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Isotopic calcium biogeochemistry of MIS 5 fossil vertebrate bones: application to the study of the dietary reconstruction of Regourdou 1 Neandertal fossil



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ABSTRACT

The calcium isotopic composition ($\delta^{44/42}\text{Ca}$) of bone and tooth enamel can be used for dietary reconstructions of extant and extinct mammals. In natural conditions, the $\delta^{44/42}\text{Ca}$ value of bone and teeth varies according to dietary intake with a constant isotopic offset of about -0.6‰ . Owing to the poor conservation of collagen, carbon (C), and nitrogen (N) isotopic compositions of the Regourdou Moustertian site (MIS 5, Dordogne, France) previously failed to provide any paleodietary information. Therefore, to reconstruct the trophic chain, we have measured calcium (Ca) isotopes from fossil bone samples of the fauna from the Regourdou site, as well as from three bone samples of the Regourdou 1 Neandertal specimen. The results show a taxon-dependent patterning of the Ca isotopic compositions: herbivores generally have higher $\delta^{44/42}\text{Ca}$ values than carnivores. All the $\delta^{44/42}\text{Ca}$ values of Regourdou 1 are low ($<-1.6\text{‰}$), placing this specimen amid carnivores. Using a bone-muscle Ca isotopic offset determined on extant animals, we further show that the $\delta^{44/42}\text{Ca}$ value of the Regourdou 1 diet, and that of most carnivores, cannot be accounted for by the consumption of meat only, as plants and meat have indistinguishable $\delta^{44/42}\text{Ca}$ values. Mass balance calculations indicate that the low $\delta^{44/42}\text{Ca}$ values of the Neandertal's carnivorous diet are explained by the ingestion of bone marrow containing as little as 1% trabecular bone. Our results show that the Regourdou 1 Neandertal consumed a mixture of various herbivorous prey, as well as trabecular bone, which probably occurred when marrow was ingested, by accident or intentionally.

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1. Introduction

The reconstruction of diet is an important aspect of paleoanthropology, archeology, and prehistory because diet is related to

numerous human behaviors such as hunting practices, social organization, and mobility (e.g., Patou-Mathis, 2000; Costamagno et al., 2006; Hublin and Richards, 2009). Paleoecological reconstructions are traditionally based on the study of faunal assemblages of preserved remains and associated anthropogenic traces (Hublin and Richards, 2009), tooth microwear (Puech et al., 1983), dental size and morphology (Jiménez-Arenas et al., 2014) or more rarely from preserved organic matter (e.g., phytoliths or

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DNA in dental calculus; Henry et al., 2011). However, the reconstruction of ancient diet may be biased due to differential transport of anatomical parts of prey, and to taphonomic processes (e.g. chemical process, scavengers, water flow), which can lead to the lack of conservation of flora and other food resources (Faith and Thompson, 2018) and can modify faunal assemblages even in well-preserved sites (Fernandez-Jalvo and Andrews, 2016).

Stable isotopes have been a useful supplementary tool for studying the diet of extinct species for over 40 years. Since the 1990s and the first isotopic studies of Neandertal remains from Marillac in Charente (France; Bocherens et al., 1991), the number of studies using collagen C and N isotopic compositions of Neandertals has continually increased (more than 40 remains analyzed having provided interpretable results), allowing researchers to make robust assumptions about the dietary habits of Neandertals. The conclusions do not vary much: Neandertals were at the top of the trophic chain with a dietary preference for large herbivores, such as horse, reindeer, red deer, bovid, rhinoceros and mammoth (Balter and Simon, 2006; Bocherens, 2013; Naito et al., 2016; Wißing et al., 2016), which is consistent with the zooarchaeological evidence, bone accumulation, and cut marks (Patou-Mathis, 2000; Hublin and Richards, 2009; Marín et al., 2017).

However, recent studies of dental calculus (Henry et al., 2011) and N isotopic composition of amino acids (Naito et al., 2016; Jaouen et al., 2019) show that some Neandertals may have consumed plants in sizeable proportion (~20% of their dietary protein source). Isotopes are now used routinely to reconstruct diet, but two main issues limit the use of the C and N isotopic systems in fossil settings. First, the extraction of a sufficient amount of collagen from tooth or bone requires the collection of between 10 mg and 500 mg of fossil material, depending on the state of preservation of the collagen (Drucker et al., 1999; Bocherens et al., 2016). This amount is a limiting factor because it represents a significant fraction, if not the whole root of a tooth, which is a problem for precious material, notably unique hominin remains. Second, while collagen can be preserved for more than 100 kyr in specific environments, cases of good preservation beyond 50 kyr are rare, preventing the use of N isotopic compositions in deep time (Melin et al., 2014). This represents a great handicap for the study of Neandertals, for whom dietary habits throughout the Paleolithic are key to understanding their cultural and biological evolution.

To overcome these difficulties, or to complement C and N isotopic composition studies, new isotopic systems have been developed as dietary proxies using the mineral phase of tooth dentine, enamel, and bone. Generally, these isotopic systems are referred as 'non-traditional' (Martin et al., 2017a) isotopes and involve transition metals including copper, zinc, and iron (respectively, $^{65}\text{Cu}/^{63}\text{Cu}$, $^{66}\text{Zn}/^{64}\text{Zn}$, $^{56}\text{Fe}/^{54}\text{Fe}$ ratios; Jaouen et al., 2012, 2013, 2017; Bourgon et al., 2020), or alkaline-earth metals including magnesium, calcium, and strontium (respectively, $^{26}\text{Mg}/^{24}\text{Mg}$, $^{44}\text{Ca}/^{42}\text{Ca}$, $^{88}\text{Sr}/^{86}\text{Sr}$ ratios; Knudson et al., 2010; Martin et al., 2014, 2015, 2017a, b, 2018; Hassler et al., 2018). Calcium is the element that has been the most studied thus far for paleodietary applications because it is the essential element of the mineral fraction of bone and teeth, i.e., hydroxylapatite. This gives the isotopic method two main advantages. First, provided that secondary Ca-bearing carbonates are absent or have been leached using dilute acid, the overprinting of a diagenetic isotopic composition on the original Ca isotopic composition is unlikely. This has been modeled using simple mass balance for Mg, Ca, Fe, Cu and Zn isotopes on continental and marine settings by Martin et al. (2017a). Based on the model, a significant diagenetic alteration of the Ca fraction of bioapatite in terrestrial settings is unlikely because the Ca concentration in groundwater is negligible (15–150 ppm of Ca) compared with that of hydroxylapatite (40%; Martin et al., 2017a). This is not

the case for the isotopic composition of trace elements (i.e., Cu, Zn, Fe, or Sr) which is more sensitive to diagenetic overprinting because the concentrations of trace elements are generally lower in bioapatite than in rivers (Martin et al., 2017a). Second, minute amounts of bone or tooth enamel (typically 100 μg) are sufficient for a routine measurement of the Ca isotopic composition. This technique can thus be applied to precious fossils while causing minimal damage (Tacaïl et al., 2019; Martin et al., 2017a).

