different procedures of serial micro-sampling. For both we use a computer-assisted micro drill device (MicroMill), allowing the sampling of 80–120  $\mu\text{g}$  hydroxylapatite by drilling holes of 350–400  $\mu\text{m}$  wide and about 400  $\mu\text{m}$  depth. Teeth selected for micro-sampling were serially sampled by micro-drilling on the enamel between the apex (i.e. the top) and the neck of the tooth (i.e. the basal most enamel zone), along the growth axis of the best preserved cusp of the lingual face. The apex is mineralizing first whereas the enamel close to the neck is the last part of the enamel to mineralize. This sampling thus maximizes the studied intra-tooth time window and allows a high temporal resolution. When possible we performed serial micro-sampling using a “edge-drilling procedure”. In this case, the tooth (either isolated or extracted from a mandible) was cut along the growth axis in the central part of the cusp by using a Buehler IsoMet Low Speed precision sectioning saw with a diamond-studded circular saw blade. The edge of the enamel was then polished using sandpaper with decreasing grain sizes prior to perform the micro sampling. Sampling zones were located close to the enamel-dentine junction (EDJ) in order to minimize the temporal lag between the mineralization of appositional structures and enamel maturation (Blumenthal et al., 2014; Green et al., 2017; Traylor and Kohn, 2017; Müller et al., 2019). Alternatively, when extracting and cutting teeth was impossible or when the targeted spatial sampling resolution was sufficiently low, enamel has been micro-drilled from the outer side (“outer-drilling procedure”). For this procedure, the one or two first hundreds of  $\mu\text{m}$  of drilled enamel were not collected, in order to collect enamel relatively close to the EDJ similarly as with the edge-drilling procedure. The choice of procedure is reported in Tables S1 and S2.

## 2.4. Thin section and enamel age model

We were allowed to make thin sections on the micro sampled surface of the M1 of AB, JVB (red deer, Bauges) and ISO M1 (reindeer, Jaurens). Teeth were then sliced and polished in order to obtain 100  $\mu\text{m}$  thick sections. Observations in transmitted light microscopy failed to reveal neonatal lines within the enamel layers of the three M1 (AB, JVB, ISO M1), a structure observed in early mineralizing teeth of humans and other mammals that would mark birth (Klevezal and Mina, 1995; Zanolli et al., 2011; Dean et al., 2019). The age model necessary to anchor our enamel  $\delta^{44/42}\text{Ca}$  data was alternatively based on general observations made on tooth mineralization timing of red deer (Brown and Chapman, 1991b). Such data were not available for reindeer, but similarities between red deer and reindeer tooth eruption timings (Miller, 1972; Brown and Chapman, 1991b; Azorit et al., 2002) allow to use red deer data to build a coarse reindeer age model. As teeth are subject to wear, we spatially anchored our age model on the neck of teeth. The mean mineralization age of each enamel  $\delta^{44/42}\text{Ca}$  data point is then estimated based on the distance between the sampling zone and the neck of the tooth, on corresponding mineralization timings (Brown and Chapman, 1991b), and on the estimated full height of unworn teeth measured on young specimens. This method assumes a constant enamel mineralization rate along the tooth growth axis and inside tooth families. This method is thus not adapted to discuss inter-individual or intra-tooth differences in tooth mineralization rates and timings but allow for a fairly good estimation considering that enamel micro-samples average a body isotopic record spanning over a month to several months (further discussed in supplementary material T1). For studied red deers, unworn teeth displayed similar crown heights between specimens. Reindeer specimens, however, had unworn molars and premolars approximately 15% smaller than red deer, which has been considered in our age model.

## 2.5. Sample dissolution and ion chromatography

Immediately after sampling, bone and tooth samples were

transferred in Teflon beakers. All further manipulations have been done exclusively in a clean lab or under a laminar flux hood. Modern bone samples were dried with 500  $\mu\text{L}$  of pure ethanol evaporated at 70  $^{\circ}\text{C}$ . No attempt of leaching procedures has been carried out for fossil enamel samples, as previous Ca isotope analyses demonstrated no isotopic differences between leached and non-leached enamel in Jaurens samples (Martin et al., 2017a). All samples were dissolved using a mix of 1 mL of 15 M ultrapure nitric acid ( $\text{HNO}_3$ ) and 300  $\mu\text{L}$  of ultrapure hydrogen peroxide (30%). Beakers were left closed at ambient temperature for 1 h, then were heated at 130  $^{\circ}\text{C}$  during 1 to 2 h more with regular degassing of nitrous fumes. When production of nitrous fumes became limited, 300  $\mu\text{L}$  of ultrapure hydrogen peroxide (30%) were added and samples were left to dry down at 90  $^{\circ}\text{C}$  for a few hours. The presence of organic matter was then tested using 100  $\mu\text{L}$  of ultrapure hydrogen peroxide (30%). No effervescence was detected for any of the samples and solutions were thus left to dry down at 90  $^{\circ}\text{C}$ . Samples selected for elemental concentration analyses were dissolved in 0.5 M ultrapure  $\text{HNO}_3$  then split. A fraction of the solution was reserved for concentration analyses and the rest was dried down at 90  $^{\circ}\text{C}$ . These dried fractions and the samples not selected for concentration analyses were kept for Ca isotope analyses, dissolved in 300–500  $\mu\text{L}$  of 6 M ultrapure hydrochloric acid (HCl) and dried down at 90  $^{\circ}\text{C}$ .

The chromatography procedure used for Ca chemical purification is derived from Tacail et al. (2014). This consists in a double column chromatography, starting with an elution on AG 50WX-12 resin with ultrapure HCl, and followed by an elution on Eichrom Sr-specific resin with an ultrapure  $\text{HNO}_3$  medium. When high level of iron was suspected (e.g. fossil samples) a third column chromatography was performed, using 1 mL of AG1 X8 resin and following a procedure derived from Tacail et al. (2014). Blanks have been realized along the digestion and the chromatography, including total procedural blanks, which have undergone all the steps previously described and chromatography blanks.

## 2.6. Analytical procedures and nomenclature

For more clarity within this paper, the  $n$  notation refers to the number of samples or specimens whereas the  $n^*$  notation specifically refers to number of measurement replicates. The “s.d.” notation refers to “standard deviation”, whereas “s.e.” refers to “standard error”. Concentrations of major and trace elements were measured respectively on an inductively coupled plasma atomic emission spectrometer (ICP-AES) (ICAP 7400 Series, Thermo Scientific) and on an inductively coupled plasma mass spectrometer (ICP-MS) (ICAP-Q, Thermo Scientific). The reliability of measurements has been controlled through a set of blanks and reference material (SRM1486), and by replicating measures to  $n^* = 2$  for each sample.

We measured the Ca isotope ratios ( $^{44}\text{Ca}/^{42}\text{Ca}$ ) using a multi-collector inductively coupled plasma mass spectrometer (MC-ICP-MS, Neptune Plus, Thermo Scientific) following the method described in Tacail et al. (2014). Prior to Ca isotope analyses, Ca purified samples were dissolved and diluted in 0.05 M  $\text{HNO}_3$  in order to set the Ca concentration at 1.25  $\text{mg}\cdot\text{L}^{-1}$ . This concentration matches that of the ICP Ca Lyon, our in-house bracketing reference material made of Specpure Ca plasma standard solution (Alfa Aesar) and described in previous studies (Tacail et al., 2014, 2017; Martin et al., 2015, 2017a). Unless explicitly mentioned, the Ca isotope compositions reported in this article are all expressed as  $\delta^{44/42}\text{Ca}$  relatively to ICP Ca Lyon using the following formula:

$$\delta^{44/42}\text{Ca} = \left( \left( \frac{(^{44}\text{Ca}/^{42}\text{Ca})_{\text{sample}}}{(^{44}\text{Ca}/^{42}\text{Ca})_{\text{ICP Ca Lyon}}} - 1 \right) \times 1000 \right)$$

