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

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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.

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

3

4 **Abstract**

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

23

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

25

26 1. Introduction

27 The reconstruction of diet is an important aspect in paleoanthropology, archaeology and
28 prehistory, because diet is related to numerous human behaviors such as hunting practices, social
29 organization and mobility (e.g., Patou- Mathis, 2000; Costamagno et al., 2006; Hublin and
30 Richards, 2009). Paleoecological reconstructions are traditionally based on the study of faunal
31 assemblages of preserved remains and associated anthropogenic traces (Hublin and Richards,
32 2009), tooth microwear (Puech et al., 1983), dental size and morphology (Jiménez-Arenas et al.,
33 2014) or more rarely from preserved organic matter (e.g., phytoliths or DNA in dental calculus;
34 Henry et al., 2011). However, the reconstruction of ancient diet may be biased due to differential
35 transport of anatomical parts of prey, and to taphonomic processes (e.g. chemical process,
36 scavengers, water flow), which can lead to the lack of conservation of flora and other food
37 resources (Faith and Thompson, 2018) and can modify faunal assemblages even in well-preserved
38 sites (Fernandez-Jalvo and Andrews, 2016).

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

49 However, recent studies of dental calculus (Henry et al., 2011) and N isotopic composition
50 of amino acids (Naito et al., 2016; Jaouen et al., 2019) show that some Neandertals may have

51 consumed plants in sizeable proportion (~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
53 systems in fossil settings. First, the extraction of a sufficient amount of collagen from tooth or
54 bone requires the collection of between 10 mg and 500 mg of fossil material, depending on the
55 state of preservation of the collagen (Drucker et al., 1999; Bocherens et al., 2016). This amount is
56 a limiting factor because it represents a significant fraction, if not the whole root of a tooth, which
57 is a problem for precious material, notably unique hominin remains. Second, while collagen can
58 be preserved for more than 100 kyr in specific environments, cases of good preservation beyond
59 50 kyr are rare, preventing the use of N isotopic compositions in deep time (Melin et al., 2014).
60 This represents a great handicap for the study of Neandertals, for whom dietary habits throughout
61 the Paleolithic are key to understanding their cultural and biological evolution.

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

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 µg) 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 $\delta^{44/42}\text{Ca}$ values
89 between bone and diet ($\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$) 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 $\delta^{44/42}\text{Ca}$ 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 $\delta^{44/42}\text{Ca}$ values close to the range of variation of plants
100 (Tacail et al., 2019). Carnivores and herbivores should therefore have similar $\delta^{44/42}\text{Ca}$ values,

101 which is not the case. Bone consumption has been proposed to explain the observed difference in
102 $\delta^{44/42}\text{Ca}$ values of herbivores and carnivores (Heuser et al., 2011).

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

108

109 2. **Materials and Methods**

110 2.1. *History of the Regourdou site*

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

120 Between 1961 and 1964, the site was excavated by E. Bonifay (Bonifay, 1964). The
121 Regourdou 1 skeleton was located in Bonifay’s stratigraphic layer 4 (Bonifay, 1964), which
122 contained lithic remains belonging to a Discoid Mousterian techno-complex with the production
123 of pseudo-Levallois points and a temperate fauna highly dominated by brown bears (*Ursus*
124 *arctos*). This layer 4 was attributed to MIS 5 based on cultural evidence and this was confirmed by
125 a recent Optically-Stimulated Luminescence date (Bonifay, 1964; Bonifay et al., 2007; Maureille

