Diverse bone-calcium isotope compositions in Neandertals suggest different dietary strategies

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ABSTRACT

Zooarcheological and geochemical evidence suggests Neanderthals were top predators, but their adherence to a strictly carnivorous diet has been questioned. Recent studies have demonstrated the potential of calcium-stable isotopes to evaluate trophic and ecological relationships. Here, we measure the $\delta^{44/42}$Ca values in bone samples from Mousterian contexts at Grotte du Bison (Marine Isotope Stage 3, Yonne, France) and Regourdou (Marine Isotope Stage 5, Dordogne, France) in two new Neanderthal individuals, associated fauna, and living local plants. We use a Bayesian mixing model to estimate the dietary composition of these Neanderthal individuals, plus a third one already analyzed. The results reveal three distinct diets: a diet including accidental or voluntary consumption of bone-based food, an intermediate diet, and a diet without consumption of bone-based food. This finding is the first demonstration of diverse subsistence strategies among Neanderthals and as such, reconciles archaeological and geochemical dietary evidence.

1. Introduction

The recent first analysis of calcium-stable isotope composition ($\delta^{44/42}$Ca) of Neanderthal remains (Dodat et al., 2021) illustrated how Ca-stable isotopes can be used to reconstruct dietary habits of Neanderthals. The results of this study agreed with traditional isotopic data (primarily nitrogen) previously obtained on Neanderthal remains (Balter and Simon, 2006; Naito et al., 2016; Wißing et al., 2016): specifically, the Regourdou 1 individual has a carnivorous diet that must have included a significant proportion of bone or bone marrow (Dodat et al., 2021). Data show that Neanderthals were highly competent hunter-gatherers; a primarily meat-based diet raises the conundrum of a diet potentially lacking essential nutrients. In nutritional terms, the consumption of a protein-based diet is an effective way to provide energy to the body but is also a diet that lacks many essential nutrients, vitamins, or carbohydrates (Hardy, 2010), creating potential deficiencies that could impact fertility, fetal mortality, or exposure to kidney failure (Fiorenza et al., 2015). In fact, humans cannot tolerate a diet composed of more than 35–40% protein no matter its origin (animal or vegetal; Cordain et al., 2000; Hardy, 2010; Fiorenza et al., 2015). Ethnographic studies have shown that if hunter-gatherers obtain more than 50% of their energy from animal sources (Cordain et al., 2000), the consumption of animal fat containing little, or no protein, limits the toxicity of such a diet. Under these conditions, the remaining energy is provided by vegetal sources (Cordain et al., 2000; Fiorenza et al., 2015). Taking these metabolic arguments into account, it is unlikely that Neanderthals had a diet of ca. 100% (primarily ungulate) meat. Rather, a proportion on the order of 60–70% of the energy coming from animal sources (meat and fat) would better fit metabolic and ethnographic data (Cordain et al., 2000).

With over 40 analyzed Neanderthal remains, results of nitrogen isotopes' research argue that there was notable homogeneity in the

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Neanderthal diet, displaying a preference for consuming large herbivores such as horse, reindeer, red deer, bovids, rhinoceroses, and mammoth (e.g., Balter and Simon, 2006; Bocherens, 2013; Naito et al., 2016; Wißing et al., 2016). This dietary preference aligns with evidence from zooarchaeology, bone accumulation, and anthropic marks on faunal remains (e.g., Patou-Mahtis, 2000; Costamagno et al., 2006; Hublin and Richards, 2009; Martin et al., 2017). Nonetheless, recent methodological developments such as dental calculus studies now allow us direct analysis of diet and reveal the consumption of a large assortment of plants by Neanderthal (Henry et al., 2011; Weyrich et al., 2017; Hardy, 2022). Additionally, recent discoveries at the Figueira Brava site on Portugal's Atlantic coast have even painted a picture of a very broad food spectrum for Neanderthals, including terrestrial (animal and vegetable) and marine resources (Zilhão et al., 2020).

Stable Ca-isotope compositions ($\delta^{44/42}$Ca) are one proxy for studying Neanderthal diet (Tacail et al., 2020; Dodat et al., 2021) mainly used to detect consumption of an enriched Ca source such as bone or milk. Unfortunately, it cannot evaluate the proportion of consumed animal soft tissues versus plant material because the $\delta^{44/42}$Ca value of these two components is similar (Tacail et al., 2019). The Ca-isotope composition is however an efficient dietary proxy when applied to predators consuming whole prey, because bone, with its extremely negative $\delta^{44/42}$Ca value, is eaten along with the soft edible parts (Martin et al., 2015; Hassler et al., 2018), resulting in a more negative $\delta^{44/42}$Ca value of the consumer relative to the prey. The situation becomes more complicated in mammals because medium- to large-sized predators do not ingest bone deliberately, except for hyenas and, to a lesser degree, canids (Skulan and DePaolo, 1999; Reynard et al., 2010; Heuser et al., 2011; Clementz, 2012; Martin et al., 2017, 2018). Bone and bone marrow have similar $\delta^{44/42}$Ca values, but because of the distinct Ca concentrations of bone marrow, meat, and fresh bone (0.01%, 0.6% and 20%, respectively), a diet with a negative $\delta^{44/42}$Ca value is indicative of accidental or voluntary bone consumption (Reynard et al., 2010; Heuser et al., 2011; Martin et al., 2017, 2018; Dodat et al., 2021). The archaeological evidence suggests that the ingestion of some trabecular bone during yellow marrow consumption, or via other culinary practice is the most likely hypothesis to explain bone consumption among human populations (Fiorenza et al., 2015; Morin, 2020a).

