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1 **New frontiers in calcium stable isotope geochemistry:**

2 **Perspectives in present and past vertebrate biology**

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18 **Abstract**

19 Beyond their established uses in Earth and Planetary sciences, calcium (Ca) isotopes
20 have a promising future in the study of the biology of present and past vertebrates, including
21 humans. Early work paved the way to the ongoing research on the potential of Ca isotopes
22 as relevant tools to disciplines other than geology, including palaeobiology, bioarchaeology
23 and biomedical research. In this article, we first review the rationale behind the cycling of Ca
24 isotopes in vertebrate organisms. We then summarize and discuss the use of Ca isotopes as
25 dietary tracers from trophic reconstructions in past vertebrate ecosystems to the tracking of
26 early life dietary transitions. Next, we review and examine the research outcomes on the
27 potential of Ca isotopes as biomarkers of bone loss in physiological and pathological
28 conditions such as bone cancers and osteoporosis. While emphasizing the needs of future
29 research in each of these applications, we suggest new potential uses of Ca isotopes in
30 vertebrate biology. Finally, we identify challenges and barriers faced when developing such
31 interdisciplinary projects and suggest how these can be overcome.

32

33 **Keywords:**

- 34 Calcium isotopes,
- 35 Vertebrate biology,
- 36 Palaeobiology,
- 37 Bioarchaeology,
- 38 Biomedical research,
- 39 Bone loss

40

41 Preliminary remarks

42 Here, we shortly introduce the concepts of stable isotope geochemistry used in this
43 review. **Isotopes** of a given element have nuclei made of a given number of protons but a
44 varying number of neutrons. Depending on the number of neutrons, the nucleus of a given
45 isotope is either stable over geological timescales or disintegrates into daughter nucleus or
46 nuclei by radioactive decay. Isotopes are commonly discussed in scientific literature in
47 connection to radiometric dating for instance in geology, archaeology or palaeontology, or as
48 artificially prepared stable or radioactive nuclides used as tracers or as radiation sources in
49 biomedical contexts for example. This paper is not concerned with any of these widely known
50 applications, but with the study of variations in natural abundances of isotopes – referred to
51 as **isotope compositions** – of elements with more than one naturally occurring isotope. The
52 isotopes of interest are either stable (e.g., here ^{40}Ca , ^{42}Ca , ^{43}Ca , ^{44}Ca and ^{46}Ca) or have half-
53 lives so long that their instability can be ignored in most contexts (e.g., ^{48}Ca). The stable
54 isotope compositions are measured as abundance ratios (e.g., $^{44}\text{Ca}/^{42}\text{Ca}$ or $^{44}\text{Ca}/^{40}\text{Ca}$) with
55 mass-spectrometers that allow separation and “counting” of accelerated ions according to
56 their atomic masses (and charge). Stable isotope compositions are often expressed as
57 deviations relative to isotope ratios measured in a reference material, by means of the
58 “**delta**” **notation** defined as follows (here for $^{44}\text{Ca}/^{42}\text{Ca}$ ratios):

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

59 where $^{44}\text{Ca}/^{42}\text{Ca}$ refer to abundance ratios of sample (*sple*) or standard (*std*, *i.e.* SRM915a in
60 this article), and $\delta^{44/42}\text{Ca}$ values are expressed in per mil (‰). Variations in the **stable**
61 **isotope compositions** of an element result from differences in their mass and nuclear
62 structure. Physical and chemical processes acting on these differences can “**fractionate**”
63 isotopes, *i.e.* selectively partition isotopes between pools, for example between reactants