Skulan and collaborators (Skulan et al., 1997; Skulan and DePaolo, 1999) first described a linear correlation between the Ca isotopic composition of diet and vertebrate tissues including bone. This observation allowed Skulan and DePaolo (1999) to show a decrease in the isotopic composition of Ca along the food chain. Several authors further assessed the extent of this decrease using different animal models, giving an average difference of the $\delta^{44/42}\text{Ca}$ values between bone and diet ($\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$) of -0.57‰ (± 0.10 , 2SE; Tacaïl et al., 2020). In mammals, the major isotopic fractionation occurs during renal reabsorption, which can be affected by many physiological aspects still poorly understood (Tacaïl, 2017; Tacaïl et al., 2020). The effect of lactation is potentially better understood and causes a significant offset of the bone $\delta^{44/42}\text{Ca}$ value (Reynard et al., 2010, 2013; Tacaïl et al., 2019). Other biological processes such as hibernation (cessation of the renal cycle), the presence of a particular digestive system such as for ruminants, or the production of mineralized growths such as antlers or horns, could also affect the bodily distribution of Ca isotopes (Tacaïl et al., 2020). Despite a body of isotopic evidence consistent with ecological information, the compilation of isotopic data on biological material cannot explain the lower isotopic composition of carnivores compared with herbivores by invoking meat consumption only. Indeed, meat displays $\delta^{44/42}\text{Ca}$ values close to the range of variation of plants (Tacaïl et al., 2019). Carnivores and herbivores should therefore have similar $\delta^{44/42}\text{Ca}$ values, which is not the case. Bone consumption has been proposed to explain the observed difference in $\delta^{44/42}\text{Ca}$ values of herbivores and carnivores (Heuser et al., 2011).

In this work, we use bone Ca isotope composition to investigate the trophic structure of a mammalian fossil assemblage including a single Neandertal individual. Our approach consists first in evaluating the Ca isotopic difference between muscle, bone, and bone marrow on modern samples and second, applying the assessed amplitude of these differences to reconstruct the past trophic chain of the Regourdou fossil assemblage.

2. Materials and methods

2.1. History of the Regourdou site

The Regourdou cave is located at the top of the Lascaux hill (Montignac-sur-Vézère, Dordogne; Supplementary Online Material [SOM] Fig. S1). Regourdou site 'excavations' began in 1954 at the landowner's (R. Constant) initiative. During the night of the 22nd to the 23rd of September 1957, part of a Neandertal skeleton was discovered (Regourdou 1, Bonifay et al., 2007; Madelaine et al., 2008; Maureille et al., 2015a). A rescue operation for recovery of the hominin remains was set up under the authority of F. Bordes during the 4th and 5th of October 1957 with the participation of E. Bonifay and G. Laplace-Jauretche as the only two excavators. This quick excavation allowed the discovery of faunal and lithic remains and about 50 identified hominin bones or bone fragments belonging to one Neandertal, Regourdou 1 (Madelaine et al., 2008).

Between 1961 and 1964, the site was excavated by E. Bonifay (Bonifay, 1964). The Regourdou 1 skeleton was located in Bonifay's stratigraphic layer 4 (Bonifay, 1964), which contained lithic remains belonging to a Discoid Mousterian techno-complex with the

production of pseudo-Levallois points and a temperate fauna highly dominated by brown bears (*Ursus arctos*). This layer 4 was attributed to MIS 5 based on cultural evidence, and this was confirmed by a recent Optically Stimulated Luminescence date (Bonifay, 1964; Bonifay et al., 2007; Maureille et al., 2017). According to E. Bonifay, the site was interpreted as a highly peculiar Mousterian symbolic cave because of the potential association of a Neandertal burial with anthropic structures (a pit and a pile of stones, or 'pierrier' in French, as well as various grave goods) and buried parts of brown bear corpses in an alleged pit closed by a several-ton flat limestone slab, along with other stone piles within layers 3 and 5 (Bonifay, 1962). However, a recent review (Madelaine et al., 2008) of the 1957 field notes and drawings, as well as the 1961 to 1964 field notebooks and collections (allowing the discovery of 73 new Regourdou 1 hominin remains; Madelaine et al., 2008; Maureille et al., 2015b), questioned the Bonifay interpretation of a unique Mousterian symbolic cave, even if the new scientific team involved in this research still considers Regourdou 1 as a Neandertal intentional burial (Madelaine et al., 2008; Maureille et al., 2015a, 2016; Pelletier et al., 2017). This is an ancient cave that was used as a den for brown bears which are represented in the lower levels (Cavanhié, 2009–2010). Subsequently the cavity collapsed, forming a trap allowing the accumulation of many fauna (Bonifay, 2002; Cavanhié, 2009–2010). The Neandertal is assumed not to be a major accumulating agent because only a small number of bones show anthropic action (Cavanhié, 2009–2010). Owing to age and taphonomic process, resulting in an insufficient quality of collagen, ancient DNA and C and N isotopic compositions failed to provide any paleogenetic or paleodietary information (Bocherens, pers. comm.).

2.2. Regourdou fossil bone samples

The Ca isotopic composition was measured for 30 samples obtained from eight different mammalian species, including four reindeer (*Rangifer tarandus*), five red deer (*Cervus elaphus*), two horses (*Equus caballus*), two wild boars (*Sus scrofa*), one cave lion (*Panthera spelaea*), one wolf (*Canis lupus*), one carnivore of indeterminate species, 11 brown bears (*U. arctos*), and three bone samples of the Regourdou 1 Neandertal individual (SOM Table S1). The epiphyseal fusion of the fossil material suggests that the specimens were mature individuals with the exception of one brown bear (R27). Not all samples were obtained from the same layer (SOM Table S1): ten are from layer 2, 16 from layer 4, including the Neandertal specimen, three from layer 5 and one from layer 7. Layers 4, 5, and 7 are thought to be contemporaneous and layer 2 younger (Bonifay et al., 2007; Cavanhié, 2009–2010). All but two of the samples were taken from cortical bone; trabecular bone was sampled from one brown bear and from the left femur of Regourdou 1. Each sample of trabecular bone was paired with a sample of cortical bone taken from an adjacent region of the same piece of bone. Each sample was obtained by excising a fragment of bone <5 mm long and 1 mm wide using a scalpel (SOM Fig. S2). Samples were preferentially taken from parts that were already damaged during excavation or during the postexcavation history or in areas already sampled for previous analyses. This sampling method allowed us to preserve as much as possible the morphological and structural integrity of the pieces.

2.3. Modern faunal bone and muscle samples

To better understand the calcium isotopic distribution in mammals, we included modern cortical and trabecular bone associated with bone marrow and muscle of domestic and wild animals (SOM Table S2). These bones were analyzed to evaluate the

bone-muscle Ca isotopic offset and to characterize the mechanism responsible for the decrease in the $\delta^{44/42}\text{Ca}$ values along food chains (SOM Table S2). The total sample of 11 samples includes three pigs (*Sus scrofa domestica*), one boar (*Sus scrofa*), four cows (three adults, one veal; *Bos taurus*), one roe deer (*Capreolus capreolus*), one chicken (*Gallus gallus*), and one rabbit (*Oryctolagus cuniculus*). As the different samples were obtained from butcher shops, no information concerning the diet of these individuals is available. However, dietary information is not needed to assess muscle-bone differences.

