

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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Pierre-Jean Dodat^{a,b,*}, Théo Tacail^e, Emmanuelle Albalat^a, Asier Gómez-Olivencia^{d,e,f}, Christine Couture-Veschambre^b, Trenton Holliday^{g,h}, Stéphane Madelaine^{i,b}, Jeremy E. Martin^a, Rebeka Rmoutilova^{b, j}, Bruno Maureille^b, Vincent Balter ^a

^aENS de Lyon, Univ Lyon 1, CNRS UMR 5276, LGL-TPE, F-69007, Lyon, France.

^bUniv. Bordeaux, CNRS, MC, PACEA, UMR 5199, F-33600 Pessac

^cBristol Isotope Group, School of Earth Sciences, University of Bristol, Wills Memorial Building, Queen's Road, Bristol, BS8 1RJ, UK

^dDepartamento de Estratigrafía y Paleontología, Facultad de Ciencia y Tecnología, Universidad del País Vasco-Euskal Herriko Unibertsitatea (UPV/EHU), Leioa, Spain

eSociedad de Ciencias Aranzadi, Donostia-San Sebastián, Spain

^fCentro UCM-ISCIII de Investigación sobre Evolución y Comportamiento Humanos, Madrid, Spain

^gTulane University; Department of Anthropology, 101 Dinwiddie Hall, New Orleans, Louisiana 70118, USA

^hUniversity of the Witwatersrand; Evolutionary Studies Institute, Private Bag 3, Wits 2050, Republic of South Africa

ⁱMusée national de Préhistoire, 1 rue du Musée, 24620 Les Eyzies-de-Tayac Sireuil, France

^jDepartment of Anthropology and Human Genetics, Faculty of Science, Charles University, Viničná 7, 128 44 Prague, Czech Republic

*Corresponding author.

E-mail address: pierre-jean.dodat@ens-lyon.fr (P.-J. Dodat)

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Author Contributions

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, TT.

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Manuscript (without Author Details)
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 dietary reconstruction of Regourdou 1 Neandertal fossil.

3

4 Abstract

The calcium isotopic composition ($\delta^{44/42}$ Ca) of bone and tooth enamel can be used for 5 dietary reconstructions of extant and extinct mammals. In natural conditions, the $\delta^{44/42}$ Ca value of 6 bone and teeth varies according to dietary intake with a constant isotopic offset of about -0.6 %. 7 Due to the poor conservation of collagen, carbon (C) and nitrogen (N) isotopic compositions of 8 the Regourdou Mousterian site (MIS 5, Dordogne, France) previously failed to provide any 9 10 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 11 bone samples of the Regourdou 1 Neandertal specimen. The results show a taxon-dependent 12 patterning of the Ca isotopic compositions: herbivores generally have higher $\delta^{44/42}$ Ca values than 13 carnivores. All the $\delta^{44/42}$ Ca values of Regourdou 1 are low (<-1.6 ‰), placing this specimen amid 14 carnivores. Using a bone-muscle Ca isotopic offset determined on extant animals, we further show 15 that the $\delta^{44/42}$ Ca value of the Regourdou 1 diet, and that of most carnivores, cannot be accounted 16 for by the consumption of meat only, as plants and meat have indistinguishable $\delta^{44/42}$ Ca values. 17 Mass balance calculations indicate that the low $\delta^{44/42}$ Ca values of Neandertal's carnivorous diet 18 are explained by the ingestion of bone marrow containing as little as 1% trabecular bone. Our 19 20 results show that the Regourdou 1 Neanderthal consumed a mixture of various herbivorous preys as well as trabecular bone, which probably occurred when marrow was ingested, by accident or 21 intentionally. 22

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24 Keywords; Paleodiet; Marrow consumption; Diet; Late Pleistocene

26 1

1. Introduction

27 The reconstruction of diet is an important aspect in paleoanthropology, archaeology and prehistory, because diet is related to numerous human behaviors such as hunting practices, social 28 organization and mobility (e.g., Patou- Mathis, 2000; Costamagno et al., 2006; Hublin and 29 30 Richards, 2009). Paleoecological reconstructions are traditionally based on the study of faunal assemblages of preserved remains and associated anthropogenic traces (Hublin and Richards, 31 2009), tooth microwear (Puech et al., 1983), dental size and morphology (Jiménez-Arenas et al., 32 2014) or more rarely from preserved organic matter (e.g., phytoliths or DNA in dental calculus; 33 Henry et al., 2011). However, the reconstruction of ancient diet may be biased due to differential 34 transport of anatomical parts of prey, and to taphonomic processes (e.g. chemical process, 35 scavengers, water flow), which can lead to the lack of conservation of flora and other food 36 resources (Faith and Thompson, 2018) and can modify faunal assemblages even in well-preserved 37 38 sites (Fernandez-Jalvo and Andrews, 2016). Stable isotopes have been a useful supplementary tool for studying the diet of extinct species 39 for over 40 years. Since the 1990s and the first isotopic studies of Neandertal remains from 40 Marillac in Charente (France; Bocherens et al., 1991), the number of studies using collagen C and 41 42 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 43 44 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, 45 bovid, rhinoceros and mammoth (Balter and Simon, 2006; Bocherens, 2013; Naito et al., 2016; 46 47 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). 48 49 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 50