Calcium is the main cationic constituent of hydroxylapatite (~40% weight), the primary mineral component of bone and dental tissues. Diagenesis has been recognized for decades to be pervasive for trace elements in fossil hydroxylapatite (e.g., Trueman and Tuross, 2002; Kohn and Moses, 2013). Trace elements generally accumulate in secondary minerals (usually calcite) or ultimately, during hydroxylapatite recrystallization subsequent to a Ca substitution process. However, the diagenesis of Ca should not be as pervasive as it is for trace elements for two main reasons. First, mass-balance calculations between a fossil bone or tooth and the diagenetic soil water predict only limited diagenesis because the Ca concentration in ground water is negligible relative to that in hydroxylapatite or because marked diagenesis would require unrealistic groundwater $\delta^{44/42}$Ca values (Martin et al., 2017). Second, the diagenetic Ca in fossil bone and dental tissues can be removed via leaching protocols based on the higher solubility of calcite relative to hydroxylapatite (Sillen and LeGeros, 1991; Lee-Thorp and van der Merwe, 1991; Balter et al., 2002). Therefore, the existence of remaining secondary Ca after a leaching protocol is unlikely, unless diagenetic Ca had been incorporated into the hydroxylapatite during recrystallization (Dodat et al., 2023). Beyond the potentially strong resistance to diagenesis of bone Ca isotopes, the fact that Ca is the main cationic constituent of hydroxylapatite offers a second advantage—a very small sample

requirement. Calcium-isotope compositions are routinely measured at 1.5 μg/mL on the multicollection inductively coupled plasma mass spectrometer (MC-ICP-MS). Three runs are generally performed, and leaching usually induces an initial sample mass loss of 30−50%, so theoretically, ca. 20 μg of bone sample is sufficient for processing. However, in practice, such a tiny amount of material cannot be handled during sample preparation. Therefore, about 200 μg of material—an amount barely visible to the naked eye—can be obtained using microsampling techniques (Tacail et al., 2019) and is easier to handle. This comes, however, with a potential flaw, which is the possible significant variability of the Ca isotope composition within mineralized tissues. Fortunately, one can take advantage of this susceptibility to variation when working with tooth enamel because the position of the microsampling can be controlled and reported as a function of dental age and because enamel does not remodel. Tacail et al. (2019) for instance use such dental age reconstructions to study the age at weaning of South African hominins. When using bone, it is not possible to infer the age at which bone is formed and eventually remodeled, and a tiny sample amount of about 1 mg (which is the typical size used in the present study) might record only a small fraction of an individual lifetime, which may not be representative of its overall diet. Thus, we are faced with the persistent issue that, in order to get a certain degree of reproducibility for an averaged value, a larger sample size is required the more heterogeneous a material is. A substantial scattering of the Ca-isotope compositions is therefore expected, which will depend on lactation, gestation, dietary shift, species-specific metabolic trait, or a combination thereof (Tacail et al., 2020).

This study presents Ca-isotope compositions measured on a Neanderthal individual and eleven different faunal taxa from the Grotte du Bison archaeological site in Bourgogne (Yonne, France) and on a second Neanderthal individual, two newly analyzed faunal taxa and fauna from the Regourdou site in Black Périgord (France; Dodat et al., 2021). We also consider another Neanderthal individual and fauna from the Regourdou site already analyzed in 2021 (Dodat et al., 2021) and then characterize the diet of these three Neanderthals using Bayesian mixing models for stable isotope analyses in R (MixSIAR) based on the measured $\delta^{44/42}$Ca values.

2. Materials and methods

2.1. Grotte du Bison fossil material

The Grotte du Bison belongs to a larger group of prehistoric caves at Arcy-sur-Cure, which forms a network of nearly 4 km, with a surface area of about 60 ha (Fig. 1A; Baffier and Girard, 1997). The site of Grotte du Bison is one of the most recently discovered in the history of the excavated sites caves of Arcy-sur-Cure. It was identified in 1958 by P. Poulain and was excavated for the first time in 1959 by him and members of A. Leroi-Gourhan's scientific team until 1963 (Leroi-Gourhan, 1961). It was later excavated by F. David (1995−2008) and finally, by one of us (M.H.) from 2011 to 2019. The excavation campaigns resulted in discovery of at least 49 to 50 Neanderthal remains (40 isolated teeth, two maxillary fragments, six skull fragments, and two/one postcranial piece/s belonging to at least nineteen different individuals considering their archaeological and spatial distribution). We note also existence of four partially digested teeth (potentially humans; Maureille et al., 2017) in two (I and J) of the lower sedimentological layers, attributed to a late Mousterian period. Following Tillier et al. (2013), two accelerator mass spectrometry radiocarbon dates place the layer I between 51 519−44 120 and 47 951−42 658 BC. Other chronological correlations of the Grotte du Bison layers are available in Roblin-Jouve et al. (2018). In 2019, an incomplete right fibula diaphysis
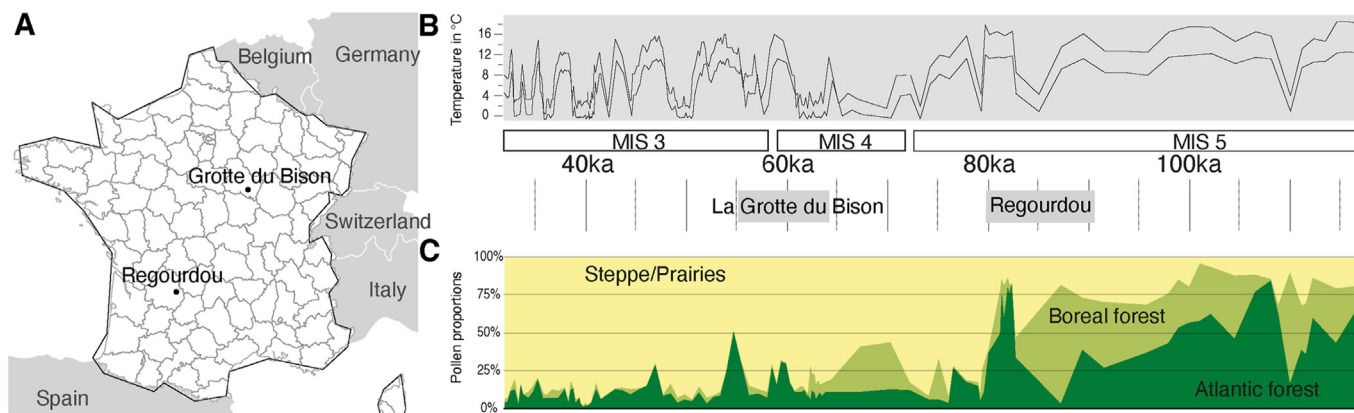
Figure 1. Correspondence between the different sites studied and palaeoclimatic and paleoenvironmental records of core MD04-2845 (Gascogne Bay) for the period 140−35 ka (Sánchez Goñi et al., 2008; Discamps, 2011). A) Location of the different sites; B) surface-water temperature in summer and winter (in °C) and absolute chronology with the different sites studied; and C) Pollen proportions according to biotope type (expressed in %).

attributed to a mature Neanderthal individual was recovered in layer J, which is related to the beginning of Marine Isotope Stage (MIS) 3. This is the specimen we sampled. The presence in layers I and J of *Crocuta crocuta* and an unusually low carnivore-to-ungulate ratio together suggest that the faunal assemblage in these layers is not the result of accumulation by hyena alone but also by Neanderthal (Pothier Bouchard, 2016). Among the three most represented herbivore taxa, at least two (*Rangifer tarandus* and *Equus caballus*) show anthropic (e.g. cut mark, fresh bone breaks) traces suggesting consumption by Neanderthals. The accumulation of bovine bone is thought to be due to *Crocuta crocuta* (Pothier Bouchard, 2016).