2.4. Analytical methods

The analyses of Ca stable isotope compositions were carried out at the Laboratoire de géologie de Lyon, Terre, Planète et Environnement at the Ecole Normale Supérieure de Lyon. All 30 bone samples from Regourdou's faunal and paleoanthropological collections (SOM Table S1) were first ground using an agate mortar and pestle before being leached with diluted ultrapure acetic acid (0.1 M) for 30 min at room temperature in an ultrasonic bath to eliminate Ca-bearing secondary carbonates, particularly calcite (Balter et al., 2002a,b). All modern samples were first freeze-dried and digested in concentrated HNO_3 acid to dissolve the organic phase.

The concentrations of major and trace elements were measured after a leaching step with acetic acid (SOM Tables S1 and S3) and diagenesis was monitored using the calcium/phosphorus, iron/calcium, manganese/calcium, and uranium/calcium ratios (Ca/P, Fe/Ca, Mn/Ca, and U/Ca, respectively; SOM Table S1) and the sum of all the rare earth elements (ΣREE ; SOM Table S3). These measurements were performed by quadrupole inductively coupled-plasma mass spectrometry (Q-ICP-MS) for trace elements and by ICP optical emission spectroscopy for major elements, respectively. Methods for the measurement of trace and major elements are briefly described here (for a complete description see Balter and Lécuyer, 2004, 2010). The concentrations of elements were calculated using calibration curves based on multielemental solutions. These solutions were also used to monitor and correct for the instrumental drift over the analytical session. Matrix effects and instrumental drift were also corrected using indium (In) and scandium (Sc) as internal standards for trace and major elements, respectively. Comparison of major and trace element was performed using a simple linear regression model and the correlation coefficient represented with an heatmap (correlation matrix). All the statistical analyses on the Ca isotopes and other elements were performed in R (V3.6.1; R Core Team, 2020).

All samples were processed to extract the bone's Ca based on the protocols established by Tacail et al. (2014). Briefly, this consists of a digestion step in concentrate HNO_3 and solubilization in 6N HCl. Then, three chromatography steps are necessary, the first to allow the recovery of Ca, Fe and Sr only, the second to isolate Sr, and the third to separate Ca from Fe. The compositions of the Ca isotopes were measured using a neptune plus multicollector ICP-MS using the standard-sample-standard-bracketing method. All Ca isotope compositions are expressed using the delta notation and were calculated using the ICP Ca Lyon standard as bracketing and reference material. A solution of the certified reference material NIST SRM 1400 (bone ash) was repeatedly purified and measured as a sample to control the accuracy of the measurements of archaeological bone and a solution of the certified reference material NIST SRM 1486 (bone meal) was repeatedly measured as a sample to control the accuracy of the measurements of modern bone and muscle samples. The SRM 1486 certified reference material yielded a value of -1.07 ± 0.049 , (2 SE, $n = 24$), which is undistinguishable from all SRM 1486 reported in other studies (-1.009 ± 0.026 , 2 SE;

Heuser and Eisenhauer, 2008; Heuser et al., 2011, 2016a, 2016b). The $\delta^{44/42}\text{Ca}$ annotation is defined according to the following equation:

$$\delta^{44/42}\text{Ca} = \left(\frac{\left(\frac{{}^{44}\text{Ca}}{{}^{42}\text{Ca}} \right)_{\text{Sample}}}{0.5 \times \left(\frac{{}^{44}\text{Ca}}{{}^{42}\text{Ca}} \right)_{\text{ICP-Ca-Lyon}}^{n-1} + 0.5 \times \left(\frac{{}^{44}\text{Ca}}{{}^{42}\text{Ca}} \right)_{\text{ICP-Ca-Lyon}}^{n+1}} - 1 \right) \times 1000$$

To facilitate comparison of our data set with other Ca isotope studies, the values obtained in this study are also expressed relative to the SRM-915a standard using a constant difference of $-0.518 \pm 0.025\%$ (2 SD).

3. Results

3.1. Calcium-normalized ratios and diagenesis

Four samples (R12, R18, R20, and R22) display significant REE enrichments together with a concomitant increase of the Fe/Ca ratio (SOM Fig. S3A) and Mn/Ca ratio (SOM Fig. S3B) but without any U enrichment (SOM Fig. S3C). The comparison of Ba/Ca ratios with REE, U/Ca, and Mn/Ca suggests a diagenetic Ba/Ca overprinting (SOM Figs. S3D and S4). The sample R11 seems to be highly impacted by Mn, U and Ba diagenetic alteration and uptake (SOM Figs. S3B–S3D). The Sr/Ca ratios seem insensitive to REE enrichment (SOM Fig. S3E), and no clear relationship is observed with the either Fe/Ca (SOM Fig. S3F) or the Mn/Ca (SOM Fig. S3G) ratio. None of the diagenesis-sensitive Ca-normalized ratios displays any correlation with the $\delta^{44/42}\text{Ca}$ values and the Ca/P ratios, when highly altered samples (i.e., R11–R12, R18, R20 and R22) are excluded (Fig. S4). Taken together, the results suggest that while the Ba/Ca ratios, and potentially the Sr/Ca ratios, are affected by some diagenetic alteration, the Ca isotopic compositions of specimens included in this study reflect original values and can be used to constrain the paleodiet of species retrieved at Regourdou. Note that the faunal pattern of the Sr/Ca ratio at Regourdou is similar to that reported for the Saint-Césaire assemblage (Balter et al., 2001, 2002a,b), which argues for a minimal diagenetic overprint of the Sr/Ca ratio at Regourdou.

3.2. Calcium isotope analyses

The observed linear correlation between the $\delta^{44/42}\text{Ca}$ and $\delta^{43/42}\text{Ca}$ values for both modern and fossil samples (SOM Fig. S5) agrees with the expected mass-dependent fractionation slope of -0.5 (Tacail et al., 2019). For modern samples, muscle always exhibits a higher $\delta^{44/42}\text{Ca}$ value than associated bone, whether cortical or trabecular (Fig. 1). Cortical and trabecular bone have similar $\delta^{44/42}\text{Ca}$ values, which are also similar to associated bone marrow in two of 4 cases (Fig. 1). For fossil samples, the $\delta^{44/42}\text{Ca}$ values of herbivorous species (with the exception of red deer) and wild boar are high in comparison with the other species, with values close to -1% (from -0.79 to -1.19% ; Fig. 2). The $\delta^{44/42}\text{Ca}$ values of herbivorous species are $\sim 0.6\%$ lower than the compiled above-ground biomass data of European grasses/herbs; the difference is smaller with the values of woody plants. Red deer exhibit much more negative $\delta^{44/42}\text{Ca}$ values (from -1.28 to -1.52% ; Fig. 2) overlapping with the range of values of carnivores, i.e. from -1.17 to -1.73% (Fig. 2). The Regourdou 1 samples display low $\delta^{44/42}\text{Ca}$ values, ranging from -1.51 to -1.67% (Fig. 2), placing the specimen in the ranges of carnivores. Despite different stratigraphic provenance, the $\delta^{44/42}\text{Ca}$ values of red deer from stratigraphic layer 4 are within the range of variation of those of red deer of stratigraphic layer 2, despite different MIS attributions. Similarly, the $\delta^{44/42}\text{Ca}$ values of brown bear from layer 2 fall in the range of variation of

those from layers 4–5 and 7. This suggests that the environmental impact on Ca isotopic composition is included in the intraindividual variability.