51 consumed plants in sizeable proportion ($\sim 20\%$ of their dietary protein source). Isotopes are now 52 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 53 bone requires the collection of between 10 mg and 500 mg of fossil material, depending on the 54 55 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 56 is a problem for precious material, notably unique hominin remains. Second, while collagen can 57 be preserved for more than 100 kyr in specific environments, cases of good preservation beyond 58 50 kyr are rare, preventing the use of N isotopic compositions in deep time (Melin et al., 2014). 59 This represents a great handicap for the study of Neandertals, for whom dietary habits throughout 60 the Paleolithic are key to understanding their cultural and biological evolution. 61

In order to overcome these difficulties, or to complement C and N isotopic composition 62 studies, new isotopic systems have been developed as dietary proxies using the mineral phase of 63 tooth dentine, enamel and bone. Generally, these isotopic systems are referred as 'non-traditional' 64 (Martin et al., 2017) isotopes and involve transition metals including copper, zinc and iron 65 (respectively, ⁶⁵Cu/⁶³Cu, ⁶⁶Zn/⁶⁴Zn, ⁵⁶Fe/⁵⁴Fe ratios; Jaouen et al., 2012, 2013, 2017; Bourgon et 66 al., 2020), or alkaline-earth metals including magnesium, calcium and strontium (respectively, 67 ²⁶Mg/²⁴Mg, ⁴⁴Ca/⁴²Ca, ⁸⁸Sr/⁸⁶Sr ratios; Knudson et al., 2010; Martin et al., 2014, 2015a, 2017, 68 2018; Hassler et al., 2018). Calcium is the element that has been the most studied thus far for 69 paleodietary applications because it is the essential element of the mineral fraction of bone and 70 teeth, i.e., hydroxylapatite. This gives the isotopic method two main advantages. First, provided 71 that secondary Ca-bearing carbonates are absent or have been leached using dilute acid, the 72 overprinting of a diagenetic isotopic composition on the original Ca isotopic composition is 73 unlikely. This has been modeled using simple mass balance for Mg, Ca, Fe, Cu and Zn isotopes on 74 continental and marine settings by Martin et al. (2017). Based on the model, a significant 75

76	diagenetic alteration of the Ca fraction of bioapatite in terrestrial settings is unlikely because the
77	Ca concentration in groundwater is negligible (15–150 ppm of Ca) compared to that of
78	hydroxylapatite (40%; Martin et al., 2017). This is not the case for the isotopic composition of
79	trace elements (i.e., Cu, Zn, Fe or Sr) which is more sensitive to diagenetic overprinting because
80	the concentrations of trace elements are generally lower in bioapatite than in rivers (Martin et al.,
81	2017). Second, minute amounts of bone or tooth enamel (typically 100 μ g) are sufficient for a
82	routine measurement of the Ca isotopic composition. This technique can thus be applied to
83	precious fossils while causing minimal damage (Tacail et al., 2019).
84	Skulan and collaborators (Skulan et al., 1997; Skulan and DePaolo, 1999), first described a
85	linear correlation between the Ca isotopic composition of diet and vertebrate tissues including
86	bone. This observation allowed Skulan and DePaolo (1999) to show a decrease in the isotopic
87	composition of Ca along the food chain. Several authors further assessed the extent of this
88	decrease using different animal models, giving an average difference of the $\delta^{44/42}$ Ca values
89	between bone and diet ($\Delta^{44/42}$ Ca _{bone-diet}) of -0.57 ‰ (±0.10, 2SE; Tacail et al., 2020). In mammals,
90	the major isotopic fractionation occurs during renal reabsorption, which can be affected by many
91	physiological aspects still poorly understood (Tacail, 2017; Tacail et al., 2020). The effect of
92	lactation is potentially better understood and causes a significant offset of the bone $\delta^{44/42}$ Ca value
93	(Reynard et al., 2010, 2013; Tacail et al., 2019). Other biological processes such as hibernation
94	(cessation of the renal cycle), the presence of a particular digestive system such as for ruminants,
95	or the production of mineralized growths such as antlers or horns, can also affect the bodily
96	distribution of Ca isotopes (Tacail et al., 2020). Despite a body of isotopic evidence consistent
97	with ecological information, the compilation of isotopic data on biological material cannot explain
98	the lower isotopic composition of carnivores compared to herbivores by invoking meat
99	consumption only. Indeed, meat displays $\delta^{44/42}$ Ca values close to the range of variation of plants
100	(Tacail et al., 2019). Carnivores and herbivores should therefore have similar $\delta^{44/42}$ Ca values,

101 which is not the case. Bone consumption has been proposed to explain the observed difference in 102 $\delta^{44/42}$ 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 value of these differences to reconstruct the past trophic chain of
 the fossil Regourdou assemblage.