2.2. Regourdou fossil material

The Regourdou cave is located at the top of Lascaux hill (Fig. 1A; Montignac-Lascaux, Dordogne, France). 'Excavations' at Regourdou began in 1954 at the landowner's (R. Constant) initiative. During the night of the 22nd to the September 23, 1957, part of a Neanderthal skeleton (Regourdou 1) was discovered under unprofessional circumstances (Maureille et al., 2013; Pelletier et al., 2017). A salvage excavation led to the discovery of some faunal and lithic remains and a set of fragments and complete bones belonging to one Neanderthal specimen, Regourdou 1 (Bonifay, 1965; Madelaine et al., 2008). The Regourdou 1 partial skeleton was recovered from Bonifay's stratigraphic layer 4 (Bonifay, 1965), which contained Discoid Mousterian lithics with the production of pseudo-Levallois points and a temperate fauna dominated by brown bears (*Ursus arctos*). Based on lithic and faunal remains, layer 4 was attributed to the first part of MIS 5 (Bonifay, 1965; Pelletier et al., 2017). Regourdou 1 skeletal conservation and preservation were interpreted as a Neanderthal burial since its discovery (Piveteau, 1959; Maureille et al., 2016; Pelletier et al., 2017). An isolated right human calcaneus, belonging to the Regourdou 2 Neandertal fossil was found near the wall opposite to the Regourdou 1 burial, and it is presumed that Regourdou 2, as Regourdou 1, came from layer 4 (Madelaine et al., 2008). Regourdou 2 lacks any archeologic context, and despite its discovery as early as 1963, it was not studied in detail until recently (Coutinho Nogueira et al., 2017), although the presence of two adult Neanderthal individuals was long suspected based on footbones present at the site (e.g. Vandermeersch and Trinkaus, 1995). A previous study based on dental macrowear and cortical bone distribution already investigated and reconstructed the ecology and diet of Regourdou 1 as a mixed diet, including both meat and plant materials (Fiorenza et al., 2019).

The Ca-isotope composition was measured for 15 samples obtained from two new mammal species (*Capreolus capreolus*, *Bos/Bison*), and seven previously analyzed species including Regourdou 2 (Supplementary Online Material [SOM] Table S1). In order to test variability within bone of a single specimen, we collected samples from two area of the same bear bone (R35 and R33).

2.3. Calcium extraction and analysis

Because cortical bone is denser and cleaner than trabecular bone, all samples were collected from cortical bone. Three cortical bone samples were paired with trabecular bone samples collected nearby on the same piece (Regourdou 1 and 2, and one from a brown bear). The samples were collected either with a scalpel in the form of a chip, about 5 mm long and 1 mm wide, or with a micromill generating a pit less than 1 mm in diameter and 1 mm deep (sample weight < 200 μg). Samples were preferentially taken in damaged areas to preserve as much as possible the morphological and structural integrity of the fossil remains. A potential drawback of using bone instead of enamel is that a false diagnosis can be made especially when a fossil piece lacks diagnostic characters, and thus we took special care to sample only well-diagnosed material.

The analyses were carried out at the Laboratoire de Géologie de Lyon. The samples (68 new plus 30 from Dodat et al., 2021, 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 minutes at room temperature in an ultrasonic bath to eliminate Ca-bearing secondary carbonates, particularly present as calcite (Lee-Thorp and van der Merwe, 1991; Sillen and LeGeros, 1991; Balter et al., 2002; Dodat et al., 2023). The residue was then digested in distilled HNO_3 (4.5 M), and about 10% of the solution was saved for concentration measurements following Balter et Lécuyer (Balter and Lécuyer, 2004). Most of the samples were not weighed to avoid sample loss that would have been critical for such small samples. The concentration of major and trace elements was measured on leached bone samples, using ICP-MS (iCap-Q, Thermo Scientific, Bremen) and an inductively coupled plasma optical emission spectrometer (iCap pro, Thermo Scientific, Bremen) for trace elements (Mn, Fe, U and rare earth elements [REEs]) and major elements (Ca, P, Mg), respectively.

All samples were successfully processed to extract and purify Ca from bone matrix according to the protocol set up by Tacail et al. (2014). Briefly, the remaining 90% of the digestion solution was taken up in distilled and titrated HCl (1.0 M), followed by three

chromatography steps: the first allows the recovery of Ca, Fe, and Sr; the second isolates Sr; and the third separates Ca from Fe. The Ca isotope compositions were measured using a Neptune Plus MC-ICP-MS (Thermo Scientific, Bremen) using the standard-sample-standard bracketing method. All Ca-isotope compositions are expressed using the delta notation, calculated as follows:

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

The ICP Ca Lyon standard, used routinely in Lyon, was used as bracketing and reference material, but the results are also expressed relative to the SRM915a-certified reference material. The certified reference material NIST SRM1400 (bone ash) was repeatedly purified and measured as a sample to control the accuracy of the $\delta^{44/42}$Ca values. During the study, six different aliquots ($n* = 6$) of the SRM1400-certified reference material were measured, which yield a value of -1.05 ± 0.07‰, (2 standard deviations [SDs], $n* = 6$, $n = 35$), indistinguishable from all SRM1400 measurements reported in other studies (-1.07 ± 0.09‰, 2 SDs, $n* = 1$, $n = 10$: Koutamanis et al., 2021; -1.06 ± 0.04‰, 2 SDs, $n* = 1$, $n = 4$: Romaniello et al., 2015; -1.02 ± 0.07‰, 2 SD, $n* = 1$, $n = 4$: Lanping et al., 2018).