64 and products in a chemical reaction. Isotope fractionation based on differences in mass
65 between isotopes is referred to as “**mass dependent fractionation**”. This implies that the
66 amplitude of a mass dependent fractionation is a function of the mass difference between the
67 considered isotopes. Isotope fractionation based on other differences, such as nuclear
68 magnetic effects, is called “**mass independent fractionation**” (e.g., Dauphas and Schauble
69 2016). All of the isotope effects discussed in this paper are mass dependent. This notably
70 implies that the $\delta^{44/40}\text{Ca}$ value of a given material is approximately twice its $\delta^{44/42}\text{Ca}$ value.
71 Mass dependent isotope fractionation between freely exchanging pools at equilibrium, such
72 as reactants and products in a reversible chemical reaction, is known as “**equilibrium**
73 **isotope fractionation**” and are notably a function of temperature at Earth’s surface. “**Kinetic**
74 **isotope fractionation**” is associated with unidirectional incomplete processes, such as rapid
75 change in phase. This paper deals with both equilibrium and kinetic effects. Readers wanting
76 to further familiarize with concepts of metal stable isotope (bio-)geochemistry can also refer
77 to several introductory reviews and book sections (e.g., Albarède, 2015, Albarède et al.,
78 2017, Martin et al., 2017a, Jaouen and Pons 2016, Wombacher et al., 2016).

80 **1. Introduction**

81 Stable isotope geochemistry has a rich history of fruitful interdisciplinary collaboration,
82 resulting in the adoption of isotope geochemical techniques and concepts in fields such as
83 ecology, forensics and archaeology. Calcium (Ca) stable isotopes have been found useful in
84 the earth and planetary sciences, for example in reconstructing secular variations in
85 seawater composition (e.g., De La Rocha and DePaolo, 2000; Fantle and DePaolo, 2005,
86 2007; Farkaš et al., 2007a, 2007b; Hinojosa et al., 2012; Jost et al., 2014; Le Houedec et al.,
87 2017; A. D. Schmitt et al., 2003). However, promising potential applications of calcium
88 isotopes also lie outside of geology. Calcium isotopes have the potential to become an
89 important tool in vertebrate biology and biomedicine, notably if challenges to their
90 widespread application can be overcome.

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91 The abundance and widely shared vital functions of Ca in vertebrate organisms
92 makes it a crucial stable isotope system in the evolution and biology of vertebrates, including
93 humans. The appearance in Cambrian marine vertebrates of calcium phosphate mineral
94 tissues, i.e. bioapatite, marks the development of revolutionary strategies in the physiological
95 regulation of Ca and inorganic phosphate, that are now widely shared among vertebrates
96 (e.g., Bouillon and Suda, 2014; Doherty et al., 2015). Calcium phosphate minerals have a
97 high preservation potential and are globally more resistant to diagenesis than carbonates.
98 This has resulted in a rich vertebrate fossil record (e.g., Armstrong et al., 2001; Barham et
99 al., 2012; Hinojosa et al., 2012; Joachimski et al., 2004; Kohn and Cerling, 2002; Luz et al.,
100 1984; Pucéat et al., 2004; Wenzel et al., 2000).

101 The pioneering work on Ca isotopes in biology was done by J.L. Skulan in the late
102 1990s (Skulan et al., 1997; Skulan and DePaolo, 1999; Skulan, 1999). Their key finding was
103 that mineralized tissues, including bone, are significantly and constantly depleted in heavy
104 isotopes of Ca when compared to Ca dietary sources. Following this early work, an
105 increasing number of investigators have explored biological and palaeobiological applications
106 of Ca stable isotopes. These explorations have proceeded in two main directions. The first
107 aims at using Ca isotopes to reconstruct the dietary habits of present and extinct vertebrates
108 in order to assess the trophic structures of past ecosystems, or the dietary behaviours of past
109 human individuals and populations. This field of research (that could be referred to as Metal
110 Stable Isotope Palaeobiology or Bioarchaeology) tackles questions relevant to
111 palaeontology, (palaeo-)ecology, archaeology and anthropology. The second topic of
112 ongoing research aims at developing the use of Ca isotopes to better understand and
113 diagnose human metabolic bone disorders, such as bone loss induced by inactivity,
114 microgravity or disease, including osteoporosis and cancers affecting bone. This field of
115 research (that could be referred to as Stable Isotope Metallomics as proposed by Albarède
116 (2015)) is at the crossroads between isotope geochemistry, fundamental physiopathology
117 and applied biomedical research.

119 However, beyond the challenges inherent in extending fundamental understanding
1 and developing practical applications, these projects also face difficulties that are common to
2 120
3
4 121 all interdisciplinary research. More than 15 years have passed by since the first recognition
5
6 122 of the potential of Ca isotopes in vertebrate biology, and yet only about 30 articles have been
7
8 123 published on the topic during this period (Figure 1). Calcium isotopes remain poorly utilized
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10
11 124 in scientific communities outside earth and planetary sciences. This raises the following
12
13 125 question: what obstacles stand in the way of more widespread applications of Ca stable
14
15 126 isotope analysis?