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
128 (a pit and a pile of stones, or ‘pierrier’ in French, as well as various grave goods) and buried parts
129 of brown bear corpses in an alleged pit closed by a several-ton flat limestone slab, along with
130 other stone piles within layers 3 and 5 (Bonifay, 1962). However, a recent review (Madelaine et
131 al., 2008) of the 1957 field notes and drawings, as well as the 1961 to 1964 field notebooks and
132 collections (allowing the discovery of 73 new Regourdou 1 hominin remains; Madelaine et al.,
133 2008; Maureille et al., 2015b), questioned the Bonifay interpretation of a unique Mousterian
134 symbolic cave, even if the new scientific team involved in this research still considers Regourdou
135 1 as a Neandertal intentional burial (Madelaine et al., 2008; Maureille et al., 2015a, 2016; Pelletier
136 et al., 2017). This is an ancient cave that was used as a den for brown bears which are represented
137 almost in the lower levels (Cavanhié, 2011). Subsequently the cavity collapsed, forming a trap
138 allowing the accumulation of many fauna (Bonifay, 2002; Cavanhié, 2011). The Neandertal is
139 assumed not to be a major accumulating agent because only a small number of bones show
140 anthropic action (Cavanhié, 2011). Due to age and taphonomic process, resulting in an insufficient
141 quality of collagen, ancient DNA and C and N isotopic compositions failed to provide any
142 paleogenetic or paleodietary information (Bocherens, pers. comm.).

143

144 2.2. *Regourdou fossil bone samples*

145 The Ca isotopic composition was measured for 30 samples obtained from eight different
146 mammalian species, including four reindeer (*Rangifer tarandus*), five red deer (*Cervus elaphus*),
147 two horses (*Equus caballus*), two wild boars (*Sus scrofa*), one cave lion (*Panthera spelaea*), one
148 wolf (*Canis lupus*), one carnivore of indeterminate species, 11 brown bears (*Ursus arctos*) and
149 three bone samples of the Regourdou 1 Neandertal individual (SOM Table S1). The epiphyseal
150 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
152 Table S1): ten are from layer 2, 16 from layer 4, including the Neandertal specimen, three from
153 layer 5 and one from layer 7. Layers 4, 5 and 7 are thought to be contemporaneous and layer 2
154 younger (Bonifay et al., 2007; Cavanhié, 2011). All but two of the samples were taken from
155 cortical bone; trabecular bone was sampled from one brown bear and from the left femur of
156 Regourdou 1. Each sample of trabecular bone was paired with a sample of cortical bone taken
157 from an adjacent region of the same piece of bone. Each sample was obtained by excising a
158 fragment of bone <5 mm long and 1 mm wide using a scalpel (SOM Fig. S2). Samples were
159 preferentially taken from parts that were already damaged during excavation or during the post-
160 excavation history or in areas already sampled for previous analyses. This sampling method
161 allowed us to preserve as much as possible the morphological and structural integrity of the
162 pieces.

163

164 2.3. *Modern faunal bone and muscle samples*

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

175

176 2.4. *Analytical methods*

177 The analyses of Ca stable isotope compositions were carried out at the Laboratoire de
178 géologie de Lyon, Terre, Planète et Environnement at the Ecole Normale Supérieure de Lyon. All
179 30 bone samples from Regourdou's faunal and paleoanthropological collections (SOM Table S1)
180 were first ground using an agate mortar and pestle before being leached with diluted ultrapure
181 acetic acid (0.1M) for 30 minutes at room temperature in an ultrasonic bath to eliminate Ca-
182 bearing secondary carbonates, particularly calcite (Balter et al., 2002). All modern samples were
183 first freeze-dried and digested in concentrated HNO₃ acid to dissolve the organic phase.

184 The concentrations of major and trace elements were measured after a leaching step with
185 acetic acid (SOM Tables S1 and S3) and diagenesis was monitored using the calcium/phosphorus,
186 iron/calcium, manganese/calcium, and uranium/calcium ratios (Ca/P, Fe/Ca, Mn/Ca, and U/Ca,
187 respectively; SOM Table S1) and the sum of all the rare earth elements (Σ REE; SOM Table S3).
188 These measurements were performed by Quadrupole Inductively-Coupled-Plasma Mass
189 Spectrometry (Q-ICP-MS) for trace elements and by Inductively-Coupled-Plasma Optical
190 Emission Spectroscopy (ICP-OES) for major elements, respectively. Methods for the
191 measurement of trace and major elements are briefly described here (for a complete description
192 see Balter and Lécuyer, 2004, 2010). The concentrations of elements were calculated using
193 calibration curves based on multi-elemental solutions. These solutions were also used to monitor
194 and correct for the instrumental drift over the analytical session. Matrix effects and instrumental
195 drift were also corrected using indium (In) and scandium (Sc) as internal standards for trace and
196 major elements, respectively. Comparison of major and trace element was performed using a
197 simple linear regression model and the correlation coefficient represented with an heatmap
198 (correlation matrix). All the statistical analyses on the Ca isotopes and other elements were
199 performed in R (V3.6.1; R Core Team, 2020).