2.4. MixSIAR modeling

Calcium-stable isotope compositions of Neanderthal individuals and potential food sources were analyzed with the Bayesian mixing model MixSIAR (Stock and Semmens, 2016; Stock et al., 2018), which estimates the relative contribution of food sources in the composition of diet, taking into account measurement uncertainty and variability. All statistical analyses were conducted with 'MixSIAR' v 3.1.12 (Stock and Semmens, 2016; Stock et al., 2018) in R v 4.0.5 (R Core Team, 2020. The SIAR Markov chain Monte Carlo algorithm was run with the predefined parameter 'extreme' Three types of data are necessary to estimate the contribution of a potential source in the diet using a Bayesian mixing model: (1) the values and associated uncertainties of the consumers of interest (Neanderthal individuals); (2) the values and associated uncertainties of potential sources (summarized in Table 1); and (3) the values and associated uncertainties of the trophic discrimination. Regarding potential animal dietary sources, we focused on the $\delta^{44/42}$Ca values of the taxa present in the archaeological sites, which were aggregated into three distinct groups (SOM Table S1; Table 1),

i.e., monogastric herbivores, including woolly rhinoceros, boar, horse, and mammoth and two distinct ruminant herbivores groups according to the family taxonomic rank, cervid, and bovid (summarized in Table 1). Regarding potential vegetal dietary sources, we have used current data obtained on European plants, in particular on the herb/grass types, or above-ground biomass (AGB), and plant leaf or stem samples collected at the vicinity of both archaeological sites. All the $\delta^{44/42}$Ca values used to calculate the AGB average and SD are given in SOM Table S2. We used a trophic discrimination value of 0.27 ± 0.1‰ for the Ca isotopic offset between bones of herbivores and carnivores (Martin et al., 2018) and a trophic discrimination value of 0.57 ± 0.1‰ for the Ca isotopic offset between plants and bones (Tacail et al., 2020).

3. Results

All results are given in SOM Table S1 and are reported in the text with two SDs (± 2 SD) unless otherwise specified. As mentioned earlier, the measurement of Ca-isotope compositions in bone necessitates only the smallest of samples. After leaching, the remaining bone residue was often so small that it was impossible to weigh it (cf. Section 2). The concentrations of major and trace elements are thus Ca-normalized.

3.1. Calcium diagenesis

The diagenesis of Ca was evaluated by testing for a correlation between the Ca/P (which could track potential excess Ca) and the $\delta^{44/42}$Ca values on the one hand, and diagenetic proxies (Fe/Ca, Mg/Ca, Mn/Ca, U/Ca and $\sum$REE/Ca) on the other. At Grotte du Bison, the $\delta^{44/42}$Ca values are negatively correlated with the $\sum$REE/Ca ratio, albeit not significantly after a Benjamini−Hochberg (BH) correction for multiple comparisons (Pearson BH-adjusted p-value = 0.087; SOM Fig. S1). At Regourdou, the Ca/P values are significantly correlated (Pearson BH-adjusted p-value = 0.017), with the $\sum$REE/Ca ratio (SOM Fig. S1). Examination of the individual relationships reveals that these correlations are induced by single values: a wolf specimen (B42; SOM Table S1; SOM Fig. S2) at Grotte du Bison and a lion specimen (R43; SOM Table S1; SOM Fig. S3) at Regourdou. The negative association between the $\delta^{44/42}$Ca value and the $\sum$REE/Ca ratio of the B42 specimen does not suggest diagenetic overprint because altered bone $\delta^{44/42}$Ca values tend to be more positive, not negative (Martin et al., 2017; Dodat et al., 2023). The Ca/P ratio of the R43 specimen (2.72; SOM Table S1) is at the upper limit of the 1.9 to 2.7 range, indicative of the stoichiometry of hydroxylapatite (Tzaphlidou and Zaichick, 2003, 2004). The $\delta^{44/42}$Ca value of the R43 specimen is also negative (-1.13‰; SOM Table S1). Taken together, these results suggest that bone Ca/P and $\delta^{44/42}$Ca values are not affected by significant diagenesis at either Grotte du Bison or Regourdou.

It is important to note that the Arcy 2015 BIS J T14 12 *Canis lupus* sample has an unexpectedly high $\delta^{44/42}$Ca value (-0.50 ± 0.10‰) and shows clear signs of partial digestion (SOM Fig. S4; SOM S1). This can be explained by the partial digestion of the bone, which

Table 1
Mean isotopic data and standard deviation used for the Bayesian mixing models for stable isotope analyses in R model grouped by sources and site share. The above-ground biomass value is the same for both sites.

Source	Site	Mean $\delta^{44/42}$Ca	SD $\delta^{44/42}$Ca	n
Bovid	Regourdou	−0.95 ‰	0.30	3
Cervid	Regourdou	−1.20 ‰	0.20	12
Monogastric	Regourdou	−0.87 ‰	0.19	5
AGB	Regourdou/Grotte du Bison	−0.49 ‰	0.26	44
Bovid	Grotte du Bison	−0.78 ‰	0.15	7
Cervid	Grotte du Bison	−0.71 ‰	0.13	8
Monogastric	Grotte du Bison	−0.60 ‰	0.08	8

Abbreviations: AGB = above-ground biomass; SD = standard deviation.

would have preferentially removed light Ca isotopes by kinetic fractionation. This sample will therefore not be further considered.

3.2. Calcium-isotope compositions

The observed linear correlation between the $\delta^{44/42}$Ca and $\delta^{43/42}$Ca values (SOM Fig. S5) agrees with the expected mass-dependent fractionation slope of ~0.5 (Tacail et al., 2014). Measurements that deviate from this line would indicate unresolved interference in the isotope ratio measurements, which is not the case here. All $\delta^{44/42}$Ca values are given in SOM Table S1 and are shown in Figure 2.