17 127 In this article, we first briefly summarize the current understanding of the Ca isotope
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19 128 cycling in vertebrates before reviewing and discussing the potential and future of Ca isotopes
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21 129 in palaeobiology and biomedical research. Finally, we identify challenges and barriers related
22
23 130 to the collaborative research necessary for the adoption of Ca isotope tools by the wider
24
25 131 scientific community.

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31 133 **2. The cycling of calcium and its isotopes in vertebrates**

33 134 The biological processing of Ca by vertebrates induces mass dependent isotope
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35 135 fractionations responsible for maintained and observable heterogeneous distributions of Ca
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37 136 isotopes amongst the different compartments of the organisms. Hence, the distribution and
38
39 137 biological functions of Ca in vertebrates are central features of the potential of Ca stable
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41 138 isotopes as tools to study their biology.

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47 140 **2.1. Distribution, functions and regulation of Ca in vertebrates**

49 141 In vertebrate organisms, the distribution of Ca²⁺ is highly compartmentalized at all
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51 142 anatomical levels (e.g., Del Valle et al., 2011; Doherty et al., 2015; Peterson and Riggs,
52
53 143 2010). Briefly, most body Ca is stored in bone mineral as a major constituent of bioapatite
54
55 144 (Ca₁₀(PO₄)₆(OH)₂). Mineralized tissues account for ca. 99 % of body Ca in vertebrates, while
56
57 145 the remaining fraction is found in non-mineralized or so-called soft tissues. The majority of
58
59 146 soft tissue Ca (around 90 %) is found inside cells (mainly in organelles), while extracellular

147 fluids including blood contain about 10 % of the soft tissue Ca. However, the free Ca^{2+} of
148 extracellular fluids is 10,000 to 20,000 times more concentrated than intracellular cytosolic
149 Ca^{2+} (Case et al., 2007). This constitutes a steep and actively maintained chemical gradient
150 between extracellular and intracellular milieus.

151 The heterogeneous and tightly regulated distribution of Ca reflects the adaptation to
152 the vital biological functions to which it contributes (see Berridge et al., 2000; Bootman, 2012;
153 Brini et al., 2013; Brown and MacLeod, 2001; Carafoli, 2002; Clapham, 1995). Calcium
154 interacts with numerous proteins, either for storage and transport or for the regulation of
155 protein activity. In multicellular organisms, Ca^{2+} is a ubiquitous intracellular second
156 messenger regulating numerous electrical, chemical, mechanical and genetic cellular
157 responses. From the cell to the whole organism, Ca^{2+} is a critical component in many vital
158 functions, including cell differentiation or death, muscle contraction or propagation of
159 electrical signals in some nerve cells. The preservation of these vital functions primarily
160 depends on the fine regulation of Ca^{2+} cycle and on the blood Ca^{2+} concentrations (Figure 2).

161 In terrestrial tetrapods, the principal source of Ca is the intestinal absorption of dietary
162 Ca, while it is also exchanged with ambient water through gills in bony and cartilaginous
163 fishes and through skin to a certain extent in amphibians (Bouillon and Suda, 2014; Doherty
164 et al., 2015; Stiffler, 1993). Blood and extracellular fluids exchange Ca^{2+} with various non-
165 excreting reservoirs, such as muscle and more importantly with bone, which acts as a
166 dynamic storage organ, especially in terrestrial vertebrates (Bouillon and Suda, 2014).
167 Various sinks balance the incoming Ca^{2+} fluxes, with contrasting contributions depending on
168 species and life stages. In mammals, skin, hair and sweat account for some of the Ca^{2+}
169 losses but the main loss occurs via urinary excretions and digestive secretions through
170 faeces in most vertebrates. Tissues playing the roles of Ca^{2+} dynamic stores (e.g., bone) and
171 interfaces exchanging with the environment (e.g., gills, intestine, kidneys) participate to the
172 regulation of the whole organism Ca cycle, called Ca homeostasis (Bouillon and Suda, 2014;
173 de Matos, 2008; Doherty et al., 2015; Flik and Verbost, 1993; Peacock, 2010; Stiffler, 1993).
174 In humans, many physiopathological conditions are associated with disrupted Ca cycles.