- 108
- 109 2. Materials and Methods

110 2.1. History of the Regourdou site

The Regourdou cave is located at the top of the Lascaux hill (Montignac-sur-Vézère, 111 Dordogne; Supplementary Online Material [SOM] Fig. S1). Regourdou site 'excavations' began 112 113 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; 114 Madelaine et al., 2008; Maureille et al., 2015a). A rescue operation for recovery of the hominin 115 remains was set up under the authority of F. Bordes during the 4th and 5th of October 1957 with 116 the participation of E. Bonifay and G. Laplace-Jauretche as the only two excavators. This quick 117 excavation allowed the discovery of faunal and lithic remains and about 50 identified hominin 118 bones or bone fragments belonging to one Neandertal, Regourdou 1 (Madelaine et al., 2008). 119 Between 1961 and 1964, the site was excavated by E. Bonifay (Bonifay, 1964). The 120 Regourdou 1 skeleton was located in Bonifay's stratigraphic layer 4 (Bonifay, 1964), which 121 contained lithic remains belonging to a Discoid Mousterian techno-complex with the production 122 of pseudo-Levallois points and a temperate fauna highly dominated by brown bears (Ursus 123 arctos). This layer 4 was attributed to MIS 5 based on cultural evidence and this was confirmed by 124 a recent Optically-Stimulated Luminescence date (Bonifay, 1964; Bonifay et al., 2007; Maureille 125

126 et al., 2017). According to E. Bonifay, the site was interpreted as a highly peculiar Mousterian 127 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 128 of brown bear corpses in an alleged pit closed by a several-ton flat limestone slab, along with 129 130 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 131 collections (allowing the discovery of 73 new Regourdou 1 hominin remains; Madelaine et al., 132 2008; Maureille et al., 2015b), questioned the Bonifay interpretation of a unique Mousterian 133 symbolic cave, even if the new scientific team involved in this research still considers Regourdou 134 1 as a Neandertal intentional burial (Madelaine et al., 2008; Maureille et al., 2015a, 2016; Pelletier 135 et al., 2017). This is an ancient cave that was used as a den for brown bears which are represented 136 almost in the lower levels (Cavanhié, 2011). Subsequently the cavity collapsed, forming a trap 137 allowing the accumulation of many fauna (Bonifay, 2002; Cavanhié, 2011). The Neandertal is 138 assumed not to be a major accumulating agent because only a small number of bones show 139 anthropic action (Cavanhié, 2011). Due to age and taphonomic process, resulting in an insufficient 140 quality of collagen, ancient DNA and C and N isotopic compositions failed to provide any 141 paleogenetic or paleodietary information (Bocherens, pers. comm.). 142

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144 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 (*Ursus 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 151 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 152 layer 5 and one from layer 7. Layers 4, 5 and 7 are thought to be contemporaneous and layer 2 153 younger (Bonifay et al., 2007; Cavanhié, 2011). All but two of the samples were taken from 154 155 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 156 from an adjacent region of the same piece of bone. Each sample was obtained by excising a 157 fragment of bone <5 mm long and 1 mm wide using a scalpel (SOM Fig. S2). Samples were 158 preferentially taken from parts that were already damaged during excavation or during the post-159 excavation history or in areas already sampled for previous analyses. This sampling method 160 allowed us to preserve as much as possible the morphological and structural integrity of the 161 pieces. 162

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2.3. Modern faunal bone and muscle samples

To better understand the calcium isotopic distribution in mammals, we included modern 165 cortical and trabecular bone associated with bone marrow and muscle of domestic and wild 166 animals (SOM Table S2). These bones were analyzed in order to evaluate the bone-muscle Ca 167 isotopic offset and to characterize the mechanism responsible for the decrease in the $\delta^{44/42}$ Ca 168 values along food chains (SOM Table S2). The total sample of 11 samples includes three pigs (Sus 169 scrofa domesticus), one boar (Sus scrofa), four cows (three adults, one veal; Bos taurus), one roe 170 deer (Capreolus capreolus), one chicken (Gallus gallus) and one rabbit (Oryctolagus cuniculus). 171 As the different samples were obtained from butcher shops, no information concerning the diet of 172 these individuals is available. However, dietary information is not needed to assess muscle-bone 173 174 differences.