200 All samples were processed in order to extract the bone's Ca according to the protocols
201 established by Tacail et al. (2014). Briefly, this consists of a digestion step in concentrate HNO₃
202 and solubilization in 6N HCl. Then, three chromatography steps are necessary, the first to allow
203 the recovery of Ca, Fe and Sr only, the second to isolate Sr, and the third to separate Ca from Fe.
204 The compositions of the Ca isotopes were measured using a Neptune Plus multi-collector ICP-MS
205 using the standard-sample-standard-bracketing method. All Ca isotope compositions are expressed
206 using the delta notation and were calculated using the inductively coupled plasma (ICP) Ca Lyon
207 standard as bracketing and reference material. A solution of the certified reference material NIST
208 SRM-1400 (bone ash) was repeatedly purified and measured as a sample to control the accuracy
209 of the measurements of archaeological bone and a solution of the certified reference material
210 NIST SRM-1486 (bone meal) was repeatedly measured as a sample to control the accuracy of the
211 measurements of modern bone and muscle samples. The SRM-1486 certified reference material
212 yielded a value of -1.07 ± 0.049 , 2 SE, $n = 24$), which is undistinguishable from all SRM-1486
213 reported in other studies (-1.009 ± 0.026 , 2 SE; Heuser and Eisenhauer, 2008; Heuser et al., 2011,
214 2016a, 2016b). The $\delta^{44/42}\text{Ca}$ annotation is defined according to the following equation:

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

216 To facilitate comparison of our dataset with other Ca isotope studies, the values obtained in this
217 study are also expressed relative to the SRM-915a standard using a constant difference of $-0.518 \pm$
218 0.025% (2 SD).

219

220 **3. Results**

221 *3.1. Calcium-normalized ratios and diagenesis*

222 Four samples (R12, R18, R20 and R22) display significant REE enrichments together with a
223 concomitant increase of the Fe/Ca ratio (SOM Fig. S3A) and Mn/Ca ratio (SOM Fig. S3B), but

224 without any U enrichment (SOM Fig. S3C). The comparison of Ba/Ca ratios with REE, U/Ca and
225 Mn/Ca suggests a diagenetic Ba/Ca overprinting (SOM Figs. S3D and S4). The sample R11 seems
226 to be highly impacted by Mn, U and Ba diagenetic alteration and uptake (SOM Figs. S3B–S3D).
227 The Sr/Ca ratios seem insensitive to REE enrichment (SOM Fig. S3E) and no clear relationship is
228 observed with the either Fe/Ca (SOM Fig. S3F) or the Mn/Ca (SOM Fig. S3G) ratio. None of the
229 diagenesis-sensitive Ca-normalized ratios displays any correlation with the $\delta^{44/42}\text{Ca}$ values and the
230 Ca/P ratios, when highly altered samples (i.e., R11–R12, R18, R20 and R22) are excluded (Fig. S4).
231 Taken together, the results suggest that while the Ba/Ca ratios, and potentially the Sr/Ca ratios, are
232 affected by some diagenetic alteration, the Ca isotopic compositions of specimens included in this
233 study reflect original values and can be used to constrain the paleodiet of species retrieved at
234 Regourdou. Note that the faunal pattern of the Sr/Ca ratio at Regourdou is similar to that reported
235 for the Saint-Césaire assemblage (Balter et al., 2001, 2003), which argues for a minimal diagenetic
236 overprint of the Sr/Ca ratio at Regourdou.