A first observation is that there is intraspecific $\delta^{44/42}$Ca variability that can be significant for some ruminant taxa, notably cervids. A second observation is that cervids have generally low $\delta^{44/42}$Ca values, comparable to carnivores. We note that there is a species-specific distribution of the Ca-isotope value at both sites, with monogastric herbivores having the highest $\delta^{44/42}$Ca values, ruminant herbivores and omnivores showing intermediate $\delta^{44/42}$Ca values, and carnivores having the lowest $\delta^{44/42}$Ca values (Fig. 2). The statistical results of pairwise comparisons, which support these observations, are given in Figure 2. The observed Ca-isotope systematic variability in the faunal assemblage is similar to that reported at Sclayn (Belgium) and Jaurens (France) and in South China (Martin et al., 2017; Hu et al., 2022). The absence of differences between herbivores and carnivores at Regourdou is due to the very low $\delta^{44/42}$Ca values of red deer (-1.34 ± 0.18‰, $n = 6$). A final observation is that the average $\delta^{44/42}$Ca values at Grotte du Bison (-0.72 ± 0.37‰, $n = 46$) are significantly higher (Wilcoxon p-value $= 10^{-10}$) than those at Regourdou (-1.09 ± 0.52‰, $n = 48$). The $\delta^{44/42}$Ca offset between the two sites is close to 0.4‰ and affects all taxa present at both sites (0.64‰ for red deer, 0.37‰ for reindeer, 0.34‰ for horse, 0.25‰ for wolf and 0.12‰ for bovid; SOM Table S1).

Cortical and trabecular bone samples were collected on the Regourdou 1 and Regourdou 2 Neanderthals (SOM Table S1). For both individuals, trabecular bone samples have a $\delta^{44/42}$Ca value (Regourdou 1: -1.56 ± 0.02‰; Regourdou 2: -0.78 ± 0.07‰) that is undistinguishable from that of cortical bone (Regourdou 1: -1.51 ± 0.10‰; Regourdou 2: -0.80 ± 0.07‰). Additionally, the

bone sampled from two areas of the cortical bone of a single brown-bear specimen (samples R35 and R33) shows no significant difference in their isotopic composition.

4. Discussion

4.1. General comments

In this study, we observe significant offset in Ca isotopic composition between the sites of Grotte de Bison and Regourdou. The $\delta^{44/42}$Ca offset observed between the two sites could be due to Ca-isotope variability at the bottom of the food web (water and/or soil). A range of 0.74‰ is measured in a global compilation of 52 water samples around the world (Tipper et al., 2010), which is compatible with the $\delta^{44/42}$Ca offset observed between Grotte du Bison and Regourdou. Several studies (e.g., Page et al., 2008; Cenki-Tok et al., 2009; Tipper et al., 2010) suggest that the Ca-isotope fractionation between plant organs is similar, whatever the species. This fractionation creates an enrichment in heavy isotopes as long as one moves up into the plant (root depleted compared to the stem, which is itself depleted compared to leaves). However, these studies also suggest that the Ca-isotope fractionation between soil and plant organs is more specific and is likely linked to the type of plant or the local soil environment (Hindshaw et al., 2013). This result suggests that the local geology and vegetation types may have an impact on the initial Ca-isotope composition of a trophic chain. To unravel the respective influence of local geology and vegetation types, we analyzed vegetal specimens (grasses, garlic mustard, common nettle and dandelion, cleavers, hazel, and oak; SOM Table S1) collected in the vicinity of the Grotte du Bison and Regourdou archaeological sites. The results reveal similar $\delta^{44/42}$Ca values at both sites, i.e., -0.67 ± 0.21‰ ($n = 10$) and -0.60 ± 0.19‰ ($n = 7$) at Grotte du Bison and Regourdou, respectively (Fig. 2; SOM Table S1), and therefore do not explain the observed bone $\delta^{44/42}$Ca offset between the two sites. A potential explanation is that these living plants are likely not representative of those that grew locally, or were consumed, tens of thousands of years ago (Fig. 1). The plant communities must have been different because Grotte du Bison is dated to the MIS 3 glacial environment, whereas Regourdou is dated to the MIS 5 interglacial environment (Fig. 1). Temperature
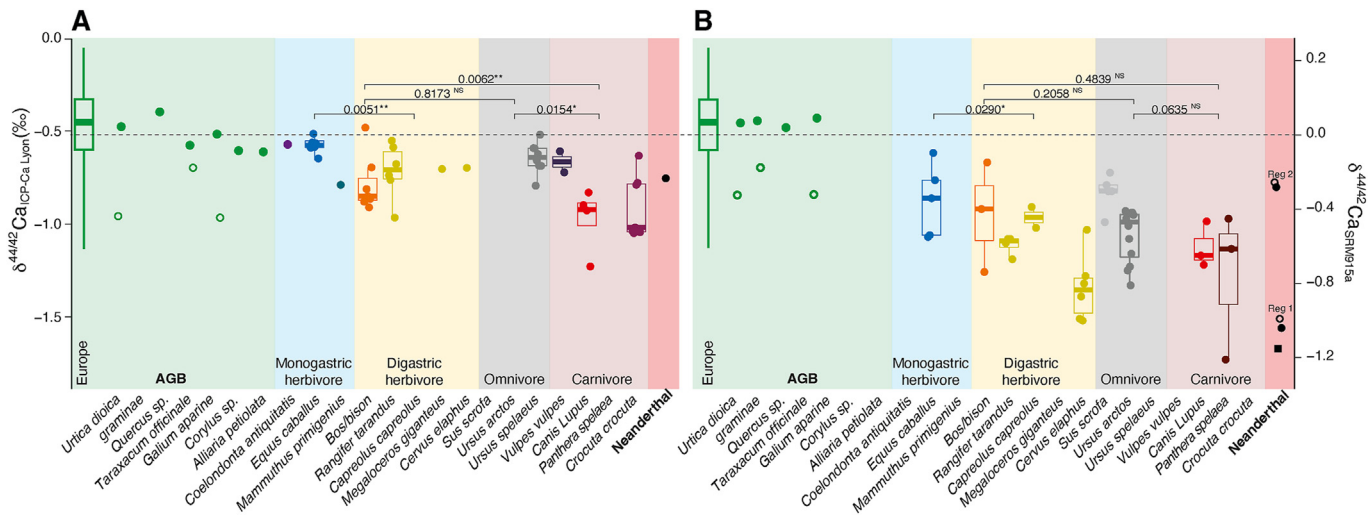
Figure 2. Box plot of species-specific distribution of $\delta^{44/42}$Ca values at the Grotte du Bison and Regourdou. The dotted line gives the correspondence between $\delta^{44/42}$Ca values relative to ICP-Ca Lyon and standard SRM915a. Open and filled symbols for vegetal samples stand for stem and leaf, respectively. Open and filled symbols for bone sample stand for spongy and cortical bone, respectively. For Regourdou 1, a circle corresponds to the femur and a square corresponds to the humerus sample. *P*-values of Wilcoxon tests are indicated with 'NS' if non-significant, with a single asterisk if $p < 0.05$, with a double asterisk if $p < 0.01$, and with a triple asterisk if $p < 0.001$. Abbreviation: AGB = above-ground biomass; ICP = inductively coupled plasma.