177 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 178 30 bone samples from Regourdou's faunal and paleoanthropological collections (SOM Table S1) 179 180 were first ground using an agate mortar and pestle before being leached with diluted ultrapure acetic acid (0.1M) for 30 minutes at room temperature in an ultrasonic bath to eliminate Ca-181 bearing secondary carbonates, particularly calcite (Balter et al., 2002). All modern samples were 182 first freeze-dried and digested in concentrated HNO₃ acid to dissolve the organic phase. 183 The concentrations of major and trace elements were measured after a leaching step with 184 acetic acid (SOM Tables S1 and S3) and diagenesis was monitored using the calcium/phosphorus, 185 iron/calcium, manganese/calcium, and uranium/calcium ratios (Ca/P, Fe/Ca, Mn/Ca, and U/Ca, 186 respectively; SOM Table S1) and the sum of all the rare earth elements (ΣREE ; SOM Table S3). 187 188 These measurements were performed by Ouadrupole Inductively-Coupled-Plasma Mass Spectrometry (Q-ICP-MS) for trace elements and by Inductively-Coupled-Plasma Optical 189 Emission Spectroscopy (ICP-OES) for major elements, respectively. Methods for the 190 measurement of trace and major elements are briefly described here (for a complete description 191 see Balter and Lécuyer, 2004, 2010). The concentrations of elements were calculated using 192 calibration curves based on multi-elemental solutions. These solutions were also used to monitor 193 and correct for the instrumental drift over the analytical session. Matrix effects and instrumental 194 drift were also corrected using indium (In) and scandium (Sc) as internal standards for trace and 195 major elements, respectively. Comparison of major and trace element was performed using a 196 simple linear regression model and the correlation coefficient represented with an heatmap 197 (correlation matrix). All the statistical analyses on the Ca isotopes and other elements were 198 performed in R (V3.6.1; R Core Team, 2020). 199

All samples were processed in order to extract the bone's Ca according to the protocols 200 established by Tacail et al. (2014). Briefly, this consists of a digestion step in concentrate HNO₃ 201 202 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. 203 204 The compositions of the Ca isotopes were measured using a Neptune Plus multi-collector ICP-MS using the standard-sample-standard-bracketing method. All Ca isotope compositions are expressed 205 using the delta notation and were calculated using the inductively coupled plasma (ICP) Ca Lyon 206 standard as bracketing and reference material. A solution of the certified reference material NIST 207 SRM-1400 (bone ash) was repeatedly purified and measured as a sample to control the accuracy 208 of the measurements of archaeological bone and a solution of the certified reference material 209 NIST SRM-1486 (bone meal) was repeatedly measured as a sample to control the accuracy of the 210 measurements of modern bone and muscle samples. The SRM-1486 certified reference material 211 vielded a value of -1.07 ± 0.049 , 2 SE, n = 24), which is undistinguishable from all SRM-1486 212 reported in other studies $(-1.009 \pm 0.026, 2 \text{ SE}; \text{Heuser and Eisenhauer, 2008; Heuser et al., 2011},$ 213 2016a, 2016b). The $\delta^{44/42}$ Ca annotation is defined according to the following equation: 214

215
$$\delta^{44/42} \text{Ca} = \left(\frac{({}^{44}Ca/{}^{42}Ca_{\text{Sample}})}{0.5 \times ({}^{44}Ca/{}^{42}Ca)_{ICP-Ca-Lyon}^{n-1} + 0.5 \times ({}^{44}Ca/{}^{42}Ca)_{ICP-Ca-Lyon}^{n+1} - 1\right) \times 1000$$

To facilitate comparison of our dataset 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).

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220 **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 224 Mn/Ca suggests a diagenetic Ba/Ca overprinting (SOM Figs. S3D and S4). The sample R11 seems 225 226 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 227 228 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 $\delta^{44/42}$ Ca values and the 229 Ca/P ratios, when highly altered samples (i.e., R11–R12, R18, R20 and R22) are excluded (Fig. S4). 230 Taken together, the results suggest that while the Ba/Ca ratios, and potentially the Sr/Ca ratios, are 231 affected by some diagenetic alteration, the Ca isotopic compositions of specimens included in this 232 study reflect original values and can be used to constrain the paleodiet of species retrieved at 233 Regourdou. Note that the faunal pattern of the Sr/Ca ratio at Regourdou is similar to that reported 234 for the Saint-Césaire assemblage (Balter et al., 2001, 2003), which argues for a minimal diagenetic 235 236 overprint of the Sr/Ca ratio at Regourdou.

237

238 *3.2.Calcium isotope analyses*

The observed linear correlation between the $\delta^{44/42}$ Ca and $\delta^{43/42}$ Ca values for both modern 239 and fossil samples (SOM Fig. S5) agrees with the expected mass-dependent fractionation slope of 240 ~0.5 (Tacail et al., 2019). For modern samples, muscle always exhibits a higher $\delta^{44/42}$ Ca value 241 than associated bone, whether cortical or trabecular (Fig. 1). Cortical and trabecular bone have 242 similar $\delta^{44/42}$ Ca values, which are also similar to associated bone marrow in two of 4 cases (Fig. 243 1). For fossil samples, the $\delta^{44/42}$ Ca values of herbivorous species (with the exception of red deer) 244 and wild boar are high in comparison with the other species, with values close to -1 ‰ (from -0.79 245 to -1.19 ‰; Fig. 2). The $\delta^{44/42}$ Ca values of herbivorous species are ~ 0.6 ‰ lower than the 246 compiled above-ground biomass data of European grasses/herbs; the difference is smaller with the 247 values of woody plants. Red deer exhibit much more negative $\delta^{44/42}$ Ca values (from -1.28 to -1.52) 248