4. Discussion

4.1. The ecological and physiological significance of calcium isotopes in herbivores

Adding the $\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$ value to bone (about -0.6% ; Tacail et al., 2020) allows us to estimate the average $\delta^{44/42}\text{Ca}$ value of the animals' diets (SOM Table S1). The remodeling of the cortical bone is very slow once growth is complete (e.g. Manolagas, 2000), with an average estimation corresponding to the diet of the last years of the individual's life. For herbivores, the reconstructed diet would correspond to plants and plant organs, for which the distribution of the $\delta^{44/42}\text{Ca}$ values is known based on several studies and previously compiled data (Martin et al., 2018; Tacail et al., 2019, 2020) which are used in the present study. Some of the plant variability is known, but a continuing effort is necessary to fully depict the Ca isotopic variability in vegetal materials. Only two European Late Pleistocene assemblages have been analyzed isotopically (Martin et al., 2017a). The specimens from Sclayn (Namur, Belgium, from layer 1A, dated >36.2 kyr and 38.7 ± 1.5 kyr BP; Gilot, 1992) and Jaurens (Corrèze, France, dated from 29.7 to 32.6 kyr BP; Guérin et al., 1979) caves are more recent than those from Regourdou, but are nevertheless used here for comparison in this study.

Horses (*E. caballus*) at Regourdou exhibit a mean $\delta^{44/42}\text{Ca}$ value of -1.06% (± 0.01 , 2SD, $n = 2$; Fig. 2), identical to that obtained at Sclayn ($-1.06 \pm 0.36\%$, 2SD, $n = 2$; Martin et al., 2017a). Additional data on horses could challenge the apparent homogeneity of the $\delta^{44/42}\text{Ca}$ values at Sclayn and Regourdou and as a consequence, raise questions about the similarity of the dietary niches. The case of cervids raises a number of questions. Here, reindeer (*R. tarandus*) have relatively high $\delta^{44/42}\text{Ca}$ values (from -1.08% to -1.19%), while red deer (*C. elaphus*) exhibit much lower values (from -1.28% to -1.51%), characteristic of carnivores. The presence of antlers in cervids is likely to represent an additional and significant reservoir of Ca, although it remains to be demonstrated that antler formation (antlerogenesis) is associated with a species-dependent Ca isotope fractionation. In the absence of such information, we have considered that diet-specific behaviors have a prominent effect on the bone $\delta^{44/42}\text{Ca}$ value compared with antler formation. Reindeer and red deer are two herbivores whose diets are highly adaptable. Reindeer are mainly grazers and sometimes browsers that consume lichen (Martin, 1982), lichen having high $\delta^{44/42}\text{Ca}$ values (Fig. 2), while red deer are more browsers and tend to consume shrubs (Martin, 1982; Hofmann, 1989; Hearney and Jennings, 2009; Discamps, 2011), with the stem and trunk generally having the highest $\delta^{44/42}\text{Ca}$ plant values (Fig. 2). The assimilation of Ca through a non-nutritive source, such as bone or antler chewing, is likely to mimic bone consumption (Kierdorf, 1994; Cáceres et al., 2011). This would be consistent with the low $\delta^{44/42}\text{Ca}$ values of modern roe deer compared to other herbivores (Fig. 1). Similar dietary behavior could be at the origin of the observed low $\delta^{44/42}\text{Ca}$ values in red deer (Fig. 2).

4.2. The ecological and physiological significance of calcium isotopes in omnivores

The wild boar (*Sus scrofa*) is the species with the highest average $\delta^{44/42}\text{Ca}$ value ($-0.89 \pm 0.28\%$, 2SD, $n = 2$) in the Regourdou faunal assemblage (Fig. 2). The main components of its current diet include rhizomes, acorns and grains (Fournier-Chambrillon et al., 1995). The consumption of buried tubers can be accompanied by