and aridity have been known to influence Ca-isotope cycling at the water−rock−vegetation interface (Page et al., 2008; Cenki-Tok et al., 2009; Holmden and Bélanger, 2010; Hindshaw et al., 2013). Furthermore, it is well established that environmental changes and food supplies vary between two climatic periods, e.g., MIS 3 and MIS 5 in our study (Fig. 1). Microwear and macrowear analysis for taxa, such as cervids and large bovids demonstrates the variability of dietary traits of herbivores that could be conditioned by the environment and food resources (Amano et al., 2016); this variability could have repercussions on calcium isotope composition.

4.2. Species-specific comments

The low $\delta^{44/42}$Ca values and associated important variability of cervids have also been observed by other authors in enamel (Martin et al., 2017; Hu et al., 2022). It is tempting to explain the cervid low $\delta^{44/42}$Ca values by the formation of ^{44}Ca-enriched antlers (Hassler et al., 2021). A pilot investigation (Hassler et al., 2021) highlighted that one antler of a modern red deer displays $\delta^{44/42}$Ca values higher than the rest of the skeleton (c.a. ≤0.2‰), but this represents a too small fractionation to account for the very low $\delta^{44/42}$Ca values of cervids relative to other mammals. Further studies are necessary to clarify the influence of antlerogenesis on cervid bone and enamel $\delta^{44/42}$Ca values. An obvious source of Ca-isotope variability for herbivores is that of the local vegetal sources. Here, we show that plant stems have lower $\delta^{44/42}$Ca values than leaves (Fig. 2), a pattern previously observed by multiple authors and compiled by Tacail et al., 2020. Because stems and roots have similar $\delta^{44/42}$Ca values (Tacail et al., 2019, 2020), the intraorgan Ca-isotope difference between leaves and roots, the edible parts of a plant, is an additional source of Ca-isotope variability among herbivores. As the accepted mechanism to explain the decrease of the $\delta^{44/42}$Ca values up the trophic chain is bone consumption (Skulan and DePaolo, 1999; Reynard et al., 2010; Heuser et al., 2011; Clementz, 2012; Martin et al., 2015, 2017, 2018; Hassler et al., 2018), the assimilation of Ca through a nonnutritive source, such as bone or antler chewing, is likely to mimic bone consumption even for cervid species (Kierdorf, 1994; Cáceres et al., 2011). The results of our MixSIAR model support the hypothesis of osteophagia to explain the low values of red deer at Regourdou (SOM Fig. S6).

Hyenas, and to a lesser degree, canids, are recognized to be regular bone crushers and are known to usually exhibit extremely negative $\delta^{44/42}$Ca values (Heuser et al., 2011; Martin et al., 2018; Hassler et al., 2021). This is the case for the hyenas at Grotte du Bison; however, the absence of hyenas at Regourdou prevents us from assessing where the bone crusher carnivore stands on the isotopic scale. The range of variation of $\delta^{44/42}$Ca values is higher for carnivores, e.g., ca. 0.7‰ for wolf at Regourdou or ca. 0.8‰ for lion at Grotte du Bison (Fig. 2). This observation has also been made in other studies on enamel (Martin et al., 2015; Hu et al., 2022); thus, the variability seen here should be attributed to the increased probability of integrating an unusual Ca-isotope signal due to the variability of possible preys and the proportion of ingested bone.

The difference between cave bear from Grotte du Bison and brown bear from Regourdou is 0.42‰. This difference is compatible with the general offset between the two sites and is not indicative of different dietary patterns, as suggested by nitrogen isotopes (Tacail et al., 2017).

4.3. Estimation of diet composition for the Grotte du Bison Neanderthal

The Grotte du Bison Neanderthal displays a $\delta^{44/42}$Ca value of 0.75 ± 0.07‰, lying in the middle of the local isotopic variation (Fig. 2). Importantly, zooarchaeological data show that among the three most common herbivore taxa (reindeer, horse, and bovid), at least two (reindeer and horse) show traces of soft-part acquisition by Neanderthals. In the MixSIAR modeling (cf. Section 2 Materials and methods), we focus on the mean of the identified dietary end-members, i.e., bone of bovid and cervid (ruminant herbivores), bone of monogastric herbivores and European plants or AGB, with the latter indistinguishable from meat. Results for the Grotte du Bison Neanderthal suggest that the contribution of each potential food source was broadly similar (Fig. 3A), ranging from 20% of bovid bone to 30% of AGB or meat. This result is consistent with a Neanderthal individual who was not far from the top of the trophic chain and also reflects the metabolic constraint that its diet cannot solely come from animal sources.

### 4.4. Estimation of diet composition for the Regourdou 1 Neanderthal

The Regourdou 1 individual has an average $\delta^{44/42}$Ca value of −1.58 ± 0.16‰ (*n* = 3), placing this Neanderthal individual at the lower end of local isotopic variability, among carnivores and out of the range of herbivores (Fig. 2). The results of the MixSIAR modeling for the Regourdou 1 Neanderthal suggest a preponderance of cervid bone consumption in the diet (51%; Fig. 3B). Bovid bone shows a modest contribution (29%), whereas monogastric bone and AGB (or meat) show a low contribution, 11% and 10%, respectively (Fig. 3B). The MixSIAR results, along with the observed consistency of the $\delta^{44/42}$Ca values between cortical and trabecular bone, suggest a specialization in the frequent consumption of cervids and bovids. The diet of the Regourdou 1 Neanderthal agrees with the traditional interpretations based on nitrogen isotopic data (Balter et al., 2002; Henry et al., 2011; Wißing et al., 2016) including recent compound-specific analysis of nitrogen-stable isotopes in individual amino acids (Naito et al., 2016; Jaouen et al., 2019).