For easing comparisons with studies using other reference materials,  $\delta^{44/42}\text{Ca}_{\text{ICP Ca Lyon}}$  values are converted to  $\delta^{44/42}\text{Ca}_{\text{SRM915a}}$  in our tables and figures. Based on 71 measures compiled in the appendix of Martin et al. (2018), we converted  $\delta^{44/42}\text{Ca}_{\text{ICP Ca Lyon}}$  values to  $\delta^{44/42}\text{Ca}_{\text{SRM915a}}$

values by adding +0.518 ‰ to  $\delta^{44/42}\text{Ca}_{\text{ICP Ca Lyon}}$  values. To express a difference of  $\delta^{44/42}\text{Ca}$  value between two Ca reservoirs we use the  $\Delta$  notation based on the following formula:

$$\Delta x - y = \delta x - \delta y.$$

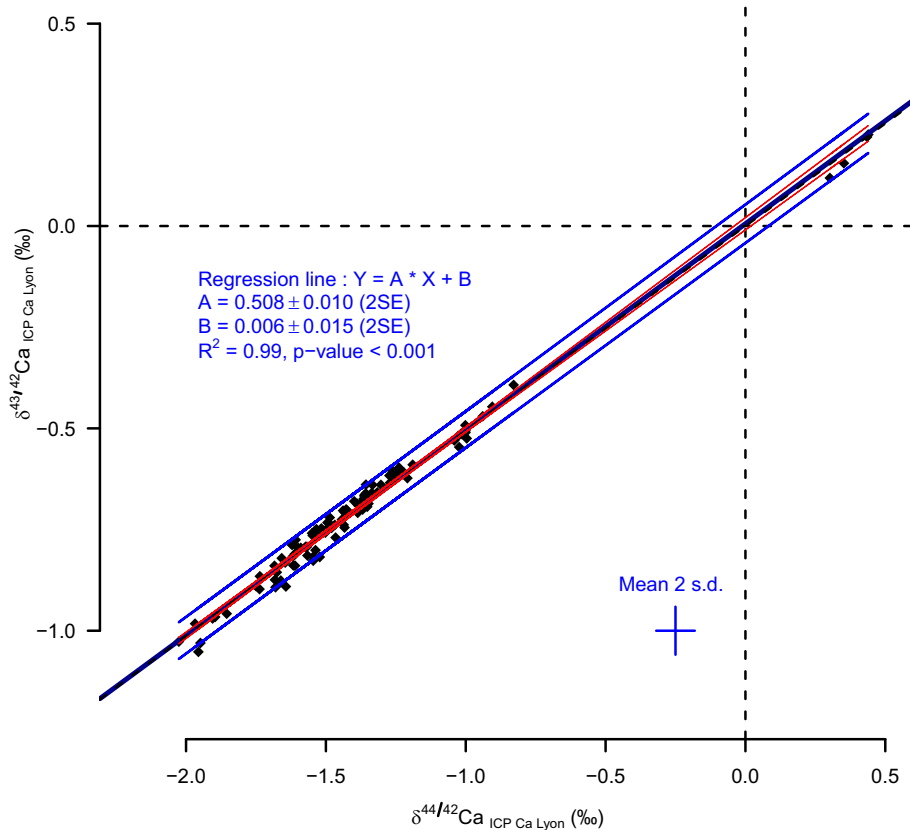
where  $x$  and  $y$  are different Ca reservoirs. Secondary reference materials of different matrix and documented Ca isotope composition have been used to ensure the accuracy of our analytical procedure and chemical purifications. The SRM1486 cow bone meal reference material (NIST) was used in that purpose, as well as the IAPSO sea water (OSIL). The Ca concentration of the blanks have also been measured with the Neptune Plus. All samples and standard measurements have been replicated to a  $n^* = 2$  or more (Tables S1 and S2). Sample Ca isotope compositions are considered different when their  $\delta^{44/42}\text{Ca}$  mean value ( $\pm 2$  s.e.) do not overlap. Unpaired Wilcoxon rank-sum tests are used to assess if  $\delta^{44/42}\text{Ca}$  values of different sample groups (e.g. male versus female  $\delta^{44/42}\text{Ca}$  values) are significantly different. These differences are also expressed in ‰ with a confidence interval calculated from a t-test (Welch’s test variant). These intervals emphasize the degree of confidence around these isotopic differences, but it has to be noted that results of Wilcoxon rank-sum tests are more appropriate to assess the statistical significance of these differences (essentially because of the small size of compared populations).

## 3. Results

### 3.1. Trueness and precision

Chromatography and total blanks display a maximum of 70 ng of Ca. This is several thousand times less than the amount of Ca in our macro-samples (e.g. bone samples) which is thus negligible. Most of our micro-samples (i.e. collected from spatial micro-sampling) contained more than 6250 ng of Ca, and in the worst cases about 3800 ng of Ca. In this case Ca from blanks is not completely negligible but its effect can be considered minimal. Indeed, a simple mass balance calculation shows that even a contamination with environmental Ca of extreme isotopic composition (close to seawater  $\approx 0.41\%$ ; Martin et al. (2015)) affecting an extremely  $^{44}\text{Ca}$ -depleted sample (e.g.  $\delta^{44/42}\text{Ca} = -2.00\%$ ) with 3800 ng of Ca would only generate a +0.04‰ difference. This unlikely +0.04‰ correspond to the maximum of contamination effect possibly expected with our blank levels for the smallest of our micro samples. Such effects can thus be neglected considering the order of magnitude of inter-sample differences discussed below.

The complete Ca isotope dataset of this study includes 104 samples and reference materials for a total of 358 accurate measurements. The correlation between  $\delta^{43/42}\text{Ca}$  and  $\delta^{44/42}\text{Ca}$  values of each of these samples/reference materials follows the predictions from the linear approximation of the exponential mass-dependent fractionation law (Fig. 1), with a slope value of  $0.508 \pm 0.010$  (2 s.e.), an intercept of  $0.006 \pm 0.015$  (2 s.e) and a  $R^2 = 0.99$  ( $p$ -value  $< 0.001$ ). This demonstrates that no mass independent fractionation or mass specific interference has affected our measurements. Along with our different sessions of analysis, the SRM1486 exhibited a mean  $\delta^{44/42}\text{Ca}$  value of  $-0.99 \pm 0.13\%$  (2 s.d. inter-session,  $n = 8$  sessions) with a mean intra-session 2 s.d. of 0.08‰ ( $n = 88$  measurements). Our other secondary reference material, the IAPSO exhibited a mean  $\delta^{44/42}\text{Ca}$  value of  $+0.38 \pm 0.12\%$  (2 s.d. inter-session,  $n = 5$  sessions) with a mean intra-session 2 s.d. of 0.06‰ ( $n = 14$  measurements). These results are both in the range of previously published data (Table S3) for the SRM1486 (Heuser and Eisenhauer, 2008; Tacail et al., 2016, 2017; Martin et al., 2018) and the IAPSO (Tacail et al., 2014; Martin et al., 2015). By considering all the measurements of samples and reference materials ( $n = 107$ ), the mean intra-sample reproducibility was of  $\pm 0.07\%$  (2 s.d.,  $n = 358$  measurements).



**Fig. 1.** Mass dependency.

Three isotope plot:  $\delta^{43/42}\text{Ca}$  as a function of  $\delta^{44/42}\text{Ca}$  (‰, expressed relatively to ICP Ca Lyon) for all samples and standards analyzed for Ca isotope compositions in this study. The regression line of these Ca isotope compositions (central blue line) has a y-axis intercept of  $0.006 \pm 0.015$  (‰, 2 s.e.), indistinguishable from theoretical 0‰ intercept. The slope value of this line is  $0.509 \pm 0.016$  (2 s.e.), indistinguishable from the 0.507 slope predicted by the exponential mass-dependent fractionation law (black dotted line). Error bars at the bottom right are average 2 s.d. for  $\delta^{43/42}\text{Ca}$  and  $\delta^{44/42}\text{Ca}$ . The two most external lines (blue) delimit the prediction interval whereas the two lines (red) around the central line correspond to the 95% confidence interval of the regression line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Bone data among modern cervids

The bone sample set from Bauges NRP displays a range of  $\delta^{44/42}\text{Ca}$  values between  $-1.62 \pm 0.19\text{‰}$  (2 s.d.,  $n^* = 3$ ) and  $-1.22 \pm 0.04\text{‰}$  (2 s.d.,  $n^* = 2$ ), with a mean 2 s.d. of  $0.09\text{‰}$ . Females red deer have a mean bone  $\delta^{44/42}\text{Ca}$  value of  $-1.36 \pm 0.24\text{‰}$  (2 s.d.,  $n = 11$ ), and males a mean bone  $\delta^{44/42}\text{Ca}$  value of  $-1.41 \pm 0.27\text{‰}$  (2 s.d.,  $n = 10$ ). The  $\delta^{44/42}\text{Ca}$  values of female individuals are statistically indistinguishable from males when considering all age classes together (Wilcoxon rank-sum test,  $p$ -value = 0.3494), but this changes when considering adult specimens only (Wilcoxon rank-sum test,  $p$ -value = 0.0499). Isolating females who most likely gave birth during the last birth season from the other females allows for an even clearer distinction (Fig. 2). These six females (later referred as lactating hinds or females) display bone  $\delta^{44/42}\text{Ca}$  values higher of  $+0.23 \pm 0.08\text{‰}$  (95% confidence interval; Wilcoxon rank-sum test :  $p$ -value < 0.01; Fig. 3) compared to the two adult females in the body mass range which suggest that they did not reproduce recently (later referred as yield hinds). Lactating hinds bone  $\delta^{44/42}\text{Ca}$  values also differ significantly from adult males (Wilcoxon rank-sum test,  $p$ -value < 0.01; Fig. 3) with a mean bone  $\delta^{44/42}\text{Ca}$  value higher of  $+0.17 \pm 0.12\text{‰}$  (95% confidence interval). Bone  $\delta^{44/42}\text{Ca}$  values of lactating hinds compared to bone  $\delta^{44/42}\text{Ca}$  values of adult males and yield hinds pulled together are higher of  $+0.19 \pm 0.09\text{‰}$  (95% confidence interval; Wilcoxon rank-sum test :  $p$ -value < 0.01). The three subadult females display a range of bone  $\delta^{44/42}\text{Ca}$  values which overlap with both lactating and yield adult hinds, with the heaviest subadult females displaying the highest bone  $\delta^{44/42}\text{Ca}$  values. Finally, the correlation between adult male body mass and bone  $\delta^{44/42}\text{Ca}$  values is virtually absent ( $r = -0.29$ ,  $p$ -value = 0.49,  $n = 8$ ). We can make no distinction between bone  $\delta^{44/42}\text{Ca}$  values of adult males and subadult males (Wilcoxon rank-sum test,  $p$ -value = 0.18) or between adult males and subadult or yield females (Wilcoxon rank-sum test,  $p$ -value = 0.62).