237

238 *3.2. Calcium isotope analyses*

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

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

257

258 **4. Discussion**

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

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

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

293

294 4.2. *The ecological and physiological significance of Calcium isotopes in omnivores*

295 The wild boar (*Sus scrofa*) is the species with the highest average $\delta^{44/42}\text{Ca}$ value ($-0.89 \pm$
296 0.28 ‰ , 2SD, $n = 2$) in the Regourdou faunal assemblage (Fig. 2). The main components of its
297 current diet include rhizomes, acorns and grains (Fournier-Chambrillon et al., 1995). The

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

305 The brown bears (*Ursus arctos*) display a large variability of their $\delta^{44/42}\text{Ca}$ values, ranging
306 from 0.9 ‰ to -1.3 ‰ (SOM Table S1). This can be explained by their highly variable and
307 seasonally omnivorous diet (Robu et al., 2013). The cave bears (*Ursus spelaeus*) at Sclayn (Martin
308 et al., 2017) do not exhibit such a high Ca isotopic variability, because their dietary niche was
309 more restricted than for brown bears. Tooth shape and N isotope suggest an herbivorous diet for
310 this taxon (Bocherens et al., 1994; Pacher and Stuart, 2009). In extant brown bears, meat
311 consumption is described as highly variable depending on the resource (Pacher and Stuart, 2009).
312 Other scavenging behaviors, including necrophagia following hibernation, and bone gnawing,
313 which can be associated with direct bone ingestion, are also reported. The Ca isotope
314 compositions of trabecular and cortical bone of the brown bear (sample D2-33; Fig. 2) support a
315 highly variable diet, their $\delta^{44/42}\text{Ca}$ values being distinct by about 0.3 ‰ (Fig. 2), indicating varying
316 Ca dietary sources during the period of bone formation. Indeed, the turnover is different between
317 trabecular and cortical bone. Patterns of bones remodeling among mammals other than humans,
318 nonhuman primates or laboratory rodents are poorly documented, but always faster for trabecular
319 than cortical bone. For humans, the bone remodeling rate has been reported to be 25% per year for
320 trabecular bone compared with 3–4% per year for cortical bone (Manolagas, 2000). In addition,
321 hibernation periods could have an impact on the bone Ca isotopic composition. Hibernation
322 induces a physiological period of reduced activity associated with a quantitative turnover of the

323 urea cycle (Barboza et al., 1997). Since the Ca fractionation is most likely driven by renal
324 physiology (Tacail et al., 2020), a possible specific isotopic fractionation could be induced by
325 hibernation. However, the inanition period due to hibernation does not induce bone loss (McGee-
326 Lawrence et al., 2015). Thus, there is little evidence showing that Ca isotope compositions of bone
327 will be affected by hibernation. Collectively, their dietary and hibernation behavior might explain
328 the high Ca isotopic variability observed in brown bears.

329

330 4.3. *The ecological and physiological significance of Calcium isotopes in carnivores*

331 The range of $\delta^{44/42}\text{Ca}$ values among carnivores (~ 0.6 ‰) is almost as great as among
332 herbivores (~ 0.8 ‰), suggesting highly variable sources of Ca for carnivores. The diet of
333 carnivores is mainly based on meat, i.e., mostly muscle, with possible incorporation of plant
334 matter in smaller quantities (Van Valkenburgh, 1996; Stanek et al., 2017; Gable et al., 2018).
335 Information on the variability of muscle $\delta^{44/42}\text{Ca}$ values is presently not as robust as for plants.
336 Pairing bone and muscle from a given specimen, considering our values and those compiled from
337 the literature (Skulan and DePaolo, 1999; Tacail et al., 2014), allows us to calculate an average
338 $\Delta^{44/42}\text{Ca}_{\text{bone-muscle}}$ offset value of -0.32 ‰ (± 0.12 ; 2SE; $n = 14$; Fig. 3). This value complements
339 the spacing between the $\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$ offset value of -0.6 ‰ (Tacail et al., 2020) and the trophic
340 level effect between bones of carnivores and herbivores of -0.3 ‰ (Tacail et al., 2020). Adding
341 0.3 ‰ from the bone $\delta^{44/42}\text{Ca}$ value of herbivores matches the diet $\delta^{44/42}\text{Ca}$ value of carnivores.