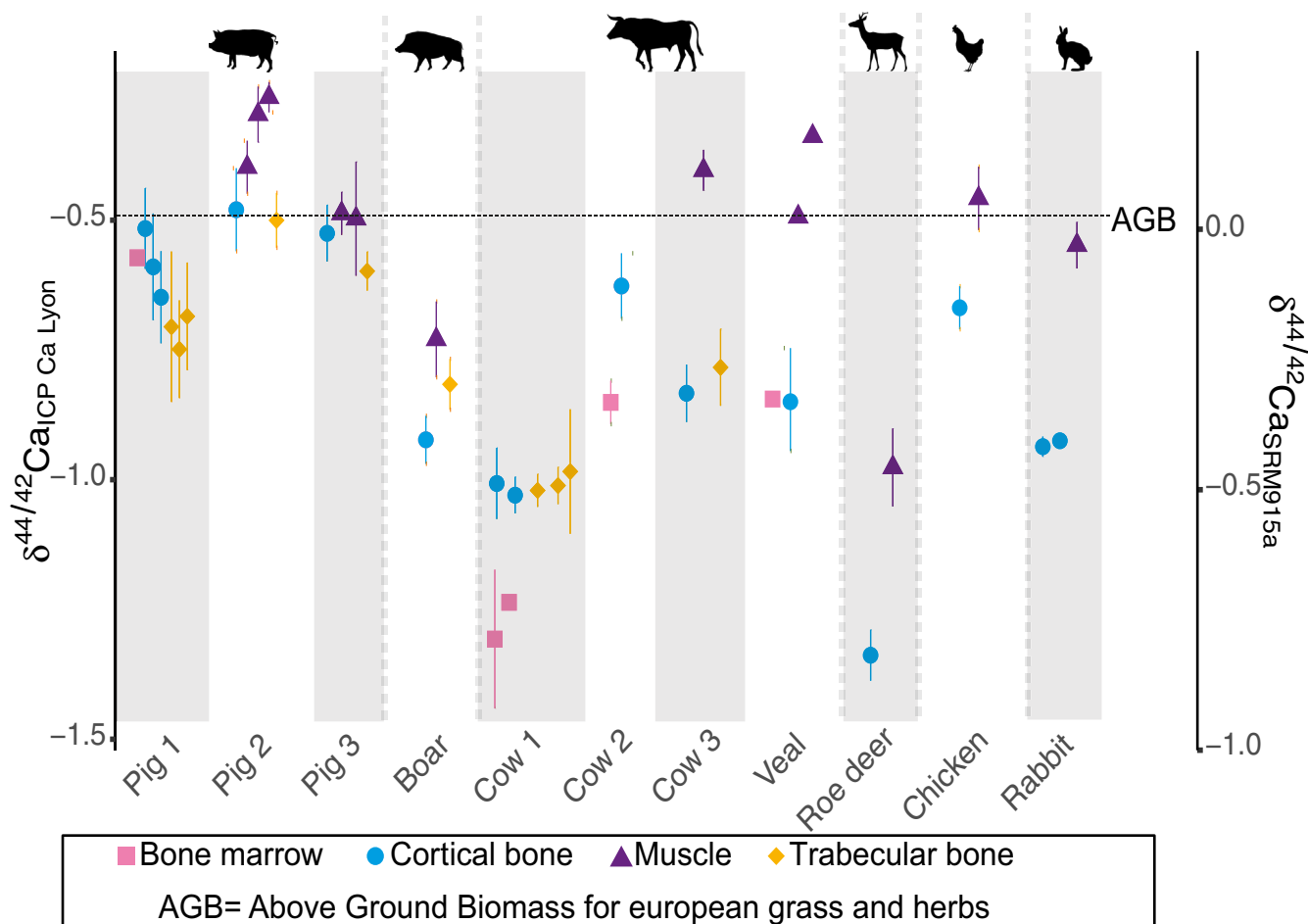


Figure 1. $\delta^{44/42}\text{Ca}$ values for modern samples (‰, relative to ICP Ca Lyon standard and SRM 915a). Individuals are grouped by species. The shape and colors correspond to the different sampled hard or soft tissues. The above-ground biomass (AGB) line is the mean of AGB for European grass and herbs compiled from Skulan and DePaolo (1999), Chu et al. (2006), Hindshaw et al. (2013), Moore et al. (2013), Christensen et al. (2018). All silhouettes are taken from www.phylopic.org (Public Domain license). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the ingestion of soluble soil particles which have an isotopic composition that is close to zero permil (Tacaïl, 2017). Fruits and vegetables have generally high $\delta^{44/42}\text{Ca}$ values (Tacaïl et al., 2019, 2020). The consumption of grains has not been considered in the present study as it is mainly related to agricultural expansion in the Neolithic. Despite a lack of information on the paleoecology of *Sus scrofa*, high $\delta^{44/42}\text{Ca}$ values in fossil wild boar at Regourdou seem to be in good agreement with current knowledge on the diet of modern boars (Fournier-Chambrillon et al., 1995).

The brown bears (*U. arctos*) display a large variability of their $\delta^{44/42}\text{Ca}$ values, ranging from 0.9‰ to -1.3 ‰ (SOM Table S1). This can be explained by their highly variable and seasonally omnivorous diet (Robu et al., 2013). The cave bears (*Ursus spelaeus*) at Sclayn (Martin et al., 2017a) do not exhibit such a high Ca isotopic variability because their dietary niche was more restricted than for brown bears. Tooth shape and N isotope suggest an herbivorous diet for this taxon (Bocherens et al., 1994; Pacher and Stuart, 2009). In extant brown bears, meat consumption is described as highly variable depending on the resource (Pacher and Stuart, 2009). Other scavenging behaviors, including necrophagia following hibernation, and bone gnawing, which can be associated with direct bone ingestion, are also reported. The Ca isotope compositions of trabecular and cortical bone of the brown bear (sample D2-33; Fig. 2) support a highly variable diet, their $\delta^{44/42}\text{Ca}$ values being distinct by about 0.3‰ (Fig. 2), indicating varying Ca dietary sources

during the period of bone formation. Indeed, the turnover is different between trabecular and cortical bone. Patterns of bone remodeling among mammals other than humans, nonhuman primates, or laboratory rodents are poorly documented but are always faster for trabecular than cortical bone. For humans, the bone remodeling rate has been reported to be 25% per year for trabecular bone compared with 3–4% per year for cortical bone (Manolagas, 2000). In addition, hibernation periods could have an impact on the bone Ca isotopic composition. Hibernation induces a physiological period of reduced activity associated with a quantitative turnover of the urea cycle (Barboza et al., 1997). Because the Ca fractionation is most likely driven by renal physiology (Tacaïl et al., 2020), a possible specific isotopic fractionation could be induced by hibernation. However, the inanition period due to hibernation does not induce bone loss (McGee-Lawrence et al., 2015). Thus, there is little evidence showing that Ca isotope compositions of bone will be affected by hibernation. Collectively, their dietary and hibernation behavior might explain the high Ca isotopic variability observed in brown bears.

4.3. The ecological and physiological significance of calcium isotopes in carnivores

The range of $\delta^{44/42}\text{Ca}$ values among carnivores (~ 0.6 ‰) is almost as great as among herbivores (~ 0.8 ‰), suggesting highly variable

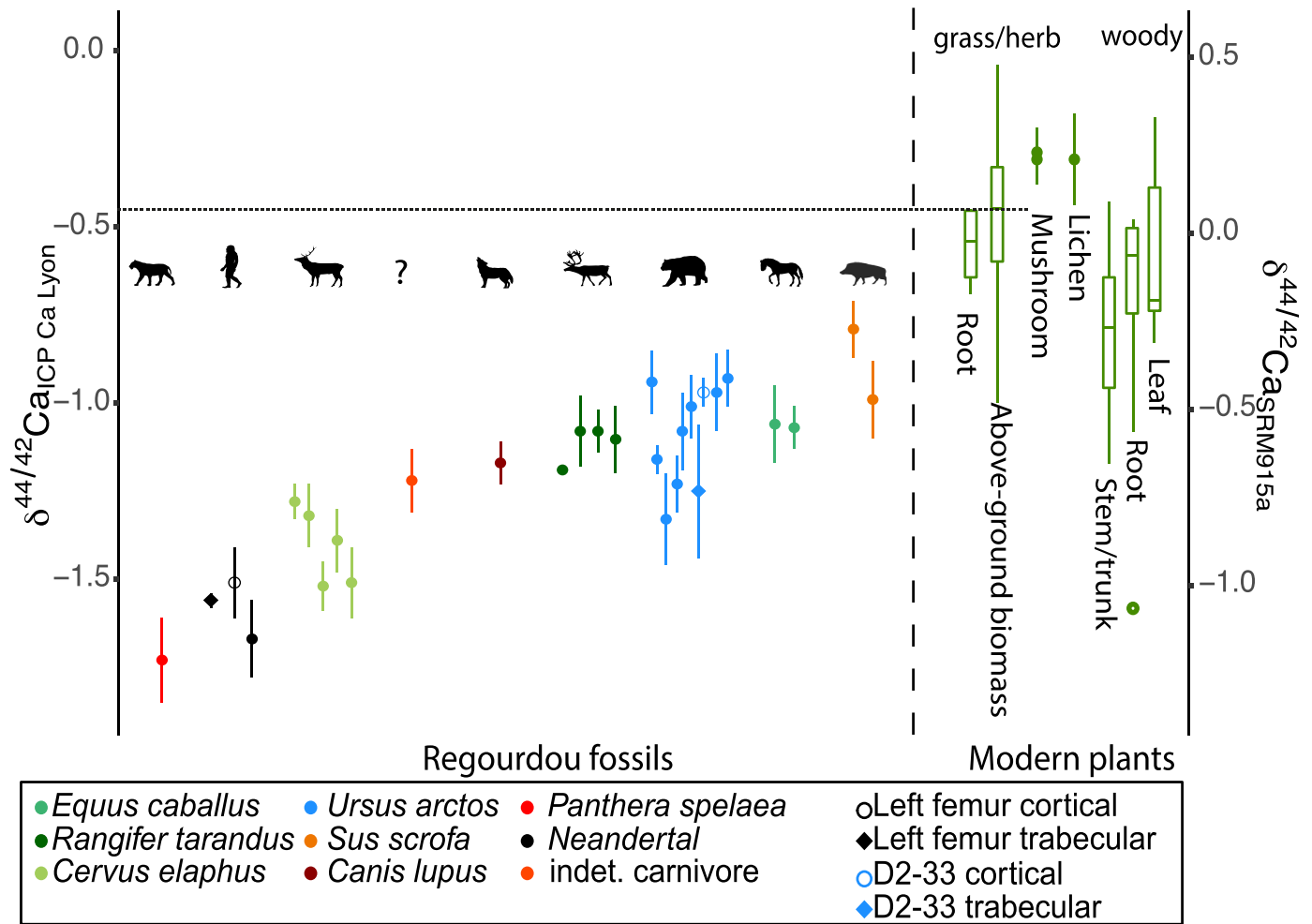


Figure 2. On the left, fossil mammal $\delta^{44/42}\text{Ca}$ values (‰ relative to the ICP Ca Lyon standard and SRM915a). Species are ranked from left to right according to their increasing average $\delta^{44/42}\text{Ca}$ values. All samples were measured at least three times; the typical error bar represents the largest 2SD. All silhouettes are taken from www.phylopic.org (Public Domain license). On the right, distribution of isotope composition of current European plants, compiled from Skulan and DePaolo (1999), Schmitt et al. (2003), Chu et al. (2006) Page et al. (2008), Bagard et al. (2013), Hindshaw et al. (2013), Moore et al. (2013), and Christensen et al. (2018).