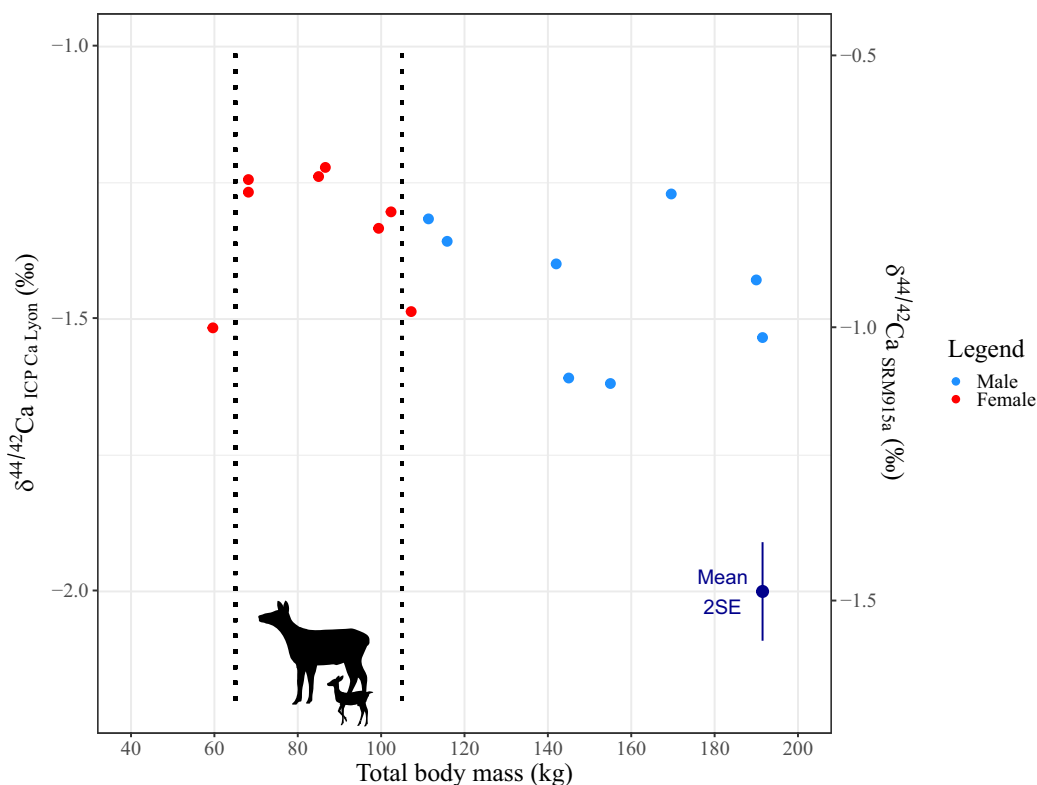
The SPB specimen displays a mandible bone  $\delta^{44/42}\text{Ca}$  value of  $-1.62$

$\pm 0.04\text{‰}$  (2 s.d.,  $n^* = 3$ ), whereas the antler bone displays  $\delta^{44/42}\text{Ca}$  values of  $-1.43 \pm 0.02\text{‰}$  (2 s.d.,  $n^* = 4$ ) at the base,  $-1.49 \pm 0.06\text{‰}$  (2 s.d.,  $n^* = 3$ ) in the middle and  $-1.38 \pm 0.06\text{‰}$  (2 s.d.,  $n^* = 4$ ) at the top of the right antler (Fig. 4). Each of the antler  $\delta^{44/42}\text{Ca}$  values is significantly higher from that of the mandible by  $0.13\text{‰}$  or more. Calcium and phosphorus concentrations decrease from the base to the tip of the antlers, whereas magnesium reaches its maximum concentration in the middle part of the antler (Table S4).

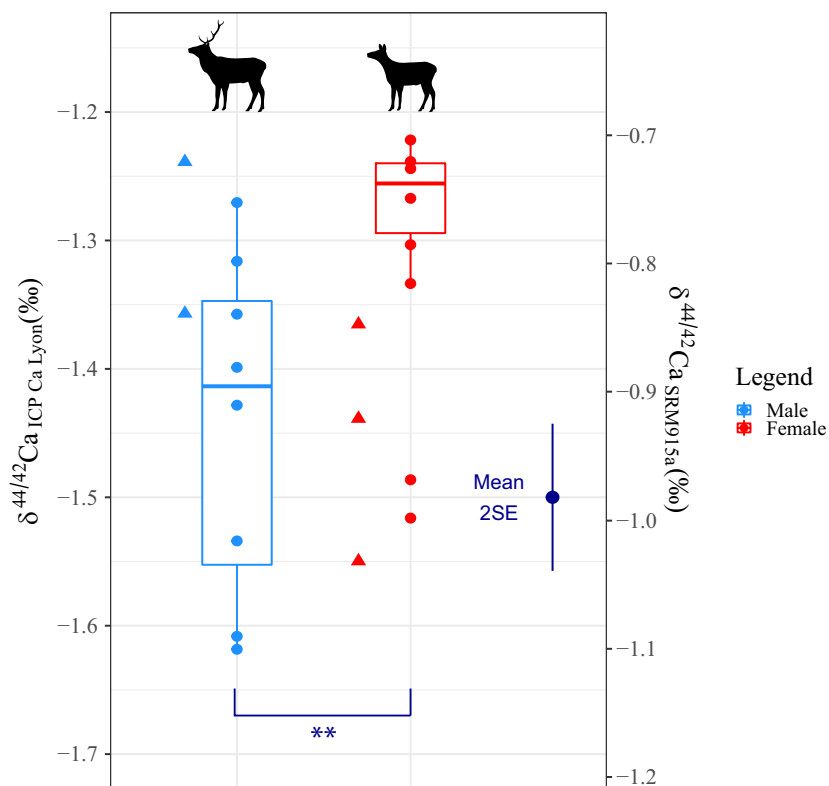
### 3.3. Enamel micro-samples

Enamel and bone Ca isotope data of specimen AB (adult male) and JVB (juvenile female) are reported in Fig. 5. Among these individuals, a majority of enamel  $\delta^{44/42}\text{Ca}$  values of most adult parts of M3 and M2 teeth are undistinguishable from bone, whereas the DP4 (when present) and the early mineralizing part (next to the crown apex) of the M1 display  $\delta^{44/42}\text{Ca}$  values which are  $-0.23\text{‰}$  to  $-0.37\text{‰}$  lower than bones. A similar difference is observed within the M1 of the AB specimen, with early mineralizing parts exhibiting a  $\delta^{44/42}\text{Ca}$  value  $-0.22\text{‰}$  lower than late mineralizing parts. Plant remains found between tooth cusps from AB and JVB display a Ca concentration of 1.83 wt% and 0.99 wt%, respectively. The complete  $\delta^{44/42}\text{Ca}$  dataset of the specimens from the Bauges NRP is reported in Table S1.

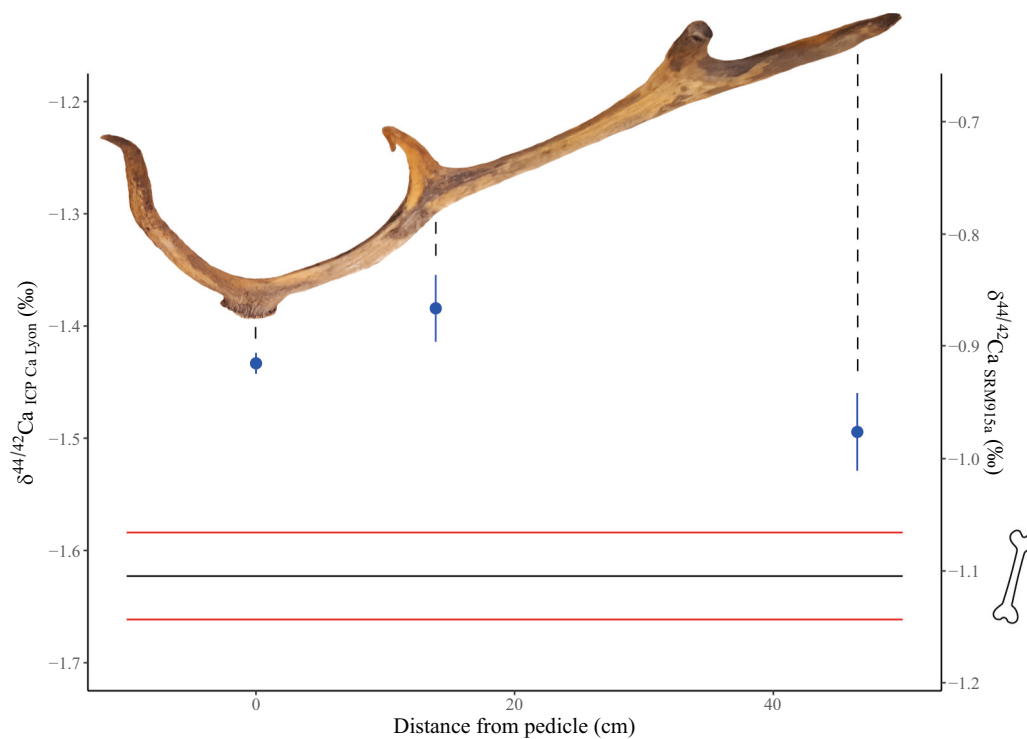
Enamel  $\delta^{44/42}\text{Ca}$  values of fossil reindeer (Fig. 6) range between  $-1.21 \pm 0.04\text{‰}$  (2 s.d.,  $n^* = 3$ ) and  $-2.03 \pm 0.08\text{‰}$  (2 s.d.,  $n^* = 2$ ). As for studied red deer, specimens with unworn M1 (JVJ and ISO M1) exhibit an important change of  $\delta^{44/42}\text{Ca}$  values between early and late mineralizing enamel. The earliest mineralizing part of the M1 displays a  $\delta^{44/42}\text{Ca}$  value lower by  $-0.82\text{‰}$  for JVJ and by  $-0.47\text{‰}$  for ISO M1 compared to the last mineralizing part of the M1. The DP4 enamel of JVJ displays a  $\delta^{44/42}\text{Ca}$  value of  $-1.94 \pm 0.06\text{‰}$  (2 s.d.,  $n = 3$ ), undistinguishable from that of the earliest mineralizing part of its M1 ( $-2.03 \pm 0.08\text{‰}$ , 2 s.d.,  $n^* = 2$ ). Fluctuations of  $\delta^{44/42}\text{Ca}$  values during the adult