‰; Fig. 2) overlapping with the range of values of carnivores, i.e. from -1.17 to -1.73 ‰ (Fig. 2). 249 The Regourdou 1 samples display low $\delta^{44/42}$ Ca values, ranging from -1.51 to -1.67 ‰ (Fig. 2), 250 placing the specimen in the ranges of carnivores. Despite different stratigraphic provenance, the 251 $\delta^{44/42}$ Ca values of red deer from stratigraphic layer 4 are within the range of variation of those of 252 red deer of stratigraphic layer 2, despite different MIS attributions. Similarly, the $\delta^{44/42}$ Ca values 253 of brown bear from layer 2 fall in the range of variation of those from layers 4–5 and 7. This 254 suggests that the environmental impact on Ca isotopic composition is included in the 255 intraindividual variability. 256

257

258 **4. Discussion**

259 4.1. The ecological and physiological significance of Calcium isotopes in herbivores

Adding the $\Delta^{44/42}$ Ca_{bone-diet} value to bone (about -0.6 %; Tacail et al., 2020) allows us to 260 estimate the average $\delta^{44/42}$ Ca value of the animals' diets (SOM Table S1). The remodeling of the 261 cortical bone is very slow once growth is complete (e.g. Manolagas, 2000), with an average 262 estimation corresponding to the diet of the last years of the individual's life. For herbivores, the 263 reconstructed diet would correspond to plants and plant organs, for which the distribution of the 264 $\delta^{44/42}$ Ca values is known based on several studies and previously compiled data (Martin et al., 265 2018; Tacail et al., 2019, 2020) which are used in the present study. Some of the plant variability 266 267 is known, but a continuing effort is necessary to fully depict the Ca isotopic variability in vegetal 268 materials. Only two European Late Pleistocene assemblages have been analyzed isotopically (Martin et al., 2017). The specimens from Sclayn (Namur, Belgium, from layer 1A, dated >36.2 269 kyr and 38.7 ± 1.5 kyr BP; Gilot, 1992) and Jaurens (Corrèze, France, dated from 29.7 to 32.6 kyr 270 271 BP; Guérin et al., 1979) caves are more recent than those from Regourdou, but are nevertheless used here for comparison in this study. 272

Horses (*Equus caballus*) at Regourdou exhibit a mean $\delta^{44/42}$ Ca value of -1.06 ‰ (± 0.01, 273 2SD, n = 2; Fig. 2), identical to that obtained at Sclayn (-1.06 ± 0.36 ‰, 2SD, n = 2; Martin et al., 274 2017). Additional data on horses could challenge the apparent homogeneity of the $\delta^{44/42}$ Ca values 275 at Sclayn and Regourdou and as a consequence, raise questions about the similarity of the dietary 276 niches. The case of cervids raises a number of questions. Here, reindeer (*Rangifer tarandus*) have 277 relatively high $\delta^{44/42}$ Ca values (from -1.08 ‰ to -1.19 ‰), while red deer (*Cervus elaphus*) exhibit 278 much lower values (from -1.28 ‰ to -1.51 ‰), characteristic of carnivores. The presence of 279 antlers in cervids is likely to represent an additional and significant reservoir of Ca, although it 280 remains to be demonstrated that antler formation (antlerogenesis) is associated with a species-281 dependent Ca isotope fractionation. In the absence of such information, we have considered that 282 diet-specific behaviors have a prominent effect on the bone $\delta^{44/42}$ Ca value compared to antler 283 formation. Reindeer and red deer are two herbivores whose diets are highly adaptable. Reindeer 284 are mainly grazers and sometimes browsers that consume lichen (Martin, 1982), lichen having 285 high $\delta^{44/42}$ Ca values (Fig. 2), while red deer are more browsers and tend to consume shrubs 286 (Martin, 1982; Hofmann, 1989; Hearney and Jennings, 2009; Discamps, 2011), with the stem and 287 trunk generally having the highest $\delta^{44/42}$ Ca plant values (Fig. 2). The assimilation of Ca through a 288 non-nutritive source, like bone or antler chewing, is likely to mimic bone consumption (Kierdorf, 289 1994; Cáceres et al., 2011). This would be consistent with the low $\delta^{44/42}$ Ca values of modern roe 290 deer compared to other herbivores (Fig. 1). Similar dietary behavior could be at the origin of the 291 observed low $\delta^{44/42}$ Ca values in red deer (Fig. 2). 292

293

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

The wild boar (*Sus scrofa*) is the species with the highest average $\delta^{44/42}$ Ca value (-0.89 ± 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 the ingestion of soluble soil particles which have an isotopic composition that is close to zero permil (Tacail, 2017). Fruits and vegetables have generally high $\delta^{44/42}$ Ca values (Tacail et al., 2019, 2020). The consumption of grains has not been considered in the current study as it is mainly related to agricultural expansion in the Neolithic. Despite a lack of information on the paleoecology of *Sus scrofa*, high $\delta^{44/42}$ Ca values in fossil wild boar at Regourdou seems in good agreement with current knowledge on the diet of modern boars (Fournier-Chambrillon et al., 1995).