sources of Ca for carnivores. The diet of carnivores is mainly based on meat, i.e., mostly muscle, with possible incorporation of plant matter in smaller quantities (Van Valkenburgh, 1996; Stanek et al., 2017; Gable et al., 2018). Information on the variability of muscle $\delta^{44/42}\text{Ca}$ values is presently not as robust as for plants. Pairing bone and muscle from a given specimen, considering our values and those compiled from the literature (Skulan and DePaolo, 1999; Tacaïl et al., 2014), allows us to calculate an average $\Delta^{44/42}\text{Ca}_{\text{bone-muscle}}$ offset value of -0.32‰ (± 0.12 ; 2SE; $n = 14$; Fig. 3). This value complements the spacing between the $\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$ offset value of -0.6‰ (Tacaïl et al., 2020) and the trophic level effect between bones of carnivores and herbivores of -0.3‰ (Tacaïl et al., 2020). Adding 0.3‰ from the bone $\delta^{44/42}\text{Ca}$ value of herbivores matches the diet $\delta^{44/42}\text{Ca}$ value of carnivores.

Wolves (*C. lupus*) are carnivores but have a generalist diet (Stanek et al., 2017; Gable et al., 2018) being occasional consumers of fish, rodents and berries (Stanek et al., 2017; Gable et al., 2018). The Ca isotopic composition of fish (marine only as there are no data on freshwater fish) and berries is close to 0‰ (Tacaïl, 2017; Tacaïl et al., 2020), and prey such as wild boars and brown bears would explain the relatively high $\delta^{44/42}\text{Ca}$ value of wolf. This holds for the indeterminate carnivore too, which might be a wolf. The

cave lion (*P. spelaea*) has the lowest Ca isotopic composition (-1.73‰) which aligns well with the low Ca isotopic composition of felidae measured by Martin et al. (2018) in the modern ecosystem of Tsavo (Kenya). These low $\delta^{44/42}\text{Ca}$ values evoke a monospecific diet for cave lions as already suggested by C and N isotopes (Bocherens et al., 2011) and could be explained at Regourdou by a diet mainly based on red deer and/or other species with low $\delta^{44/42}\text{Ca}$ values, although this must remain speculative as none of these other species are represented in our faunal assemblage.

4.4. The diet of the Regourdou 1 Neandertal

The Regourdou 1 samples display low $\delta^{44/42}\text{Ca}$ values ($-1.58 \pm 0.16\text{‰}$; 2SD; $n = 3$), placing the specimen amid carnivores and supporting the commonly accepted idea that Neandertals mainly relied on meat consumption. Applying a $\Delta^{44/42}\text{Ca}_{\text{bone-muscle}}$ ($-0.32 \pm 0.06\text{‰}$; SOM Table S1) offset value to bone of herbivores to evaluate their muscle $\delta^{44/42}\text{Ca}$ value, and a $\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$ (-0.6‰ ; SOM Table S1) offset value to the Neandertal, allows us to obtain the theoretical $\delta^{44/42}\text{Ca}$ value of the Regourdou 1 diet. Results of this estimate show a diet based on red deer meat and potentially brown bear, wolf and reindeer meat. Although rare, cut marks and