**Fig. 2.** Sex and body mass control over bone  $\delta^{44/42}\text{Ca}$  values. Bone  $\delta^{44/42}\text{Ca}$  values of adult red deer specimens ( $\geq$  three years old) from Bauges NRP plotted in function of their total body mass. The average double standard error of these measures (Mean 2SE in the graph) is represented at the bottom right of the graph. The two vertical dotted lines mark the limits of body mass (65 and 105 kg) expected from females which supposedly gave birth and lactate during the last birth season preceding their death. Red deer silhouettes are modified from pictures of the public domain accessible in the following website: [www.phylopic.org](http://www.phylopic.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Sex and age control over bone  $\delta^{44/42}\text{Ca}$  values. Bone  $\delta^{44/42}\text{Ca}$  values of red deer from Bauges NRP grouped by sex and age categories. Adult red deer ( $\geq$  three years old) are represented by dots and sub-adults (two years old) by triangles. Boxplots are plotted from adult male and lactating female data. The degree of significance of the difference between these two groups is represented with stars (\*), with two stars indicating a Wilcoxon rank-sum test:  $p$ -value  $< 0.01$ . The average double standard error of these measures (Mean 2SE in the graph) is represented at the bottom right of the graph. Red deer silhouettes are modified from pictures of the public domain accessible in the following website: [www.phylopic.org](http://www.phylopic.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Antler Ca isotopic composition. Intra-individual variability of mandible and antler bone  $\delta^{44/42}\text{Ca}$  values from the SPB specimen. In this graph antler micro-samples are plotted in function of their original position from the sampled antler displayed in photo. Blue error bars represent the 2 s.e. intervals of each sample. The horizontal black and red lines represent respectively the mean mandible bone  $\delta^{44/42}\text{Ca}$  value of the specimen and the limits of its confidence interval ( $\pm 2$  s.e.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

life of reindeer are also observed within the M3 and the P4 of the AJ specimen. The mean  $\delta^{44/42}\text{Ca}$  value measured on these two teeth is  $-1.56 \pm 0.19\text{‰}$  (2 s.d.,  $n = 3$  for each tooth) with a total range of  $0.22\text{‰}$ . The complete  $\delta^{44/42}\text{Ca}$  dataset of the specimens from Jaurens is reported in Table S2.

All three DP4 teeth display a pattern of low  $\delta^{44/42}\text{Ca}$  values, relatively stable from the apex to the neck ( $\Delta_{\text{max-min}} = 0.11\text{‰}$ ; intra-teeth 2 s.d. =  $0.11\text{‰}$ ,  $n = 7$ ). The M1 teeth display a different pattern, with AB, JVJ and ISO M1 specimens exhibiting a high amplitude change from low to high  $\delta^{44/42}\text{Ca}$  values from the apex to the neck ( $\Delta_{\text{max-min}} = 0.50\text{‰}$ ; intra-teeth 2 s.d. =  $0.35\text{‰}$ ,  $n = 3$ ). The M1 of JVB displays more stable low  $\delta^{44/42}\text{Ca}$  values ( $\Delta_{\text{max-min}} = 0.17\text{‰}$ ; intra-teeth 2 s.d. =  $0.12\text{‰}$ ,  $n = 7$ ), whereas the M1 of AJ displays stable  $\delta^{44/42}\text{Ca}$  values close to its M2 and M3  $\delta^{44/42}\text{Ca}$  values ( $\Delta_{\text{max-min}} = 0.11\text{‰}$ ; intra-teeth 2 s.d. =  $0.12\text{‰}$ ,  $n = 3$ ).

## 4. Discussion

### 4.1. Comparability of bone and enamel Ca isotope composition

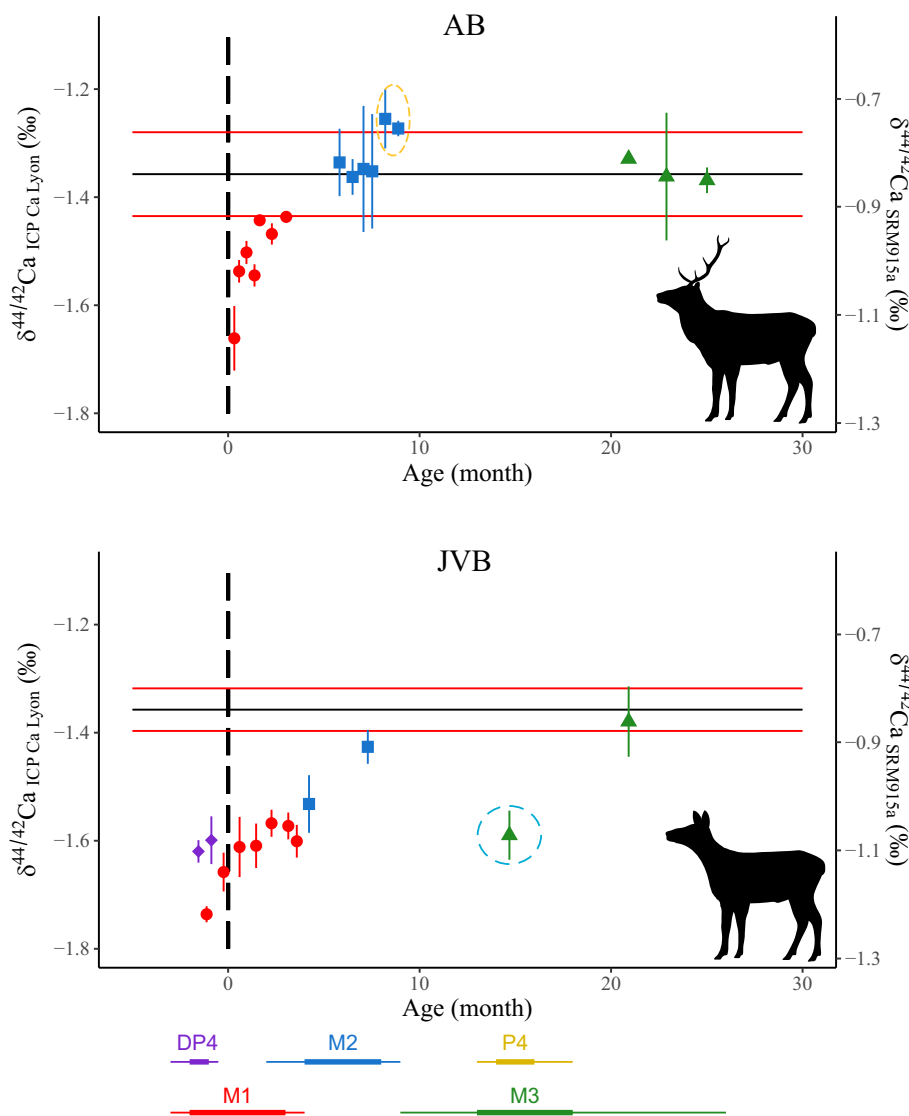
We observe some offsets in  $\delta^{44/42}\text{Ca}$  bone and enamel values in the M2 and M3 teeth of AB and JVB specimens (Fig. 5), but these offsets tend to be rarer after 5–6 months. The fact that the late mineralizing enamel of the M2 and M3 of red deer specimens AB and JVB display a  $\delta^{44/42}\text{Ca}$  value predominantly identical to their bones (Fig. 5), suggests that a similar Ca isotope fractionation occurs during bone and enamel mineralization (i.e.  $\alpha_{\text{blood-bone}} \approx \alpha_{\text{blood-enamel}}$  and  $\alpha_{\text{diet-bone}} \approx \alpha_{\text{diet-enamel}}$ ), despite that different cellular processes are involved. A similar suggestion arises from human populations who exhibit M3 teeth with a  $\Delta^{44/42}\text{Ca}_{\text{diet-enamel}}$  value similar to the general mammal  $\Delta^{44/42}\text{Ca}_{\text{diet-bone}}$  (Tacaïl et al., 2017), which suggests that this could be a general feature among mammals or even vertebrates. These data consequently support that a  $\Delta^{44/42}\text{Ca}_{\text{bone-enamel}}$  offset, when observed (Heuser et al., 2011; Tacaïl et al., 2014; Martin et al., 2015, 2017a), is more likely caused by diagenetic alteration (for fossil tissues), or by the different mineralization timings of enamel and bone. Indeed, in contexts of variable body Ca isotope composition (e.g. forced by seasonal dietary variations), these

different mineralization timings generate different bone and enamel  $\delta^{44/42}\text{Ca}$  values, with bone sections averaging years of body Ca isotope compositions and enamel micro-samples reflecting week to month periods (see supplementary material T1 for further discussion of the length of these mineralization periods).