342 Wolves (*C. lupus*) are carnivores but have a generalist diet (Stanek et al., 2017; Gable et al.,
343 2018) being occasional consumers of fish, rodents and berries (Stanek et al., 2017; Gable et al.,
344 2018). The Ca isotopic composition of fish (marine only as there are no data on freshwater fish)
345 and berries is close to 0‰ (Tacail, 2017; Tacail et al., 2020), and prey such as wild boars and
346 brown bears would explain the relatively high $\delta^{44/42}\text{Ca}$ value of wolf. This holds for the
347 indeterminate carnivore too, which might be a wolf. The cave lion (*Panthera spelaea*) has the

348 lowest Ca isotopic composition (-1.73 ‰) which aligns well with the low Ca isotopic composition
349 of felidae measured by Martin et al. (2018) in the modern ecosystem of Tsavo (Kenya). These low
350 $\delta^{44/42}\text{Ca}$ values evoke a monospecific diet for cave lions as already suggested by C and N isotopes
351 (Bocherens et al., 2011) and could be explained at Regourdou by a diet mainly based on red deer
352 and/or other species with low $\delta^{44/42}\text{Ca}$ values, though this must remain speculative as none of these
353 other species are represented in our faunal assemblage.

354

355 4.4. *The diet of the Regourdou 1 Neandertal*

356 The Regourdou 1 samples display low $\delta^{44/42}\text{Ca}$ values (-1.58 ± 0.16 ‰; 2SD; $n = 3$), placing
357 the specimen amid carnivores and supporting the commonly accepted idea that Neandertals
358 mainly relied on meat consumption. Applying a $\Delta^{44/42}\text{Ca}_{\text{bone-muscle}}$ (-0.32 ± 0.06 ‰; SOM Table S1)
359 offset value to bone of herbivores to evaluate their muscle $\delta^{44/42}\text{Ca}$ value, and a $\Delta^{44/42}\text{Ca}_{\text{bone-diet}}$ ($-$
360 0.6 ‰; SOM Table S1) offset value to the Neandertal, allows us to obtain the theoretical $\delta^{44/42}\text{Ca}$
361 value of the Regourdou 1 diet. Results of this estimate show a diet based on red deer meat and
362 potentially brown bear, wolf and reindeer meat. Although rare, cut marks and anthropic fractures
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
365 environment of Regourdou 1. Although possible consumption or exploitation of carnivores has
366 been documented at Mousterian sites (Auguste, 1995; Costamagno et al., 2009; Gómez-Olivencia
367 et al., 2018), the generalized consumption of carnivores such as brown bear and wolf seems
368 unlikely given the difficulties of hunting carnivores and the generally low abundance of carnivores
369 in the faunal spectrum of anthropic accumulation. The specialized consumption of red deer is
370 consistent with the C and N isotope studies that have placed Neandertals at the top of the trophic
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 $\delta^{44/42}\text{Ca}$ 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 $\delta^{44/42}\text{Ca}$ value of the
376 trabecular bone sample ($-1.56\text{‰} \pm 0.02$; 2SD) has a $\delta^{44/42}\text{Ca}$ value that is undistinguishable from
377 that of the cortical bone sample ($-1.51\text{‰} \pm 0.10$; 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 $\delta^{44/42}\text{Ca}$ 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.

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

396

397

398 4.5. *Calcium isotope trophic shift due to bone consumption*

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

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

423 presence of reindeer in the diet (Figs. 4A–C). In contrast, the $\delta^{44/42}\text{Ca}$ values of the wolf and the
424 indeterminate carnivore $\delta^{44/42}\text{Ca}$ 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 $\delta^{44/42}\text{Ca}$ 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 $\delta^{44/42}\text{Ca}$
430 values, bone consumption thus appears to be the key parameter explaining the generalized
431 decrease in food chains of the $\delta^{44/42}\text{Ca}$ 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 $\delta^{44/42}\text{Ca}$ 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).