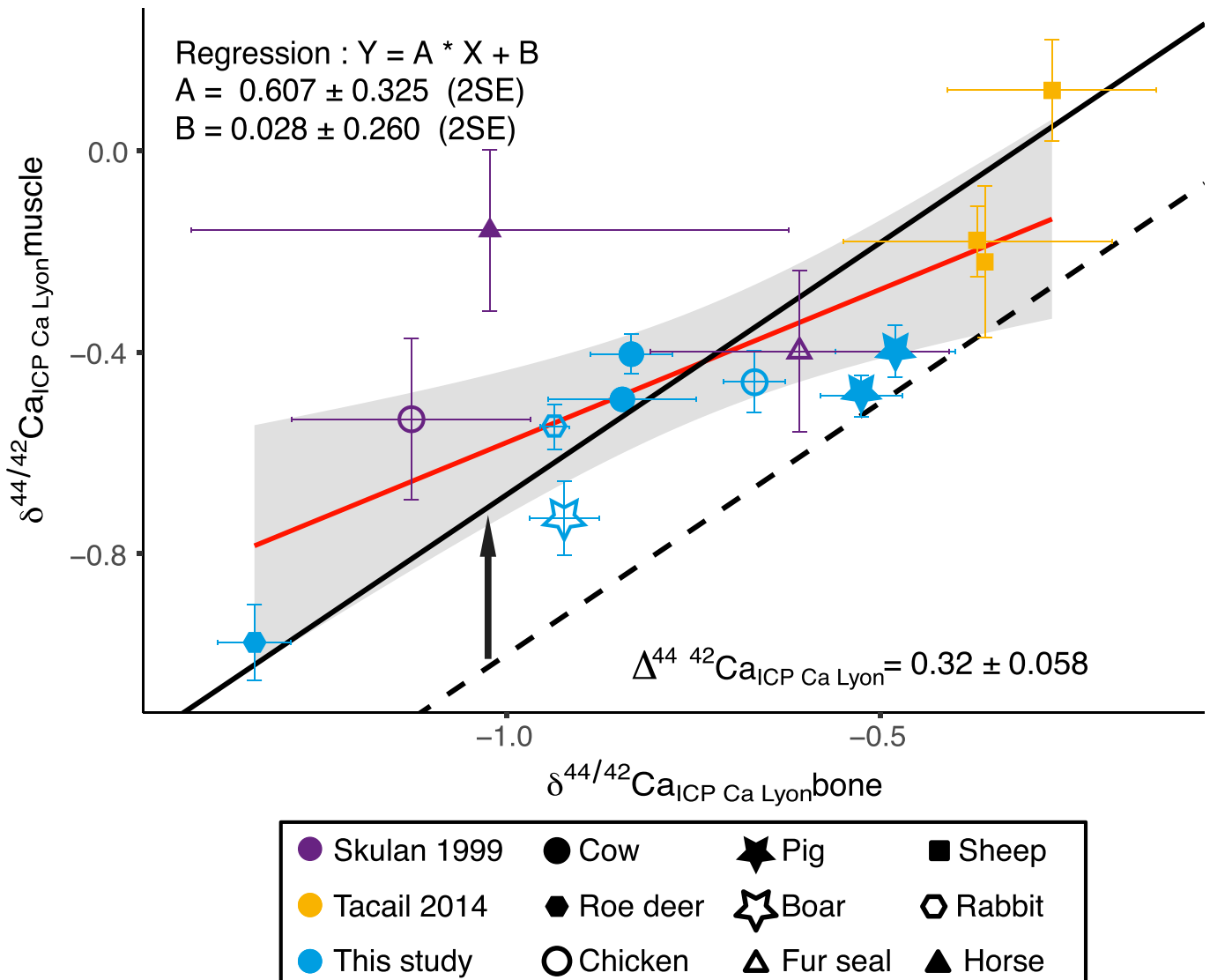


Figure 3. Bone $\delta^{44/42}\text{Ca}$ values versus muscle $\delta^{44/42}\text{Ca}$ values (‰, relative to ICP Ca Lyon standard). The red line corresponds to the regression line (slope = 0.607 ± 0.325 ; 2SE, intercept = 0.028 ± 0.260 ; 2SE) calculated for all samples analyzed in this study and compiled from the literature (Skulan and DePaolo, 1999; Tacail et al., 2014). The gray area represents the 95% confidence interval for the linear regression. The dotted line is 1:1 and the black line represents the straight line of the equation $y = x + 0.32$, corresponding to the average offset between cortical bone and its paired muscle. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

anthropic fractures of bones of reindeer, red deer and brown bear bones support this estimation (Cavanhié, 2009–2010). Reindeer consumption is excluded because they are only present in layer 2 and absent from the environment of Regourdou 1. Although possible consumption or exploitation of carnivores has been documented at Mousterian sites (Auguste, 1995; Costamagno et al., 2009; Gómez-Olivencia et al., 2018), the generalized consumption of carnivores such as brown bear and wolf seems unlikely given the difficulties of hunting carnivores and the generally low abundance of carnivores in the faunal spectrum of anthropic accumulation. The specialized consumption of red deer is consistent with the C and N isotope studies that have placed Neandertals at the top of the trophic chain and with a dietary preference for large herbivores (Bocherens et al., 2001; Naito et al., 2016).

The fact that the three Neandertal bone samples have similar $\delta^{44/42}\text{Ca}$ values is consistent with the bones belonging to the same individual (Madelaine et al., 2008; Gómez-Olivencia et al., 2013; Maureille et al., 2015b). It is interesting to note, however, that the

$\delta^{44/42}\text{Ca}$ value of the trabecular bone sample ($-1.56\text{‰} \pm 0.02$; 2SD) has a $\delta^{44/42}\text{Ca}$ value that is undistinguishable from that of the cortical bone sample ($-1.51\text{‰} \pm 0.10$; 2SD), contrasting with the large difference for the brown bear. Considering that cortical and trabecular bone tissues have different rates of turnover (Manolagas, 2000), this indicates a relatively stable diet at Regourdou 1 during the time of remodeling of cortical bone and that of trabecular bone. Pairing cortical and trabecular modern bone samples across animal species (Fig. 1; SOM Table S2 and SOM Fig. S6) shows that both tissues have very close $\delta^{44/42}\text{Ca}$ values. This is not surprising because all of these animals, with the exception of wild boars and roe deer, were fed on farms with presumably similar diets. Future studies measuring trabecular and cortical bone from wild mammals will be more informative for testing the existence of possible isotopic heterogeneities that may be characteristic of seasonal dietary adaptations. This could lead to a renewed interest in isotopic signatures of trabecular bone, which reflect recent variation in diet due to its higher rate of remodeling compared with cortical bone

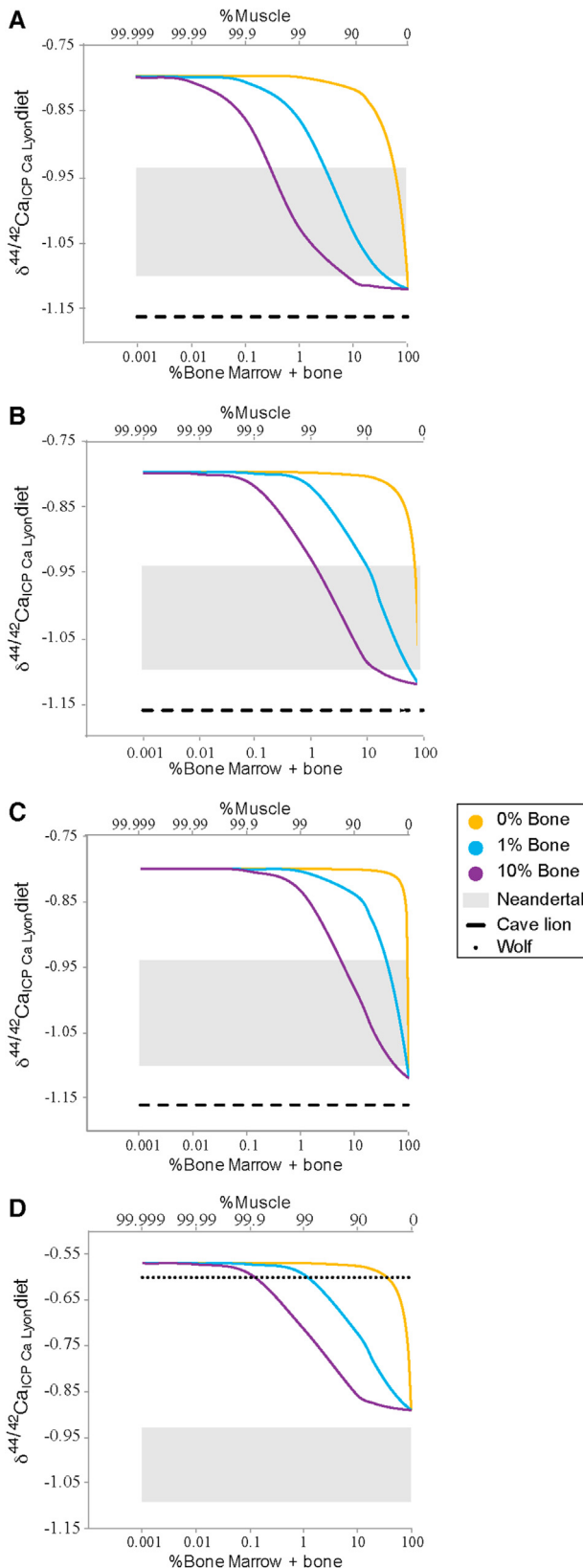


Figure 4. Dietary $\delta^{44/42}\text{Ca}$ values (‰, relative to ICP Ca Lyon) as a function of the amount of bone marrow and bone (%) relative to meat, as calculated with a mixed model similar to Heuser et al. (2011). The yellow curve is for 0% by weight of bone in the marrow, the blue curve is for 1% by weight of bone in the marrow and the purple curve is for 10% by weight of bone in the marrow. The gray area represents the expected range of diet $\delta^{44/42}\text{Ca}$ values for the Regourdou 1 Neandertal individual. The

(Manolagas, 2000), contributing further to our understanding of variation in dietary and hunting behavior.

The great asymmetry between the right and left human femurs found in the Regourdou site raises the possibility that at least two individuals were present (Madelaine et al., 2008; Maureille et al., 2015a). The similarity of the $\delta^{44/42}\text{Ca}$ and the Sr/Ca values of the three samples from the left femur and the left humerus of the Regourdou 1 Neandertal argue for a single individual or implies that both individuals had the same diet. However, there is currently no data on the variability of the Ca isotopic composition for humans at the individual level.