### 4.2. Physiological control on $\delta^{44/42}\text{Ca}$ values in adult tissues

#### 4.2.1. Gestation effects

In the red deer population from Bauges NRP, adult females who recently gave birth (referred as lactating hinds) display a mean bone  $\delta^{44/42}\text{Ca}$  value of  $+0.17 \pm 0.12\text{‰}$  (95% confidence interval) higher than adult males (Fig. 3). This is similar to what was previously reported for domestic sheep (Reynard et al., 2010), in which females ( $n = 8$ ) displayed a bone  $\delta^{44/42}\text{Ca}$  value significantly higher of  $+0.14 \pm 0.08\text{‰}$  (95% confidence interval) compared to males ( $n = 16$ ). The two factors pointed to be the cause of this sexual driven difference in sheep were the accretion of bone during gestation and the excretion of milk (Reynard et al., 2010), as only these two Ca fluxes were thought to be associated with Ca isotope fractionation at this time. However, the very low enamel  $\delta^{44/42}\text{Ca}$  values we record in cervid teeth mineralizing in utero (DP4, early M1, Figs. 5 and 6) suggests that during gestation a mother will preferentially transfer light Ca isotopes through placental Ca transfer. This is also suggested by data from pig umbilical blood (Hassler et al., 2021) and human deciduous teeth (Tacaïl et al., 2017, 2019), although other data point out that it could be representative of the last stage of gestation only (Li et al., 2020). Such trapping of isotopically light Ca by the fetus would have comparatively enriched mother tissues in heavy Ca isotopes (including mineralizing bone or enamel). The most simplistic box model of gestation (a 2 box model with a  $\Delta^{44/42}\text{Ca}_{\text{fawn-hind}}$  of  $-0.25\text{‰}$  and a hind giving 10% of her Ca to her fawn) gives a  $\delta^{44/42}\text{Ca}$  shift of  $+0.02$  to  $+0.03\text{‰}$  for the hind, but it has to be noted that this model is very simplistic and amplifies the shift compared to reality (notably by neglecting Ca transfers from the fetus to the hind and the Ca intake of the hind). Among other changes, gestation also triggers higher Ca urinary excretions (Giesemann et al., 1998; Kovacs and Fuleihan, 2006; Reynard et al., 2010), a Ca flux which contributes to lower blood



**Fig. 5.** Enamel and bone  $\delta^{44/42}\text{Ca}$  variability of modern red deer.

The  $\delta^{44/42}\text{Ca}$  values from bone and enamel micro-samples of AB and JVB specimens (modern red deer, Bauges NRP). The horizontal black and red lines represent respectively the mean bone  $\delta^{44/42}\text{Ca}$  value of each specimen and the limits of their confidence interval ( $\pm 2$  s.e.). Micro-samples from DP4, M1, M2 and M3 teeth are respectively represented by purple diamonds, red points, blue squares and green triangles. Dashed orange and cyan ellipses respectively emphasize periods during which mineral licking and osteophagia events are suspected. Error bars represent the 2 s.e. intervals for each sample. The age model used for temporally anchor enamel micro-samples is described in Section 2.4 and is extrapolated from Brown and Chapman (1991a), with the only exception of enamel mineralizing in utero for which ages are arbitrary. Teeth mineralization timings which served this model are displayed at the bottom of the graph. Note that despite our micro-sampling procedure time averaging likely affects each of these enamel micro-samples (see supplementary material T1). Red deer silhouettes are modified from pictures of the public domain accessible in the following website: [www.phylopic.org](http://www.phylopic.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

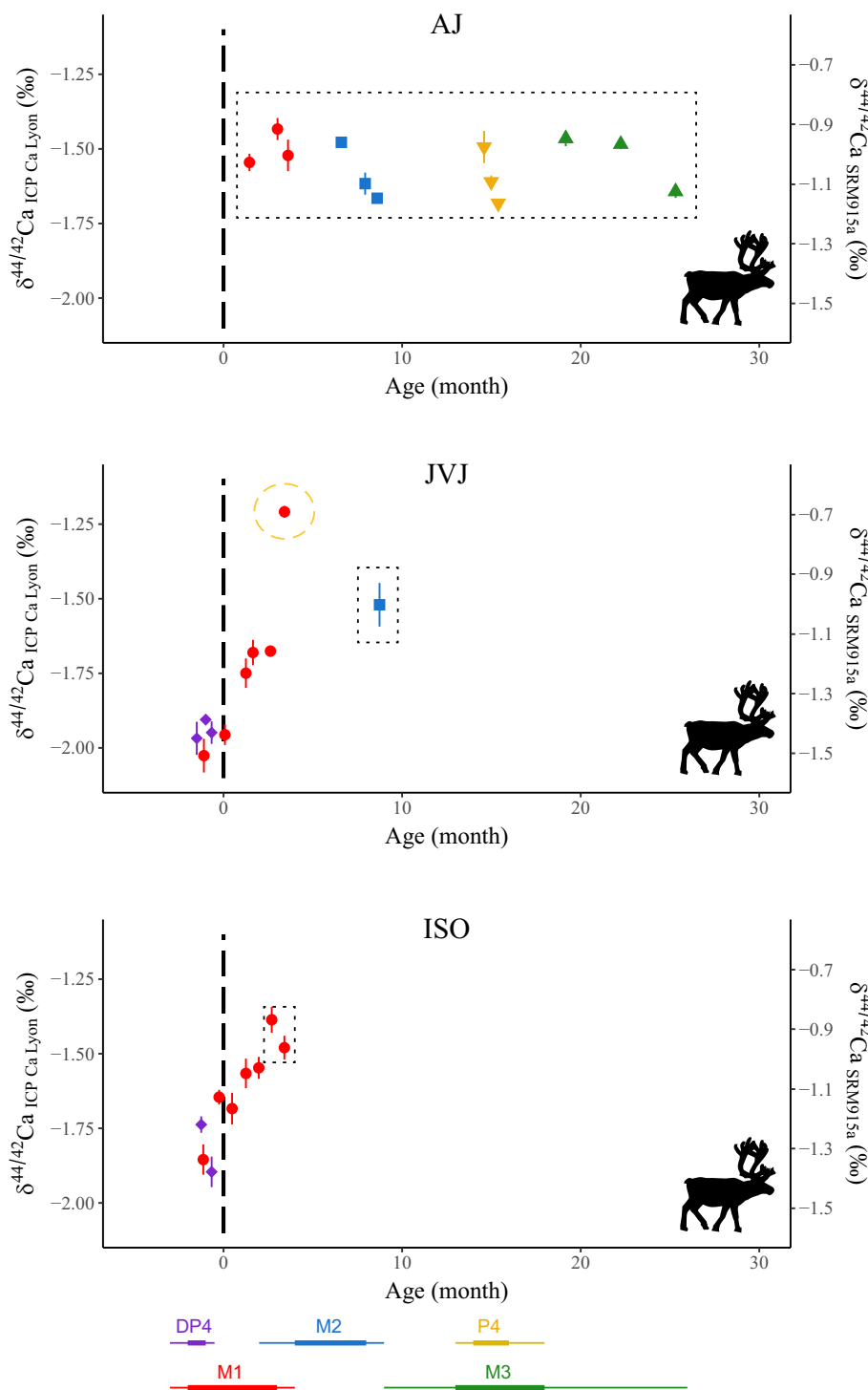
$\delta^{44/42}\text{Ca}$  values by preferentially exporting heavy Ca isotopes in urines (Skulan et al., 2007; Heuser and Eisenhauer, 2010; Morgan et al., 2012; Tacail et al., 2014; Channon et al., 2015; Heuser et al., 2016, 2019; Tacail, 2017; Eisenhauer et al., 2019). Thus, the consequences of gestation have opposite influences on blood Ca isotope composition, and therefore limit the record of a gestation signal in bone and enamel. Moreover, the recent questioning about the  $\alpha_{\text{blood-bone}}$  amplitude (Tacail, 2017; Tacail et al., 2020; Hassler et al., 2021) also questions the base of the bone accretion effect. Further investigations are necessary on this topic but at the moment these sparse data suggest that differences of bone  $\delta^{44/42}\text{Ca}$  values between male and female red deer (this study) and sheep (Reynard et al., 2010) have another origin than gestation. Following the same logic, it seems unlikely that gestation effects can significantly disrupt the trophic clustering of adult bone and enamel Ca isotope compositions.