443

444 **5. Conclusions**

445 The use of Ca isotopes allows for the first time the dietary reconstruction of a MIS 5
446 Neandertal fossil from Southwest of France, Regourdou 1, in the absence of collagen. We show
447 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
449 Regourdou 1 Neandertal was predominantly carnivorous, consuming a mixture of various
450 herbivorous prey along with the associated consumption of trabecular bone likely occurring during
451 the ingestion of marrow. The same conclusion can be drawn for the cave lion, with a higher
452 proportion of red deer or other prey with similar $\delta^{44/42}\text{Ca}$ values not preserved at Regourdou. In
453 contrast, we show that the wolf and the indeterminate carnivore have a different diet, more
454 enriched in heavy Ca isotopes. The $\delta^{44/42}\text{Ca}$ values of the fauna exhibit an ecological patterning
455 that awaits further elucidation. Some explanations will probably come from a better knowledge of
456 plant $\delta^{44/42}\text{Ca}$ variability, notably in fruits and nuts. Other explanations will arise from an
457 improved understanding of the physiological processes involving Ca metabolism, e.g., kidney
458 function in hibernating species and the influence of rumination and antlerogenesis.

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

469

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729 **Figure Legends**

730

731 **Figure 1.** $\delta^{44/42}\text{Ca}$ values for modern samples (‰, relative to ICP Ca Lyon). Individuals are grouped by
732 species. The shape and colors correspond to the different sampled hard or soft tissues. The above ground
733 biomass (AGB) line is the mean of AGB for European grass and herbs compiled from Skulan and
734 DePaolo (1999), Chu et al. (2006), Hindshaw et al. (2013), Moore et al. (2013), Christensen et al. (2018).
735 All silhouettes are taken from www.phylopic.org (Public Domain license).

736

737 **Figure 2.** On the left, fossil mammal $\delta^{44/42}\text{Ca}$ values (‰ relative to the ICP Ca Lyon standard). Species
738 are ranked from left to right according to their increasing average $\delta^{44/42}\text{Ca}$ values. All samples were
739 measured at least three times; the typical error bar represents the largest 2SD. All silhouettes are taken
740 from www.phylopic.org (Public Domain license). On the right, distribution of isotope composition of
741 current European plants, compiled from Skulan and DePaolo (1999), Schmitt et al. (2003), Chu et al.
742 (2006) Page et al. (2008), Bagard et al. (2013), Hindshaw et al. (2013), Moore et al. (2013), and
743 Christensen et al. (2018).

744

745 **Figure 3.** Bone $\delta^{44/42}\text{Ca}$ values versus muscle $\delta^{44/42}\text{Ca}$ values (‰, relative to ICP Ca Lyon standard). The
746 red line corresponds to the regression line (slope = 0.607 ± 0.325 ; 2SE, intercept = 0.028 ± 0.260 ; 2SE)
747 calculated for all samples analyzed in this study and compiled from the literature (Skulan and DePaolo,
748 1999; Tacail et al., 2014). The gray area represents the 95% confidence interval for the linear regression.
749 The dotted line is 1:1 and the black line represents the straight line of the equation $y = x + 0.32$,
750 corresponding to the average offset between cortical bone and its paired muscle.

751 **Figure 4.** Dietary $\delta^{44/42}\text{Ca}$ values (‰, relative to ICP Ca Lyon) as a function of the amount of bone
752 marrow and bone (%) relative to meat, as calculated with a mixed model similar to Heuser et al. (2011).
753 The yellow curve is for 0% by weight of bone in the marrow, the blue curve is for 1% by weight of bone

754 in the marrow and the purple curve is for 10% by weight of bone in the marrow. The gray area represents
755 the expected range of diet $\delta^{44/42}\text{Ca}$ values for the Regourdou 1 Neandertal individual. The dashed line
756 represents the expected dietary isotopic composition of the lion. A concentration of 100 ppm of Ca has
757 been used for marrow and a concentration of 40% of Ca for bone. A. Mixed diet with equal proportion of
758 horse, red deer and wild boar meat with 163 ppm of Ca. B. Mixed diet with equal proportion of horse, red
759 deer and wild boar meat with 633 ppm of Ca. C. Mixed diet with equal proportion of horse, red deer and
760 wild boar meat with 3228 ppm of Ca. D. 100 % boar diet, meat with 633ppm of Ca. The average of each
761 species is used for the calculation.