The brown bears (*Ursus arctos*) display a large variability of their $\delta^{44/42}$ Ca values, ranging 305 from 0.9 ‰ to -1.3 ‰ (SOM Table S1). This can be explained by their highly variable and 306 seasonally omnivorous diet (Robu et al., 2013). The cave bears (Ursus spelaeus) at Sclayn (Martin 307 et al., 2017) do not exhibit such a high Ca isotopic variability, because their dietary niche was 308 more restricted than for brown bears. Tooth shape and N isotope suggest an herbivorous diet for 309 this taxon (Bocherens et al., 1994; Pacher and Stuart, 2009). In extant brown bears, meat 310 consumption is described as highly variable depending on the resource (Pacher and Stuart, 2009). 311 Other scavenging behaviors, including necrophagia following hibernation, and bone gnawing, 312 313 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 314 highly variable diet, their $\delta^{44/42}$ Ca values being distinct by about 0.3 % (Fig. 2), indicating varying 315 Ca dietary sources during the period of bone formation. Indeed, the turnover is different between 316 trabecular and cortical bone. Patterns of bones remodeling among mammals other than humans, 317 nonhuman primates or laboratory rodents are poorly documented, but always faster for trabecular 318 than cortical bone. For humans, the bone remodeling rate has been reported to be 25% per year for 319 trabecular bone compared with 3–4% per year for cortical bone (Manolagas, 2000). In addition, 320 hibernation periods could have an impact on the bone Ca isotopic composition. Hibernation 321 induces a physiological period of reduced activity associated with a quantitative turnover of the 322

urea cycle (Barboza et al., 1997). Since the Ca fractionation is most likely driven by renal
physiology (Tacail 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 (McGeeLawrence 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.

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

The range of $\delta^{44/42}$ Ca values among carnivores (~0.6 ‰) is almost as great as among 331 herbivores (~0.8 ‰), suggesting highly variable sources of Ca for carnivores. The diet of 332 carnivores is mainly based on meat, i.e., mostly muscle, with possible incorporation of plant 333 matter in smaller quantities (Van Valkenburgh, 1996; Stanek et al., 2017; Gable et al., 2018). 334 Information on the variability of muscle $\delta^{44/42}$ Ca values is presently not as robust as for plants. 335 Pairing bone and muscle from a given specimen, considering our values and those compiled from 336 the literature (Skulan and DePaolo, 1999; Tacail et al., 2014), allows us to calculate an average 337 $\Delta^{44/42}$ Cabone-muscle offset value of -0.32 ‰ (± 0.12; 2SE; n = 14; Fig. 3). This value complements 338 the spacing between the $\Delta^{44/42}$ Ca_{bone-diet} offset value of -0.6 % (Tacail et al., 2020) and the trophic 339 level effect between bones of carnivores and herbivores of -0.3 ‰ (Tacail et al., 2020). Adding 340 0.3 % from the bone $\delta^{44/42}$ Ca value of herbivores matches the diet $\delta^{44/42}$ Ca value of carnivores. 341 Wolves (C. lupus) are carnivores but have a generalist diet (Stanek et al., 2017; Gable et al., 342 2018) being occasional consumers of fish, rodents and berries (Stanek et al., 2017; Gable et al., 343 2018). The Ca isotopic composition of fish (marine only as there are no data on freshwater fish) 344 and berries is close to 0‰ (Tacail, 2017; Tacail et al., 2020), and prey such as wild boars and 345 brown bears would explain the relatively high $\delta^{44/42}Ca$ value of wolf. This holds for the 346 indeterminate carnivore too, which might be a wolf. The cave lion (Panthera spelaea) has the 347

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 $\delta^{44/42}$ 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 $\delta^{44/42}$ Ca values, though this must remain speculative as none of these other species are represented in our faunal assemblage.