175 Chronic perturbation of Ca and phosphate homeostasis may lead to metabolic bone disease
176 (e.g., osteoporosis) or result from disease (e.g., cancers affecting bone, c.f. Peacock, 2010).

177 In reproductive contexts, Ca^{2+} cycles are modified especially in female organisms. In
178 mammals, pregnancy and lactation are associated with modified Ca homeostasis (Del Valle
179 et al., 2011; Doherty et al., 2015; Kalkwarf, 1999; Kovacs and Fuleihan, 2006). For instance,
180 a significant Ca amount is lost to the foetus skeleton during pregnancy, while breastfeeding
181 involves significant Ca daily output. Vertebrates that lay eggs with heavily mineralized shells
182 may also lose significant fractions of Ca via secretion and formation of shell Ca carbonate as
183 well as yolk and albumen.

184 To conclude, as a major bio-essential metal in vertebrates, calcium lies at the
185 crossroads of important scientific questions relevant to the evolution of biology and ecology
186 of present and past vertebrates as well as to crucial modern health issues in humans.

187

188 **2.2. Cycling of Ca isotopes in vertebrates**

189 The main and first described feature of the Ca isotopes cycle in bony vertebrates is
190 the significantly ^{44}Ca -depleted isotope composition of bone when compared to dietary Ca
191 (Skulan and DePaolo, 1999). Compilation of literature data in 6 species of mammals and one
192 bird (Figure 3) shows that Ca of bone systematically displays lower $\delta^{44/42}\text{Ca}$ values than
193 dietary Ca, by about $-0.57 \pm 0.10 \text{ ‰}$ (2SE, $\sim -1.14 \text{ ‰}$ in $\delta^{44/40}\text{Ca}$) (Chu et al., 2006; Heuser et
194 al., 2016; Hirata et al., 2008; Skulan and DePaolo, 1999; Tacail et al., 2014). Despite the
195 various represented species in literature, no significant relationship between the extent of this
196 isotope effect and the organism physiology has been identified yet. A diet-bone isotopic
197 offset is well conserved within amniotes, suggesting a phylogenetically shared mechanism
198 resulting in Ca isotope fractionation during biological processing. In teleost and
199 elasmobranch fishes, the amplitude of the offset between diet and mineralized tissues
200 remains to be more thoroughly explored in controlled conditions but is possibly not as
201 marked. Seawater ingestion and osmoregulation involving seawater filtering through gills

202 could possibly attenuate or buffer these effects, as suggested by studies of marine
203 ecosystems (Clementz et al., 2003; Martin et al., 2015).

204 According to the comparison of soft tissue and blood Ca with bone of the same
205 animals (Skulan and DePaolo, 1999), this ⁴⁴Ca-depleted composition of bone primarily
206 results from a major isotope fractionation occurring during mineralisation of bone. On the
207 contrary, Ca loss from bone, during bone remodelling for instance, does not fractionate Ca
208 isotopes. The amplitude of the mineralization isotope effect is thought to be the same as the
209 diet-bone difference (Channon et al., 2015; Heuser and Eisenhauer, 2010; Morgan et al.,
210 2012; Reynard et al., 2010; Skulan and DePaolo, 1999; Skulan et al., 2007), i.e. -0.57‰,
211 although more recent comparison of bone and blood of sheep (Tacail et al., 2014) and pig
212 (Heuser et al., 2016) suggest that fractionation during mineralisation could be less
213 pronounced.

214 The processing of Ca by kidneys also induces a significant isotope effect, as
215 observed in various mammals. The Ca excreted in urine is systematically enriched in heavy
216 isotopes when compared to blood Ca, by ca. +1.2‰ in $\delta^{44/42}\text{Ca}$ (ca. +2.4‰ in $\delta^{44/40}\text{Ca}$), in
217 human (Channon et al., 2015; Eisenhauer et al., 2019; Heuser and Eisenhauer, 2010;
218 Skulan et al., 2007), sheep (Tacail et al., 2014) and pig (Heuser et al., 2016; Morgan et al.,
219 2012). The relationship between the amount of excreted urinary Ca and its isotope
220 composition in human suggests a Rayleigh isotope distillation process resulting from the
221 preferential reabsorption of Ca light isotopes from primary urine to blood along the nephron
222 (Heuser et al., 2019; Heuser and Eisenhauer, 2010; Morgan et al., 2012).