762

Figure 1

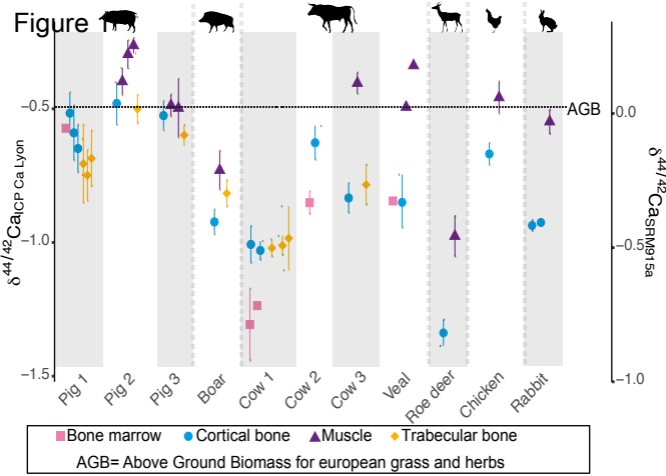
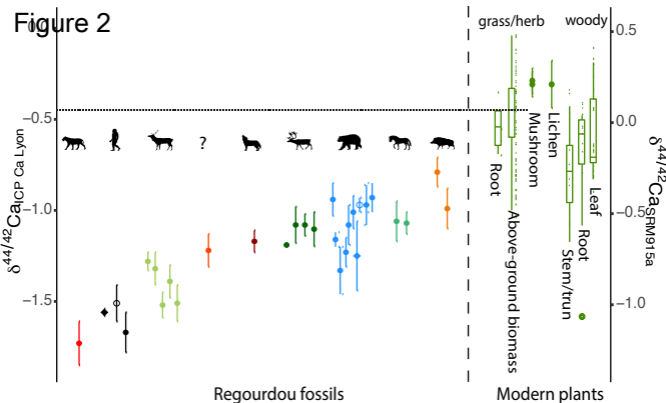


Figure 2



- | | | | |
|----------------------------|-----------------------|---------------------------|-------------------------|
| ● <i>Equus caballus</i> | ● <i>Ursus arctos</i> | ● <i>Panthera spelaea</i> | ○ Left femur cortical |
| ● <i>Rangifer tarandus</i> | ● <i>Sus scrofa</i> | ● <i>Neandertal</i> | ◆ Left femur trabecular |
| ● <i>Cervus elaphus</i> | ● <i>Canis lupus</i> | ● indet. carnivore | ○ D2-33 cortical |
| | | | ◆ D2-33 trabecular |

Figure 3

Regression : $Y = A * X + B$
 $A = 0.607 \pm 0.325$ (2SE)
 $B = 0.028 \pm 0.260$ (2SE)

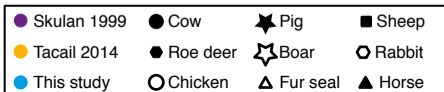
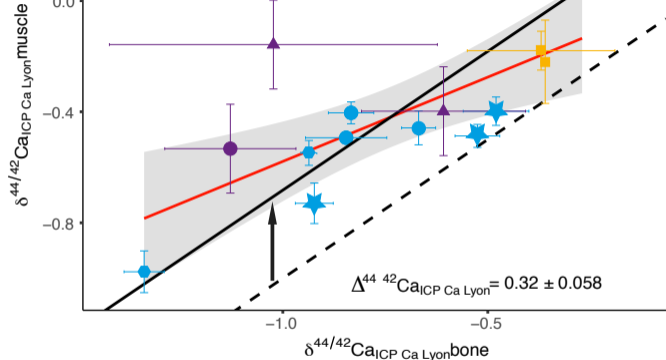


Figure 4