4.5. Calcium isotope trophic shift due to bone consumption

The use of the abovementioned $\Delta^{44/42}\text{Ca}_{\text{bone-muscle}}$ and $\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$ offset values suggest a highly specialized diet, based primarily on red deer, for the Regourdou 1 Neandertal. This monospecific regime is dubious from the standpoint of hunting strategy and other taxa must have been consumed as well (Delpech and Grayson, 2007). Another component needs to be considered to match the very negative $\delta^{44/42}\text{Ca}$ values of the cave lion and the Neandertal. Bone marrow and bone are the most likely candidates (Heuser et al., 2011) and would be consistent with the bone fracture pattern observed at Mousterian sites (Costamagno, 2013; Costamagno and Rigaud, 2013). Using the data obtained on associated bone, bone marrow, and muscle (Fig. 11; SOM Table S2), it is possible to estimate the diet $\delta^{44/42}\text{Ca}$ value resulting from a mixture of meat and bone or bone marrow (Fig. 4).

The $\delta^{44/42}\text{Ca}$ value of the dietary source was estimated using a simplified mixing model and compared with the average isotopic composition of the main diet source of Ca of Regourdou 1 ($-1.01\text{‰} \pm 0.16$; 2SD) illustrated by the grayed area in Figure 4. We used the minimum, maximum and average Ca concentrations in muscle ($[\text{Ca}]_{\text{min}} = 163 \text{ ppm}$, Fig. 4A; $[\text{Ca}]_{\text{mean}} = 633 \text{ ppm}$, Fig. 4B; $[\text{Ca}]_{\text{max}} = 3228 \text{ ppm}$, Fig. 4C); on a diet composed with equal proportions of horse, red deer and wild boar, mixed with marrow containing different proportions of bone (0%, 1% and 10% of weight). Changes in the respective contributions of these three taxa will not substantially change the conclusion but will slightly affect the proportion of bone required to explain the Regourdou 1 diet. The consumption of trabecular bone in small proportion (only 0.1 or 1%, being accidental or related to the hominin diet; Costamagno and Rigaud, 2013) during marrow consumption, can explain the isotopic composition of Regourdou 1 and greatly limits the contribution of marrow (<10%) and monospecific hunting. A mixed diet including bone marrow and accidental bone consumption is also probable for the lion. Its lower $\delta^{44/42}\text{Ca}$ value compared with Regourdou 1, and its stratigraphic position in layer 2, however, suggests a higher proportion of red deer and the presence of reindeer in the diet (Fig. 4A–C). In contrast, the $\delta^{44/42}\text{Ca}$ values of the wolf and the indeterminate carnivore $\delta^{44/42}\text{Ca}$ values are incompatible with a mixed diet made up of red deer (Fig. 4A–C). The consumption of prey like boar, brown bear or prey with similar isotope compositions, together with the absence of bone and low marrow ingestion, are more in agreement with the

dashed line represents the expected dietary isotopic composition of the lion. A concentration of 100 ppm of Ca has been used for marrow and a concentration of 40% of Ca for bone. (A) Mixed diet with equal proportion of horse, red deer, and wild boar meat with 163 ppm of Ca. (B) Mixed diet with equal proportion of horse, red deer and wild boar meat with 633 ppm of Ca. (C) Mixed diet with equal proportion of horse, red deer, and wild boar meat with 3228 ppm of Ca. (D) 100% boar diet, meat with 633 ppm of Ca. The average of each species is used for the calculation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

bone $\delta^{44/42}\text{Ca}$ values of the wolf but not the Regourdou 1 Neandertal (Fig. 4D).

Based on the state of our current knowledge, the Ca isotopic signatures do not allow us to differentiate between meat and plant consumption. As plants and meat have similar $\delta^{44/42}\text{Ca}$ values, bone consumption thus appears to be the key parameter explaining the generalized decrease in food chains of the $\delta^{44/42}\text{Ca}$ values between herbivores and carnivores. The consumption of entire animal prey implies de facto the ingestion of a large part of the skeleton, which is partly digested in the digestive tract of the predator (Massare, 1987; Baquedano et al., 2012). Regarding large mammal carnivores (such as hyena, wolf or lion), the prey is rarely ingested all at once, but bony parts are most often accidentally ingested by the predator along with muscle and organs. For humans, negative $\delta^{44/42}\text{Ca}$ values will be observed in the case of accidental bone consumption. Archaeological evidence suggests that the ingestion of some trabecular bone during marrow consumption is the most likely explanation. However, the ingestion of other bone parts due to cutting, gnawing or for any social/culinary aspects cannot be excluded. Plant cannot be excluded from the Neanderthal diet (Henry et al., 2011; Hardy et al., 2012) and could replace meat in any proportion, the only constraint being that about 1–10% of the diet must be composed of bone marrow (Fig. 4A–C).

5. Conclusions

The use of Ca isotopes allows for the first time the dietary reconstruction of a MIS 5 Neandertal fossil from Southwest of France, Regourdou 1, in the absence of collagen. We show that the Ca isotopic compositions are not affected by diagenesis despite postmortem diagenetic alteration of trace elements (REE, Ba, U, and Mn) of the fossil bones. The results show that the Regourdou 1 Neandertal was predominantly carnivorous, consuming a mixture of various herbivorous prey along with the associated consumption of trabecular bone likely occurring during the ingestion of marrow. The same conclusion can be drawn for the cave lion, with a higher proportion of red deer or other prey with similar $\delta^{44/42}\text{Ca}$ values not preserved at Regourdou. In contrast, we show that the wolf and the indeterminate carnivore have a different diet, more enriched in heavy Ca isotopes. The $\delta^{44/42}\text{Ca}$ values of the fauna exhibit an ecological patterning that awaits further elucidation. Some explanations will probably come from a better knowledge of plant $\delta^{44/42}\text{Ca}$ variability, notably in fruits and nuts. Other explanations will arise from an improved understanding of the physiological processes involving Ca metabolism, e.g., kidney function in hibernating species and the influence of rumination and antlerogenesis.

The two main advantages of Ca isotopes over the use of isotopic ratios of trace elements are that the diagenesis of Ca isotopes is unlikely to occur, allowing for the analysis of trabecular bone, and the fact that minute amounts of sample are sufficient for the measurement of the Ca isotopic composition, allowing for collection of material without observable damage to bone and teeth. The potential for renewed interest in trabecular bone isotopic signatures lies in the fact that it could carry information about seasonal dietary changes when studied in comparison with cortical bone. Our preliminary results of cortical versus trabecular bone Ca isotope differences for brown bears suggesting dietary flexibility must be confirmed by further studies on other omnivorous species. Collectively, these advantages are likely to turn Ca isotopes into an essential interpretative tool for studying the evolution of diet in the human lineage.

Declaration of competing interest

The authors declare no conflict of interest.

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VB and BM designed the project. PJD, TT, and EA designed the isotopic analysis. BM, SM, and AGO helped for sampling. BM, TH, and AGO participated to the global study of Regourdou 1 Neandertal. PJD, BM, and VB wrote the manuscript with contributions from AGO, JEM, and TT.

Supplementary Online Material

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