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4.4. The diet of the Regourdou 1 Neandertal

The Regourdou 1 samples display low $\delta^{44/42}$ Ca values (-1.58 ± 0.16 ‰; 2SD; *n* = 3), placing 356 the specimen amid carnivores and supporting the commonly accepted idea that Neandertals 357 mainly relied on meat consumption. Applying a $\Delta^{44/42}$ Cabone-muscle (-0.32 ± 0.06‰; SOM Table S1) 358 offset value to bone of herbivores to evaluate their muscle $\delta^{44/42}$ Ca value, and a $\Delta^{44/42}$ Ca_{bone-diet} (-359 0.6 %; SOM Table S1) offset value to the Neandertal, allows us to obtain the theoretical $\delta^{44/42}$ Ca 360 value of the Regourdou 1 diet. Results of this estimate show a diet based on red deer meat and 361 potentially brown bear, wolf and reindeer meat. Although rare, cut marks and anthropic fractures 362 363 of bones of reindeer, red deer and brown bear bones support this estimation (Cavanhié, 2011). 364 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 365 been documented at Mousterian sites (Auguste, 1995; Costamagno et al., 2009; Gómez-Olivencia 366 et al., 2018), the generalized consumption of carnivores such as brown bear and wolf seems 367 unlikely given the difficulties of hunting carnivores and the generally low abundance of carnivores 368 in the faunal spectrum of anthropic accumulation. The specialized consumption of red deer is 369 consistent with the C and N isotope studies that have placed Neandertals at the top of the trophic 370 371 chain and with a dietary preference for large herbivores (Bocherens et al., 2001; Naito et al.,

372 2016).

373	The fact that the three Neandertal bone samples have similar $\delta^{44/42}$ Ca values is consistent
374	with the bones belonging to the same individual (Madelaine et al., 2008; Gómez-Olivencia et al.,
375	2013; Maureille et al., 2015b). It is interesting to note, however, that the $\delta^{44/42}$ Ca value of the
376	trabecular bone sample (-1.56 $\% \pm 0.02$; 2SD) has a $\delta^{44/42}$ Ca value that is undistinguishable from
377	that of the cortical bone sample (-1.51‰ \pm 0.10; 2SD), contrasting with the large difference for the
378	brown bear. Considering that cortical and trabecular bone tissues have different rates of turnover
379	(Manolagas, 2000), this indicates a relatively stable diet at Regourdou 1 during the time of
380	remobilization of cortical bone and that of trabecular bone. Pairing cortical and trabecular modern
381	bone samples across animal species (Fig. 1; SOM Table S2 and SOM Fig. S6) shows that both
382	tissues have very close $\delta^{44/42}$ Ca values. This is not surprising because all of these animals, with the
383	exception of wild boars and roe deer, were fed on farms with presumably similar diets. Future
384	studies measuring trabecular and cortical bone from wild mammals will be more informative for
385	testing the existence of possible isotopic heterogeneities that may be characteristic of seasonal
386	dietary adaptations. This could lead to a renewed interest in isotopic signatures of trabecular bone,
387	which reflect recent variation in diet due to its higher rate of remodeling compared with cortical
388	bone; Manolagas, 2000), contributing further to our understanding of variation in dietary and
389	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 $\delta^{44/42}$ 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.

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398 4.5. Calcium isotope trophic shift due to bone consumption

The use of the above-mentioned $\Delta^{44/42}$ Ca_{bone-muscle} and $\Delta^{44/42}$ Ca_{bone-diet} offset values suggest a 399 highly specialized diet, based primarily on red deer, for the Regourdou 1 Neandertal. This 400 monospecific regime is dubious from the standpoint of hunting strategy and other taxa must have 401 been consumed as well (Delpech and Grayson, 2007). Another component needs to be considered 402 to match the very negative $\delta^{44/42}$ Ca values of the cave lion and the Neandertal. Bone marrow and 403 bone are the most likely candidates (Heuser et al., 2011) and would be consistent with the bone 404 fracture pattern observed at Mousterian sites (Costamagno, 2013; Costamagno and Rigaud, 2013). 405 Using the data obtained on associated bone, bone marrow, and muscle (Fig. 11; SOM Table S2), it 406 is possible to estimate the diet $\delta^{44/42}$ Ca value resulting from a mixture of meat and bone or bone 407 marrow (Fig. 4). 408

The $\delta^{44/42}$ Ca value of the dietary source was estimated using a simplified mixing model and 409 compared to the average isotopic composition of the main diet source of Ca of Regourdou 1 (-1.01 410 $\% \pm 0.16$; 2SD) illustrated by the grayed area in Fig. 4. We used the minimum, maximum and 411 average Ca concentrations in muscle ([Ca]_{min} = 163 ppm, Fig. 4A; [Ca]_{mean} = 633 ppm, Fig 4B; 412 $[Ca]_{max} = 3228 \text{ ppm}$, Fig. 4C) on a diet composed with equal proportions of horse, red deer and 413 wild boar, mixed with marrow containing different proportions of bone (0%, 1% and 10% of 414 weight). Changes in the respective contributions of these three taxa will not substantially change 415 the conclusion but will slightly affect the proportion of bone required to explain the Regourdou 1 416 diet. The consumption of trabecular bone in small proportion (only 0.1–1%, being accidental or 417 related to the hominin diet; Costamagno and Rigaud, 2013) during marrow consumption, can 418 explain the isotopic composition of Regourdou 1 and greatly limits the contribution of marrow 419 (<10%) and monospecific hunting. A mixed diet including bone marrow and accidental bone 420 consumption is also probable for the lion. Its lower $\delta^{44/42}$ Ca value compared to Regourdou 1, and 421 its stratigraphic position in layer 2, however, suggests a higher proportion of red deer and the 422