4.5. Estimation of diet composition for the Regourdou 2 Neanderthal

The Regourdou 2 samples display an average $\delta^{44/42}$Ca value of 0.79 ± 0.04‰ (*n* = 2), placing this Neanderthal individual at the upper end of the local variability, among herbivores and out of the carnivore range (Fig. 2). The results of the MixSIAR modeling for the Regourdou 2 Neanderthal suggest that the consumption of AGB (or meat) and bovid bone constitute, respectively, 38% and 35% of the diet (Fig. 3C). Bone of monogastric animals shows a medium contribution (19%), whereas cervid bone provides a low contribution (9%). This result is unexpected, considering the previous studies on Neanderthal diet based on nitrogen isotopic data (Balter and Simon, 2006; Richards and Trinkaus, 2009; Naito et al., 2016; Wißing et al., 2016) or other isotope systems chemical proxies (e.g., Sr/Ca; Balter et al., 2001; $\delta^{66/64}$Zn; Jaouen et al., 2022), even if the AGB contribution is overestimated and instead reflects meat consumption. It suggests that animal resources contribute less to the Regourdou 2 diet than plant resources, with a less extensive exploitation of carcasses bone marrow. This result is even more surprising when one considers that the Regourdou 1 and Regourdou 2 individuals are supposed to be attributed to the same sedimentary layer 4 (Madelaine et al., 2008), as this would suggest that they may have been pene-contemporaries. Even if we cannot ascertain that they were contemporaries, they most probably lived in similar environments (Fig. 1B and C).

4.6. Dietary diversity among Neanderthals

This study presents Ca-isotope compositions for three Neanderthal individuals, showing they were probably occupying distinct
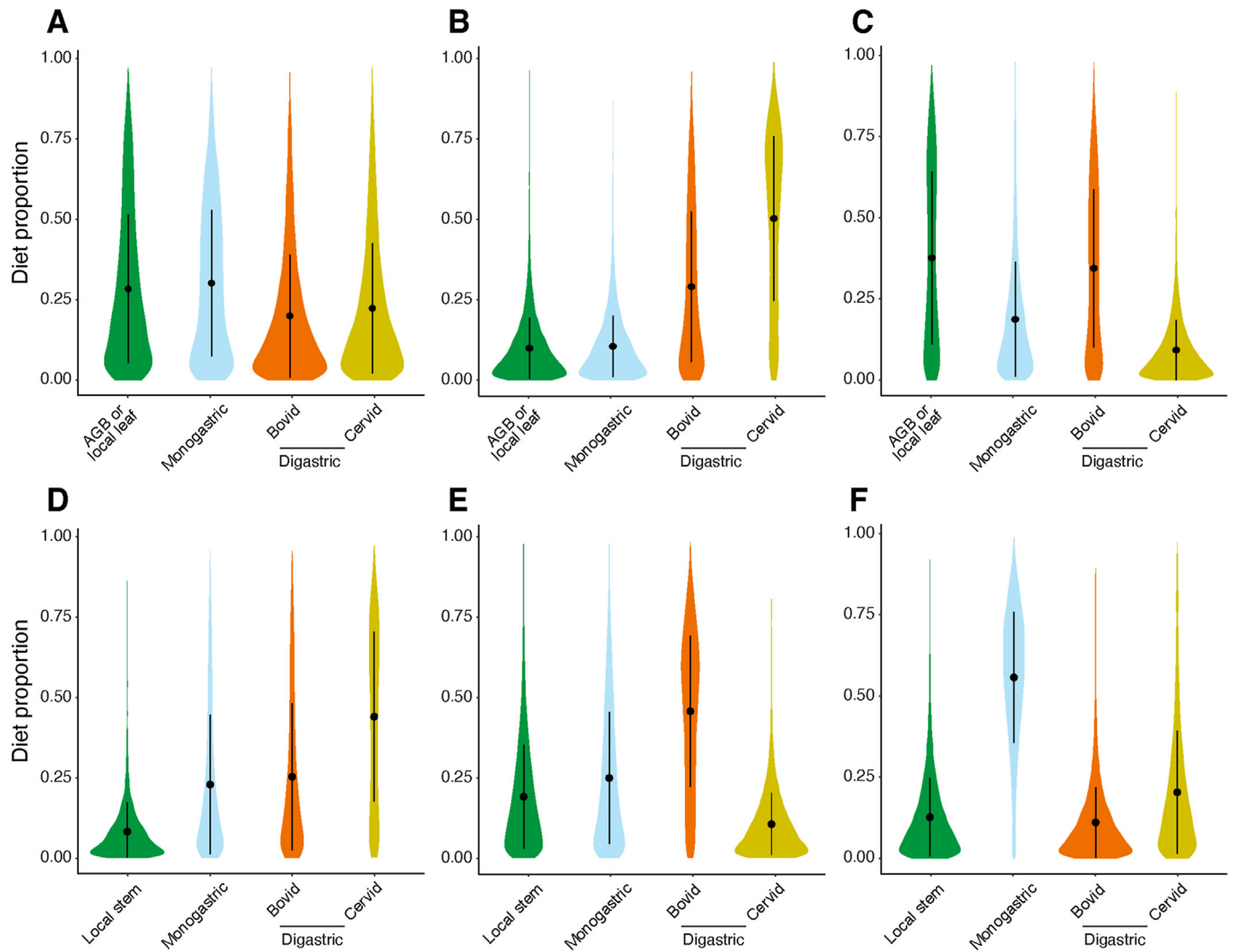
Figure 3. Violin plots representing diet proportion estimates for the three Neanderthal individuals using two different vegetation sources: Grotte du Bison (A), Regourdou 1 (B), and Regourdou 2 (C) using $\delta^{44/42}$Ca values of fossil bone and above-ground biomass (AGB) average from actual European plants or local leaf $\delta^{44/42}$Ca values. Grotte du Bison (D), Regourdou 1 (E), and Regourdou 2 (F) using $\delta^{44/42}$Ca values of fossil bone and local stem (as a proxy for root) $\delta^{44/42}$Ca values. Dots indicate the probable mean value.