#### 4.2.2. Lactation effects

The amount of Ca provided by the hind to its fawn during the 3 to 8 months of nursing is huge, as a fawn grows to about the half of the hind body mass between its birth and weaning (Table 1; Table S1; Mitchell et al., 1976; Jones et al., 2009). Comparatively, a juvenile red deer usually weighs about 8 kg at birth (Mitchell et al., 1976; Jones et al., 2009) which represents about 8.6% of the mean body mass of adult

hinds from our dataset (Table S1) and consequently implies far less Ca transfers from the hind to the fawn than lactation does. As milk is very depleted in heavy Ca isotopes ( $\Delta^{44/42}\text{Ca}_{\text{milk-diet}} = -0.6\text{‰}$ ; Chu et al., 2006; Gussone and Heuser, 2016; Heuser, 2016; Tacail, 2017; Hassler et al., 2021), its excretion can lead to enrich lactating females in heavy Ca isotopes (Reynard et al., 2010; Hassler et al., 2021). Along with important Ca excretion through milk, lactating mammals are subject to higher Ca absorption in the digestive tract, to bone loss and to changes in Ca urinary excretion (Ramberg Jr et al., 1970; Cross et al., 1995; Giesemann et al., 1998; Karlsson et al., 2001; Wysolmerski, 2002; Vanhouten and Wysolmerski, 2003; Kovacs and Fuleihan, 2006; Tacail, 2017). However, such changes in Ca fluxes only secondarily affect the lactation signal, as these changes stay minimal compared to the order of magnitude of milk Ca excretion (Hassler et al., 2021). The lactation should therefore be characterized by higher body  $\delta^{44/42}\text{Ca}$  values in hinds, including within their bones if the nursing lasts for several months (supplementary material T1). In this context, the high bone  $\delta^{44/42}\text{Ca}$  values reported in lactating hinds (Figs. 2 and 3) seems very compatible with the manifestation of a lactation effect, similarly to what is observed for domestic sheep (Reynard et al., 2010). The important residence time of Ca in bones certainly heavily damps this lactation signal (see supplementary material T1 and Hassler et al., 2021), although the extensive bone remodeling and bone losses usually





**Fig. 6.** Enamel  $\delta^{44/42}\text{Ca}$  variability of fossil reindeer. The  $\delta^{44/42}\text{Ca}$  values from bone and enamel micro-samples of AJ, JVJ, ISO M1 and ISO DP4 specimens (fossil reindeer). Note that ISO M1 and ISO DP4 are two distinct specimens that have been merged in one graph only for visual convenience. Micro-samples from DP4, M1, M2, M3 and P4 teeth are respectively represented by purple diamonds, red points, blue squares, green triangles and inverted yellow triangles. The dashed orange ellipse emphasizes a period during which mineral licking events are suspected. Dotted boxes contain points identified as providing accurate trophic information (see Section 4.3). Error bars represent the 2 s.e. intervals of each sample. The age model used for temporally anchor enamel micro-samples is described in Section 2.4 and is extrapolated from Brown and Chapman, 1991a, with the only exception of enamel mineralizing in utero for which ages are arbitrary. Teeth mineralization timings which served this model are displayed at the bottom of the graph. Note that despite our micro-sampling procedure time averaging likely affects each of these enamel micro-samples (see supplementary material T1). Reindeer silhouettes are pictures from the public domain accessible in the following website: [www.phylopic.org](http://www.phylopic.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recorded during lactation decreases this residence time and improves the potential of the signal to be recorded (Giesemann et al., 1998; Vanhouten and Wysolmerski, 2003; Kovacs and Fuleihan, 2006; Hassler et al., 2021). Looking at subadult hinds for such a signal is more difficult, as their reproductive success is much more variable and lower than in females of three years and more.. However, higher body mass also suggests higher chance that they gave birth to a fawn during the last birth season (Mitchell et al., 1976; Clutton-Brock et al., 1982; Albon et al., 1986; Pellerin et al., 2014). Thus, the fact that bone  $\delta^{44/42}\text{Ca}$  values of subadult females overlap with both lactating and yield adult hinds suggest that this group includes both nulliparous and primiparous

females (Fig. 3). This is also consistent with the fact that the heaviest subadult female displays the highest  $\delta^{44/42}\text{Ca}$  values, suggesting that this specimen gave birth to a fawn during the last birth season.

Interestingly, the female with the highest body mass in our dataset displays lower bone  $\delta^{44/42}\text{Ca}$  values despite having an age and body mass suggesting lactating periods in previous birth seasons, as high body mass mean higher reproduction chances (Albon et al., 1986; Pellerin et al., 2014). This suggests that skeletal Ca turnover is intense enough to conceal lactation signals from one year to another in this species, at least within the cortical part of the dentary bone (the bone part analyzed in this study). We lack precise data about bone remodeling rate for red deer

**Table 1**  
Body mass data of red deer from Bauges NRP (in kg).

Sex	Age	Mean	sd	n	min	max
F	A	98.05	12.82	447	81.58	114.52
F	J	52.00	8.75	223	40.73	63.27
F	SA	77.21	9.30	102	65.15	89.27
M	A	150.71	27.90	457	114.87	186.56
M	J	56.40	8.70	218	45.20	67.60
M	SA	96.05	13.66	187	78.44	113.66

Note. This table shows the total body mass data [kg] recorded on 1634 specimens of red deer from Bauges NRP. Age classes referred as adult (A), subadult (SA) and juvenile (J).

dentary bone. However, this fast Ca turnover seems odd considering that the total skeleton would need about 9 years to remove 75% of a lactation signal (see box model described in supplementary material T1 and Fig. S1). The post-lactation bone accretion and the relatively small male/female  $\delta^{44/42}\text{Ca}$  difference observed so far might help to achieve a quicker concealing of the lactation signal, but further studies are necessary to confirm this dynamic as only one individual suggests this tendency in our dataset. If bone Ca isotope composition proves to be that dynamic, the time interval between last lactation and death, as well as the time interval between consecutive lactations might be critical to preserve a lactation signal within bone Ca isotope composition. Nevertheless, the amount of produced milk, the duration of lactation periods and the size of the skeleton Ca reservoir also likely modulate this sex-driven Ca isotopic difference among mammal species (Hassler et al., 2021). Those factors are probably a key to explain why human populations studied by Reynard et al. (2010, 2013) display non-significant isotopic clustering between sexes, along with the diversity of possible food sources which characterize humans. Such lactation signal is in theory less dampened in enamel than in bones (see supplementary material T1). However, looking for these signals in adult teeth of red deer females seems challenging, as the enamel of the last tooth to mineralize (i.e. the M3) has generally completed its mineralization around 26 months (Brown and Chapman, 1991b), while all females of two-year-old have not yet achieved their first reproduction. We cannot be certain of that concerning the reindeer population of Jaurens. However, our data suggest that any record of a lactation signal within their enamel would result in higher enamel  $\delta^{44/42}\text{Ca}$  values, whereas these reindeers exhibit suspiciously low  $\delta^{44/42}\text{Ca}$  values. We thus conclude that lactation effects on body Ca isotope composition have to be considered when studying mammal populations but cannot be invoked to explain anomalously low bone or enamel  $\delta^{44/42}\text{Ca}$  values.

#### 4.2.3. Antlerogenesis effects and antler records

We report the first comparison between Ca isotope compositions of antlers and the rest of the skeleton (Fig. 4). This pilot investigation highlights that antlers display  $\delta^{44/42}\text{Ca}$  values significantly higher than the rest of the skeleton. As for enamel and bones, the key is to assess whether this difference is the result from differences in Ca isotope fractionation coefficients at mineralization or the result of a different mineralization timing. These data call for further studies, as comparing antlers with bone and enamel could add an additional dimension for studying cervid life history traits using Ca isotopes, allowing to further investigate diet seasonality for example.

With the only data presented in Fig. 4, it seems that antlers could preferentially trap heavy Ca isotopes from blood, although physiological processes generally favor light Ca isotopes (Tacaïl, 2017). If further confirmed, this would indicate that antlerogenesis could lower body Ca isotope composition. Indeed, antlers act primarily as a Ca sink working similarly as lactation and gestation by isolating Ca from the rest of the body, although antlers are also subject to bone remodeling (Bélanger et al., 1967; Muir et al., 1987a; Rolf and Enderle, 1999). However, the fact that lactation proved to have a relatively tenuous influence on bone  $\delta^{44/42}\text{Ca}$  values, despite involving far bigger Ca fluxes and Ca isotope

fractionation coefficients than antlerogenesis (Mitchell et al., 1976; Muir et al., 1987a, 1987b; Harris et al., 2002; Thomas and Barry, 2005; Li, 2013), suggests that antlerogenesis will not be detectable within the skeleton Ca isotope composition. This is supported by the complete overlap we observe between male red deer, yeld and immature hinds bone  $\delta^{44/42}\text{Ca}$  values (Fig. 3). An overlap which occurs despite the fact that studied specimens have been slaughtered just when the antlers of males finished their growth (Mitchell et al., 1976), so just when antlerogenesis could had a strong influence on skeleton Ca isotope composition. Although further tests are necessary to prove that enamel stays unaffected too, the end of enamel mineralization occurs before large antlers can grow (Kruuk et al., 2002; Thomas and Barry, 2005) which suggests that enamel will stay largely unaffected by antlerogenesis. In conclusion, none of our data suggests that antlerogenesis could be an important driver of body Ca isotope compositions, or could cause a significant mismatch of Ca isotope compositions and trophic data.