423	presence of reindeer in the diet (Figs. 4A–C). In contrast, the $\delta^{44/42}$ Ca values of the wolf and the
424	indeterminate carnivore $\delta^{44/42}$ Ca values are incompatible with a mixed diet made up of red deer
425	(Figs. 4A–C). The consumption of prey like boar, brown bear or prey with similar isotope
426	compositions, together with the absence of bone and low marrow ingestion, are more in agreement
427	with the bone $\delta^{44/42}$ Ca values of the wolf but not the Regourdou 1 Neandertal (Fig. 4D).
428	Based on the state of our current knowledge, the Ca isotopic signatures do not allow us to
429	differentiate between meat and plant consumption. As plants and meat have similar $\delta^{44/42}$ Ca
430	values, bone consumption thus appears to be the key parameter explaining the generalized
431	decrease in food chains of the $\delta^{44/42}$ Ca values between herbivores and carnivores. The
432	consumption of entire animal prey implies de facto the ingestion of a large part of the skeleton,
433	which is partly digested in the digestive tract of the predator (Massare, 1987; Baquedano et al.,
434	2012). Regarding large mammal carnivores (like hyena, wolf or lion), the prey is rarely ingested
435	all at once, but bony parts are most often accidentally ingested by the predator along with muscle
436	and organs. For humans, negative $\delta^{44/42}$ Ca values will be observed in the case of accidental bone
437	consumption. Archaeological evidence suggests that the ingestion of some trabecular bone during
438	marrow consumption is the most likely explanation. However, the ingestion of other bone parts
439	due to cutting, gnawing or for any social/culinary aspects cannot be excluded. Plant cannot be
440	excluded from the Neanderthal diet (Henry et al., 2011; Hardy et al., 2012) and could replace meat
441	in any proportion, the only constraint being that about 1–10% of the diet must be composed of
442	bone marrow (Figs. 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

448 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 449 herbivorous prey along with the associated consumption of trabecular bone likely occurring during 450 the ingestion of marrow. The same conclusion can be drawn for the cave lion, with a higher 451 proportion of red deer or other prey with similar $\delta^{44/42}$ Ca values not preserved at Regourdou. In 452 contrast, we show that the wolf and the indeterminate carnivore have a different diet, more 453 enriched in heavy Ca isotopes. The $\delta^{44/42}$ Ca values of the fauna exhibit an ecological patterning 454 that awaits further elucidation. Some explanations will probably come from a better knowledge of 455 plant $\delta^{44/42}$ Ca variability, notably in fruits and nuts. Other explanations will arise from an 456 improved understanding of the physiological processes involving Ca metabolism, e.g., kidney 457 function in hibernating species and the influence of rumination and antlerogenesis. 458

The two main advantages of Ca isotopes over the use of isotopic ratios of trace elements are 459 460 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 461 composition, allowing for collection of material without observable damage to bone and teeth. 462 The potential for renewed interest in trabecular bone isotopic signatures lies in the fact that it 463 could carry information about seasonal dietary changes when studied in comparison with cortical 464 bone. Our preliminary results of cortical versus trabecular bone Ca isotope differences for brown 465 bears suggesting dietary flexibility must be confirmed by further studies on other omnivorous 466 species. Collectively, these advantages are likely to turn Ca isotopes into an essential 467 interpretative tool for studying the evolution of diet in the human lineage. 468

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729 Figure Legends

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Figure 1. δ^{44/42}Ca values for modern samples (‰, relative to ICP Ca Lyon). 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).

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Figure 2. On the left, fossil mammal $\delta^{44/42}$ Ca values (‰ relative to the ICP Ca Lyon standard). Species are ranked from left to right according to their increasing average $\delta^{44/42}$ 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).

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Figure 3. Bone $\delta^{44/42}$ Ca values versus muscle $\delta^{44/42}$ 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.

Figure 4. Dietary d^{44/42}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 754 the expected range of diet d^{44/42}Ca values for the Regourdou 1 Neandertal individual. The dashed line 755 represents the expected dietary isotopic composition of the lion. A concentration of 100 ppm of Ca has 756 been used for marrow and a concentration of 40% of Ca for bone. A. Mixed diet with equal proportion of 757 horse, red deer and wild boar meat with 163 ppm of Ca. B. Mixed diet with equal proportion of horse, red 758 deer and wild boar meat with 633 ppm of Ca. C. Mixed diet with equal proportion of horse, red deer and 759 wild boar meat with 3228 ppm of Ca. D. 100 % boar diet, meat with 633ppm of Ca. The average of each 760 761 species is used for the calculation.