trophic positions relative to the local fauna, thus suggesting different dietary strategies. This finding reconciles metabolic constraints (e.g., Cordain et al., 2000; Fiorenza et al., 2015), ethnographic studies (e.g., Speth and Spielmann, 1983; Speth, 2010), archaeological evidence (e.g., Henry et al., 2011), and geochemical results based on nitrogen isotopes (e.g., Balter and Simon, 2006; Richards and Trinkaus, 2009; Naito et al., 2016; Wiβing et al., 2016) or other isotopes or geochemical proxies (e.g., Sr/Ca; Balter et al., 2001; $\delta^{66/64}$Zn; Jaouen et al., 2022). A Bayesian mixing model used to uncover probable vs improbable diet composition shows that we should consider bone and AGB (or meat) as potential dietary sources to match Neanderthal bone $\delta^{44/42}$Ca values, at least for the Grotte du Bison and Regourdou 2 individuals. Despite the similarity of the AGB and meat $\delta^{44/42}$Ca values, this study is the first to consider a vegetal component for paleodietary reconstructions based on Ca-stable isotopes. Using an AGB $\delta^{44/42}$Ca mean value of -0.49 ± 0.26‰, it is possible to estimate a dietary plant fraction of ~10%, ~30%, and ~40% for the Regourdou 1, Grotte du Bison, and Regourdou 2 individuals, respectively. Using a local living-leaf $\delta^{44/42}$Ca mean value of -0.53 ± 0.09‰ and -0.49 ± 0.02‰ for Grotte du Bison and Regourdou, respectively, does not change the overall

picture because these $\delta^{44/42}$Ca values are similar to that of AGB (Fig. 2). However, when local present stem $\delta^{44/42}$Ca values (-0.89 ± 0.13‰ and -0.79 ± 0.08‰ for Grotte du Bison and Regourdou, respectively; Fig. 2) are used as a proxy for root consumption (Tacail et al., 2019, 2020), the dietary reconstruction is different for the three Neanderthal individuals (Fig. 3D; 3E.; 3F.). The plant proportion increases to 20% for the Regourdou 1 Neanderthal (Fig. 3E), whereas it decreases or the two other individuals, i.e., 8% and 13% for the Grotte du Bison and Regourdou 2 Neanderthals, respectively (Fig. 3D; 3F). These results show that whatever the plant $\delta^{44/42}$Ca value (or that of meat), it is not possible to obtain matching proportions of dietary sources for the three studied Neanderthal individuals. Therefore, we conclude these three individuals had diverse dietary strategies.

Although bone balance is a complex process, trabecular bone is known to have a faster turnover rate than cortical bone (18%/year and 8%/year, respectively; Parfitt, 2002). The absence of a difference between trabecular and cortical bone for Regourdou 1 and Regourdou 2 suggests that the average diet, as measured in the $\delta^{44/42}$Ca value, during the shortest bone lifetime ($0.18^{-1} = 5.5$ year for trabecular bone) is the same as during the longest bone lifetime

$(0.08^{-1} = 12.5$ year for cortical bone). In other words, the similar trabecular and cortical bone $\delta^{44/42}$Ca values for these two Neanderthals are indicative of a relatively stable diet with a duration of several years. Given that variability in bone turnover rate may be high, especially between the axial and appendicular skeleton (Parfitt, 2002), this conclusion remains preliminary. Nevertheless, the possibility to track dietary changes using small pieces of bone with contrasting turnover rates is now a distinct possibility, given the low sample amounts needed for Ca-isotope measurements.

Metabolic arguments mandate that Neanderthals could not have had a diet of 100% meat; their diet must have included fat. Animal fat is a highly sought-after resource for hunter-gatherer populations (Speth and Spielmann, 1983; Cordain et al., 2000; Speth, 2010; Morin, 2020a). In ungulates, fat is found as body fat directly beneath the skin or around organs, or as skeletal fat (Morin, 2020b). In winter, hunter-gatherers may have a special interest in skeletal fat because it is kept in reserve even when animals are in poor physical conditions (Morin, 2020a, 2020b). Skeletal fat is found in yellow marrow stored in the medullary cavity of long bones and as bone grease in the spongy bone. However, the consumption of meat or yellow marrow is insufficient to induce a negative isotopic shift of the dietary $\delta^{44/42}$Ca value; this comes only with the presence of bone in the diet (Heuser et al., 2011; Martin et al., 2017; Dodat et al., 2021). How might this have happened? It could be the case that trabecular bone is present in the medullary cavity of long bones along with yellow marrow. The collection/recovery of yellow marrow does not require sophisticated techniques to break open long bones. Archaeologically, yellow marrow consumption is well documented since at least the emergence of the genus *Homo* and persists through Mesolithic (Sahnouni et al., 2013). In this case, the ingestion of small amounts of bone would have been accidental but likely inevitable. In contrast, the consumption of bone grease requires more processing. Specifically, the epiphyses tend to be comminuted, with the resulting powder boiled to extract grease (Morin, 2020a, 2020b). The consumption of bone grease, as grease loaves or soup, could also result in the ingestion of a significant quantity of bone. In this case, the consumption of a bone-based food would have been voluntary. This is a provocative interpretation because boiling as a technique is thought by some to have only originated in the Upper Paleolithic (Speth, 2015; Morin and Soulier, 2017).

The current work suggests the existence of diverse diets among Neanderthals, whatever their proportions of plant foods and/or fat. This is one of the first potential demonstrations of substantial diversity in individual dietary behaviors among Neanderthals using stable isotopes, two of which come from the same site. Our results concur with those on dental calculus (Henry et al., 2011; Weyrich et al., 2017; Hardy, 2022) suggesting that there is a greater heterogeneity in feeding strategies within or between Neanderthal groups than previously thought, particularly regarding the variability of plants resources in the diet. Whether the differences between these dietary strategies were sociocultural, symbolic, seasonal, or personal in nature (or due to some unknown reason) remains an open question. These observations support the hypothesis that Neanderthals' dietary strategies are substantially influenced by their surroundings and indicate a great degree of ecological plasticity (Fiorenza et al., 2020; Rivals et al., 2022). The use of new isotopic methods enables us to broaden our investigation into the Neanderthal diet.

CRediT authorship contribution statement

Pierre-Jean Dodat: Writing − review & editing, Writing − original draft, Methodology, Funding acquisition, Formal analysis. **Emmanuelle Albalat:** Methodology. **Vincent Balter:** Writing −

review & editing, Writing − original draft, Supervision, Project administration, Funding acquisition. **Christine Couture-Veschambre:** Resources. **Maurice Hardy:** Resources. **Juliette Henrion:** Resources. **Trenton Holliday:** Writing − original draft. **Bruno Maureille:** Writing − review & editing, Writing − original draft, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

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