223
224 Vertebrate reproduction is also associated with significant Ca isotope effects. In
225 mammals, the isotope composition of milk was found to be remarkably ⁴⁴Ca-depleted in
226 comparison with that of the mother's diet (Chu et al., 2006). The amplitude of this difference
227 in $\delta^{44/42}\text{Ca}$ values averages at around -0.6‰ from diet to milk as observed in milk of ewes,
228 cows and humans (Chu et al., 2006; Gussone and Heuser, 2016; Heuser, 2016; Tacail,
229 2017). Prolonged milk production by lactating females could also result in a ⁴⁴Ca-enriched

230 composition of their bone when compared to males, as suggested by observations in modern
1
2 231 sheep (Reynard et al., 2010). In birds, despite limited datasets, Ca isotope compositions of
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4 232 eggs inner parts and carbonate shells were found to display remarkably extreme isotopic
5
6 233 patterns (see Skulan and DePaolo, 1999 and Figure 5). While the isotopic composition of
7
8 234 eggshell appears to be comparable to diet and significantly enriched in ^{44}Ca in comparison
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11 235 with bone of egg-laying hens, white albumen, and yolk to a lesser extent, display
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13 236 compositions that are even more enriched in heavy isotopes than for eggshell and diet. Such
14
15 237 a ^{44}Ca -enriched composition of eggshells likely relates to the high demand in Ca for its
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17 238 formation, resulting in high fluxes of poorly fractionated diet-like Ca to the eggshell (Skulan et
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20 239 al., 1997).

21
22 240 This understanding of the Ca isotope cycle led to two main hypotheses that have
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24 241 been since then further tested and exploited:

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26 242 (i) as diet directly influences the systematically shifted isotope compositions of mineralized
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28 243 tissues, Ca isotopes can be used to reconstruct dietary behaviours in past vertebrates
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31 244 (ii) as Ca isotopes are fractionated during bone mineralisation, Ca isotope compositions of
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33 245 blood and urine vary with the balance between bone formation and resorption. Calcium
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35 246 isotopes could be used to monitor variations of bone mineral balance in human subjects
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38 247 suffering from bone disorders.

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41 42 249 **2.3. Future research**

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44 250 New research is needed in order to i) further characterize the distribution of Ca isotopes
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46 251 in the various reservoirs of vertebrate organisms, ii) identify factors of variability (e.g.,
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49 252 species-specific physiological effects) and iii) improve our understanding of the mechanisms
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51 253 responsible for the major biologically induced isotope fractionations.

52
53 254 These remaining aspects would benefit from various approaches. At the scale of the
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55 255 whole organism, the results of controlled feeding experiments done on species from various
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58 256 clades of vertebrates could help further identify physiological factors of variability. This type
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60 257 of approach would help refine the identification of Ca fluxes associated with isotope effects

258 potentially affecting the whole organism Ca isotope distribution (e.g., Balter et al., 2013).
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2 259 Isotope box modelling of whole organism at steady-state and in dynamic conditions will then
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4 260 allow us to test the proposed mechanisms and investigate the potential of Ca isotopes for
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6 261 identified and future applications (e.g., Jaouen et al., 2019). Various physiological conditions
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8 262 could notably be explored, such as the age-related changes in the balance between bone
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10 263 anabolism and catabolism or the cycling of Ca isotopes between pregnant mammals and
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12 264 their foetus. In order to better understand the cellular and molecular mechanisms at play, it
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14 265 will also be possible to compare the results of experimental approaches on cellular in vitro
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16 266 models with controlled expression of cross-membrane protein transporters (e.g., Cadiou et
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18 267 al., 2017) and estimations of molecular equilibrium fractionation with numerical *ab initio*
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20 268 calculations (e.g., Moynier and Fujii, 2017). All these approaches will greatly benefit both
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22 269 palaeobiology and modern biomedical applications and potentially further reveal other yet to
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24 270 be identified applications.