#### 4.3. Nutrition control over bone and enamel $\delta^{44/42}\text{Ca}$ values in modern and fossil cervids

##### 4.3.1. Neonatal life history traits

We discussed how gestation, lactation and antlerogenesis could affect adult bone and enamel  $\delta^{44/42}\text{Ca}$  values, and neither of these phenomena can convincingly explain the low  $\delta^{44/42}\text{Ca}$  values found in reindeer populations (Martin et al., 2017a). However, gestation and lactation not only affect adult Ca isotope composition, they also control juvenile Ca intakes and the Ca isotope composition of their tissues. For the five studied cervids with a preserved DP4 or unworn M1 (AB, JVB, JVJ, ISO M1, ISO DP4), enamel mineralizing in utero systematically exhibits the lowest  $\delta^{44/42}\text{Ca}$  values recorded (Figs. 5, 6). DP4 teeth exhibit stable and low  $\delta^{44/42}\text{Ca}$  values relative to third molars (M3) and adult bones, whereas M1 teeth exhibit various rates of transition from early ontogenetic low  $\delta^{44/42}\text{Ca}$  values to late ontogenetic high  $\delta^{44/42}\text{Ca}$  values. A similar pattern of Ca isotope composition has been previously described in human deciduous teeth, in which a clear link between weaning practices and  $\delta^{44/42}\text{Ca}$  values has been described (Tacaïl et al., 2017). We thus propose that the transition from low  $\delta^{44/42}\text{Ca}$  values in DP4 and in the apex of M1, to high  $\delta^{44/42}\text{Ca}$  values in the neck of the M1, in the M2, M3 and adult bones is characteristic of the transition from maternal Ca transfer (during the gestation then the nursing) to post-weaning Ca intake (adult-like diet). The mineralization timing and enamel  $\delta^{44/42}\text{Ca}$  values of red deer and reindeer are consistent with the 3–8 months of weaning age of these species (Miller, 1972; Mitchell et al., 1976; Brown and Chapman, 1991b; Jones et al., 2009). The specimens AB, JVB and ISO M1 all reached the adult Ca isotope composition at the neck of the M1. This suggests that they were weaned before four months considering the length of enamel mineralization (Figs. 5, 6 and supplementary material T1). Data from AJ (Fig. 6) also suggest a relatively early weaning, although the wear of the M1 limits the record of pre-weaning conditions. The JVB specimen constitutes an exception to this early transition in  $\delta^{44/42}\text{Ca}$  values in the M1. For this individual, the increase of  $\delta^{44/42}\text{Ca}$  values within the M1 seems flattened (see Fig. 5;  $\Delta_{\text{max-min}} = 0.17\%$ ; intra-tooth 2 s.d. = 0.12‰, n = 7), and enamel  $\delta^{44/42}\text{Ca}$  values only approach bone-like values within the M2 and M3 late mineralizing sections. This suggests a later weaning for this individual (around 8 months), or alternatively an earlier tooth mineralization. The fact that these weaning ages seem consistent with the observations of these species in the wild, supports that Ca isotopes are accurate for detecting in utero, milk feeding and weaning periods among mammals. These data also highlight that some individuals (like JVB) could display a delayed dietary transition to a purely adult diet (i.e. without milk), in a way that the transition is not yet complete when M2 teeth mineralize. This supports that milk consumption could have affected the previously published data of cervids from Jaurens (Martin et al., 2017a) and caused their suspiciously low enamel  $\delta^{44/42}\text{Ca}$  values. For these cervid species,

we consequently advise to favor M3 enamel sampling to infer purely adult trophic information (when accessible). Note that in M3, enamel can then display higher  $\delta^{44/42}\text{Ca}$  values in case of an early breeding and subsequent lactation (see 4.2.2 section). When possible, we thus advise to analyze enamel zones which mineralized after weaning and prior to female sexual maturity. As the position of these zones are difficult to predict, especially for fossil species, we advise to use serial micro-sampling in order to identify early post-weaning periods. This approach provides useful complementary information for ecological and physiological inferences and minimizes the potential of lactating females and nursing to affect trophic inferences based on  $\delta^{44/42}\text{Ca}$  data.

#### 4.3.2. Osteophagia and mineral licks

Osteophagia (i.e. bone consumption) and the consumption of natural mineral licks are two common sources of mineral supplementation that cervids, and more generally artiodactyls, use during periods of high mineral requirement (e.g. lactation, juvenile growth, antlerogenesis), notably to cope with phosphorus (P) or other element deficiency (Cowan and Brink, 1949; Kjos-Hanssen, 1973; Krausman and Bissonette, 1977; Marie, 1982; Grasman and Hellgren, 1993; Cáceres et al., 2011; Gambín et al., 2017). There is yet no direct measure of mineral supplementation effects on enamel Ca isotope composition in the literature. However, we can confidently assume that, because bones are generally  $^{44}\text{Ca}$ -depleted compared to plant products (Gussone and Heuser, 2016), antler or other bone consumption will result in lower dietary  $\delta^{44/42}\text{Ca}$  values (Gussone and Heuser, 2016; Martin et al., 2017a, 2018). We can roughly estimate that this change of dietary  $\delta^{44/42}\text{Ca}$  value will be of 0 to  $-2\%$ , depending on the amount of consumed bones and their Ca isotope compositions (Heuser et al., 2011; Gussone and Heuser, 2016). Foraging on mineral licks would have the opposite effect on dietary  $\delta^{44/42}\text{Ca}$  value (about 0 to  $+1\%$ ), because soils and rocks are generally  $^{44}\text{Ca}$ -enriched compared to plant products (Gussone and Heuser, 2016). These modifications of the dietary Ca isotope composition logically affect the  $\delta^{44/42}\text{Ca}$  values of growing enamel, with an intensity that will depend upon the rate of enamel growth and the duration of the mineral supplementation.

The resort to mineral supplementation by red deer from Bauges NRP has not been quantified. However, we know from a red deer population from Spain that about two hundred individuals can consume about 966 g of antlers over 7 months, mainly during lactation, weaning and antlerogenesis periods (Estevez et al., 2008; Gambín et al., 2017). If we consider that antlers contained a maximum of 15% of Ca (Table S4), this would represent about 0.7 g of Ca intake per individual. Considering that mineral supplementations were enhanced by the low P availability in the plants of the study site in Spain (Estévez et al., 2009; Gambín et al., 2017), we can expect a lower to similar intensity of mineral supplementations within the Bauges NRP red deer population. Such Ca intakes seem negligible over a year compared to plant and water sources, which suggests that mineral supplementations likely have a negligible influence on bone  $\delta^{44/42}\text{Ca}$  values. However, few individuals may have been responsible for most of this bone consumption (individuals were not identified in Gambín et al. (2017)), and cervids from this study likely had access to other bones and mineral sources than monitored antler stacks (Gambín et al., 2017). Therefore, it seems plausible to expect at least short excursions in enamel  $\delta^{44/42}\text{Ca}$  values (negative for osteophagia, positive for mineral licking) for individuals experiencing periods of important supplementation (i.e. lactation, weaning, antlerogenesis). Following this postulate, we suspect that the outlier  $\delta^{44/42}\text{Ca}$  value in the M3 of the JVB specimen has been influenced by osteophagia. This data point is characterized by an isolated low  $\delta^{44/42}\text{Ca}$  value, localized far after weaning in enamel formed around 15 months of age (Fig. 5), a period which could match the peak of supplementation reported in September by Gambín et al. (2017). Finally, we note that the two latest M2  $\delta^{44/42}\text{Ca}$  values of AB (at 10 months) seem slightly higher than the average M2, M3 and bone  $\delta^{44/42}\text{Ca}$  values of this specimen (Fig. 5). It is possible that this results from Ca intakes influenced by

mineral licking, but the isotopic difference is tenuous, possibly artefactual, and does not match with the common periods of mineral supplementation recorded in Spain (Gambín et al., 2017).

Reindeer are also known to seasonally resort to mineral supplementation, likely to compensate for the low P, Ca and sodium content of the plants and lichens they consume (Grasman and Hellgren, 1993). Frequent antler chewing are reported in spring during the calving season (Kjos-Hanssen, 1973; Marie, 1982), as well as mineral licks, notably in the summer (Cowan and Brink, 1949). We have no data to quantify these supplementations, but it seems reasonable to suspect they can influence enamel  $\delta^{44/42}\text{Ca}$  values. A possible example is found within the M1 tooth of the JVJ specimen at 5 months (Fig. 6). This enamel zone constitutes the terminal part of the weaning transition, and is characterized by a higher  $\delta^{44/42}\text{Ca}$  value than within the M2 of the specimen (or of any other enamel sample from Jaurens specimens). Because this critical period favors mineral supplementations in cervids (see previous paragraph), we suspect that this positive  $\delta^{44/42}\text{Ca}$  excursion is the result of mineral licking, although the very limited post-weaning JVJ dataset provides limited evidence for this.

If the effects of mineral supplementation on body Ca isotope composition are further confirmed, this proxy could be used to study this crucial survival behavior among modern and fossil mammals. As such, mineral supplementations however complicate the relation between trophic level and enamel Ca isotope composition. Thus, we emphasize the importance of considering mineral supplementation behaviors when using  $\delta^{44/42}\text{Ca}$  data for trophic interpretations. We notably suggest a specific attention toward this behavior for the study of artiodactyls from arid or tropical environments, because these environments are generally marked by a low P availability in plants, a trigger for mineral supplementations (Grasman and Hellgren, 1993).

#### 4.3.3. Plant Ca isotopic variability

Identifying and excluding enamel  $\delta^{44/42}\text{Ca}$  data affected by pre-weaning, lactation and short term mineral supplementation periods allow, with some limits (see previous section), to identify Ca isotope data that are representative of trophic level. However, the important variability of plant Ca isotope compositions can also limit the trophic clustering of enamel and bone  $\delta^{44/42}\text{Ca}$  data, by generating isotopic variability among herbivores (Martin et al., 2018). Plant Ca isotope compositions combine a variability between plant organs and between plant taxa (Holmden and Bélanger, 2010; Gussone and Heuser, 2016; Schmitt, 2016; Moynier and Fujii, 2017; Martin et al., 2018; Griffith et al., 2020). The mean offset of  $-0.31 \pm 0.14\%$  observed between the leaves of dicotyledons and monocotyledons (e.g. grasses) is notably suspected to cause the documented difference of  $-0.18 \pm 0.10\%$  between the enamel of browser herbivores (i.e. with a leaf-dominated diet) and of grazer herbivores (i.e. with a grass-dominated diet) (Martin et al., 2018). Red deer is described as a mixed feeder, changing seasonally or regionally between more browser to more grazer diets (Hearney and Jennings, 1983; Gebert and Verheyden-Tixier, 2001; Storms et al., 2008; Berlioz et al., 2019). In the Bauges NRP, the grass proportion in the red deer diet has been estimated to change between 20% and 40% from summer to winter (Redjadj et al., 2014), and could be lower during late winter, spring and early summer according to the tendencies observed at the limits of monitored periods (Redjadj et al., 2014). Changing diet proportions from 40% grass and 60% leaves to 10% grass and 90% leaves would change the diet  $\delta^{44/42}\text{Ca}$  value by about 0.09‰, a result which is relatively close to the post-weaning range of 0.11‰ observed in the enamel record of the AB specimen (the red deer specimen with the most extensive post-weaning enamel record, Fig. 5). Our isotopic data are thus compatible, at the first order, with the known diet variability of this cervid population, although this estimation of 0.09‰ is built upon coarse approximations (notably that consumed grass  $\delta^{44/42}\text{Ca}$  value was exactly  $-0.31\%$  lower than non-grass food  $\delta^{44/42}\text{Ca}$  value) and needs further investigations. A higher resolution regarding the post-weaning enamel record, and a better constrain of the Ca isotope composition of

food items available at Bauges NRP (especially evergreen trees which are an important food source for these red deer, Redjadj et al. (2014)) would provide  $\delta^{44/42}\text{Ca}$  data that will be more comparable with  $\delta^{13}\text{C}$  records, dental micro-wear and stomach content data collected so far for this red deer population (Redjadj et al., 2014; Merceron et al., 2021). Such further studies could notably help resolving some discrepancies between dental micro-wear and stomach content estimations (i.e. higher versus lower grass content in the diet, Merceron et al. (2021)), and provide independent complementary information regarding  $\delta^{13}\text{C}$  data (e.g. diet and ecological niche).

Reindeers are also mixed-feeders, but consume lichen during winter and have a generally more browser diet compared to red deer (Hofmann, 1989; Mathiesen et al., 2000), an observation which is notably consistent with the higher  $\delta^{44/42}\text{Ca}$  values of reindeers compared to red deers when both co-exist within the same environment (Dodat et al., 2021). After weaning, the AJ specimen (the reindeer with the most extensive post-weaning enamel record) exhibits a  $\delta^{44/42}\text{Ca}$  range of 0.25‰ (Fig. 6). As for red deer, this isotopic variability is compatible, at the first order, with the consequences expected from changing proportions of grass and other plant items in the diet. Within this browser to grazer-like diet continuum, the lowest enamel  $\delta^{44/42}\text{Ca}$  values would correspond to more grazer-like diet periods, whereas periods with the highest enamel  $\delta^{44/42}\text{Ca}$  values would correspond to more browser-like diet periods. A limit to this hypothesis is the potential occurrence of mineral supplementations which can modify the dietary Ca isotope composition independently of the plants (see Section 4.3.3). In this regard, combining  $\delta^{44/42}\text{Ca}$  data and  $\delta^{13}\text{C}$  data within a multi-proxy approach (e.g. Martin et al., 2018) would be a solid option to further investigate diet and mineral supplementation seasonality among these cervids.

#### 4.3.4. Trophic position of Pleistocene fossil cervids

The previous discussion sections detail how to identify tooth and enamel parts that should be favored for trophic inter-specific comparisons (by identifying pre- and post-weaning periods, by detecting sections potentially disrupted by short term mineral supplementation, and by providing insight about intra-tooth  $\delta^{44/42}\text{Ca}$  variability). We applied this technique on three subadult to adult reindeers from the Late Pleistocene locality of Jaurens (AJ, JVJ and ISO M1). The most suited enamel data points for trophic inter-specific comparisons are indicated in Fig. 6. Unfortunately, we do not have access to an extensive M2 and M3 dataset for JVJ and ISO M1 specimens (Fig. 6). Therefore, we lack insight to determine if the enamel data points we selected for trophic inter-specific comparisons are entirely posterior to the weaning transition. Nevertheless, the selected data points of these specimens consistently range within the enamel Ca isotope compositions of AJ, which supports that they are representative of a post-weaning diet. Altogether, the weighted average (per specimen) of selected enamel  $\delta^{44/42}\text{Ca}$  values is +0.18‰ higher than previously published measures (Martin et al., 2017a). They consequently plot within the range of woolly rhinoceroses (*Coelodonta antiquitatis*,  $n = 3$ ) (Wilcoxon rank-sum test,  $p$ -value = 0.1) and steppe bisons (*Bison priscus*,  $n = 3$ ; Wilcoxon rank-sum test,  $p$ -value = 0.2) published by Martin et al. (2017a). This demonstrates that dismissing the data obtained from enamel affected by short term mineral supplementation or milk consumption, based on a serial micro-sampling approach, efficiently resolve the anomaly of  $^{44}\text{Ca}$  depletion previously reported in the enamel of reindeers from Jaurens. Serial micro-sampling is time and resource consuming and thus can hardly be used at the scale of a whole faunal assemblage, but this study proves that this technique is particularly suited to discuss diet and behavior at the individual scale, or when some  $\delta^{44/42}\text{Ca}$  data are at odds with independently inferred trophic positions or diet.

## 5. Conclusions

In this paper we discussed the behavioral and physiological events likely to affect mammalian enamel and bone Ca isotope compositions,

with two species of cervids as models (*Cervus elaphus*, *Rangifer tarandus*). Our results highlight that lactation is an effective source of Ca isotope variability as this phenomenon produced  $^{44}\text{Ca}$ -enriched isotope composition in bones of lactating females. Our study, however, failed to identify comparable gestation effects, likely because of long Ca residence time in cervid bones, of partial overprinting by lactation effects, and because gestation involves smaller changes in Ca fluxes compared to lactation or less intense Ca isotope fractionation coefficients associated to these fluxes. Similarly, antlerogenesis did not prove to be a significant driver of body Ca isotope composition in red deer. However, our data highlight that antlers display a different Ca isotopic signature than the rest of the skeleton, encouraging further studies. The Ca isotopic composition of enamel mineralizing in utero suggests that Ca transferred from the cervid mother to the fetus during gestation has a similar isotope composition than milk, at least during the closest period prior the birth. The transition from pre-parturition and pre-weaning Ca intakes to adult diet is clearly observed within the enamel of most of the studied individuals. Calcium isotopes can then be efficiently used for assessing weaning ages, providing the fact that no osteophagia is involved early in life, as this phenomenon is also able to generate low bone and enamel  $\delta^{44/42}\text{Ca}$  values (at the opposite of what is expected from mineral licks foraging). Finally, this study demonstrates that serial micro-sampling of enamel is an appropriate method to disentangle part of ecological, physiological and environmental Ca isotope signals. This method allowed to accurately extract trophic information from the enamel  $\delta^{44/42}\text{Ca}$  values of the reindeers from the Pleistocene deposit of Jaurens. When possible, we thus encourage the use of this method in order to accurately retrieve diet and trophic signals from enamel  $\delta^{44/42}\text{Ca}$  data. Such an approach would greatly benefit to paleoecology fields, as it makes Ca isotope data more readily comparable with other diet proxy such as carbon and nitrogen isotopes. Further studies are needed to investigate other cases of discrepancy between  $\delta^{44/42}\text{Ca}$  data and trophic level, as well as to assess the variability of lactation, gestation and mineral supplementation signals among mammals. However, these results open great perspectives for the study of mammal physiology in addition to clarify the trophic inferences achievable with Ca isotopes.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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