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30 31 272 **3. Calcium isotopes in palaeobiology and bioarchaeology**

32
33 273 Stable isotopes and trace elements enable the development of quantitative
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35 274 approaches to reconstruct ecological, physiological and environmental characteristics that
36
37 275 otherwise remain unseen. Unravelling the partitioning of resources notably helps to better
38
39 276 understand past and present ecosystem dynamics and biodiversity. Geochemical proxies are
40
41 277 useful means of complementing inferences of morphofunctional and microwear analyses of
42
43 278 osteological remains (e.g., Martin et al., 2017a). The same applies to the study of past
44
45 279 human societies, where such tools are valuable to complement the conclusions of
46
47 280 archaeology and anthropology (Jaouen, 2018; Jaouen and Pons, 2016). Following the
48
49 281 example of other more established proxies, such as collagen carbon and nitrogen stable
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51 282 isotopes or Sr/Ca and Ba/Ca elemental ratios (Balter and Lécuyer, 2004; Koch, 2007;
52
53 283 Newsome et al., 2010; Peek and Clementz, 2012), Ca stable isotopes of fossil bioapatite
54
55 284 have a high potential for the study of past vertebrate ecosystems and human communities
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57 285 for three main reasons.
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286 First, as discussed above, the Ca isotope composition of diet primarily determines the
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2 287 composition of vertebrate mineralized tissues. This early finding came along with the
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4 288 hypothesis that such a trophic effect could propagate along food chains. The trophic level
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6 289 index, ranging from primary producer to apex predators and decomposers, describes the
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8 290 position of species within trophic chains and thus allows synthesizing the energetic pathways
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10 291 within ecosystems (Polis and Strong, 1996). Early datasets from both marine and terrestrial
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12 292 environments supported this trophic level effect (Clementz et al., 2003; DePaolo, 2004;
13
14 293 Skulan et al., 1997; Skulan and DePaolo, 1999). The biological processing of Ca from a
15
16 294 trophic level to another would thus lead to a stepwise decrease of the $\delta^{44/42}\text{Ca}$ values.
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20 295 Second, as a major constituent of a stable mineralogical phase (~ 40 wt.% Ca of
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22 296 bioapatite), the post-mortem diagenetic processes tend not to alter skeletal bioapatite Ca and
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24 297 its isotope compositions especially in the denser and less porous tooth enamel (Heuser et
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26 298 al., 2011; Melin et al., 2014; Martin et al., 2017; Martin et al., 2018). The determination of Ca
27
28 299 isotope compositions in osteological remains makes it possible to explore deep past
29
30 300 ecosystems. So far, studies of vertebrate fossils ecology with Ca isotopes focused on
31
32 301 assemblages dating back to Late Devonian (Balter et al., 2019), Triassic to Cretaceous
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34 302 (Hassler et al., 2018; Heuser et al., 2011; Martin et al., 2017b) or Pliocene and Pleistocene
35
36 303 (Martin et al., 2018, 2015, Tacail et al., 2019). Contrastingly, the conservation of pristine
37
38 304 nitrogen isotope compositions of bone collagen primarily depends on the preservation of the
39
40 305 organic phase, the degradation of which rarely allows investigating on trophic relationships
41
42 306 beyond Holocene or Late Pleistocene (Koch, 2007). On the other hand, the preservation of
43
44 307 biogenic Sr/Ca and Ba/Ca trace element ratios strongly depends on burial conditions (e.g.,
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46 308 Martin et al., 2018; Reynard and Balter, 2014).
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51 309 Finally, the abundance of Ca in osteological remains makes it possible to sample
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53 310 minute amounts of material (down to 20 μg or less). Such sample sizes allow studying
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55 311 precious fossils, without significantly altering their physical and structural integrity. It also
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57 312 motivates the development of high resolution sampling techniques involving mechanical
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59 313 sampling of tooth enamel by micro-drilling (Tacail et al., 2016, 2017) and laser cutting (Li et
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314 al., 2016). *In situ* laser ablation isotope analyses were shown to be possible in tooth enamel
315 (Tacail et al., 2016; Zhang et al., 2019), although these approaches could greatly benefit
316 from the implementation of technological innovations such as the currently developing use of
317 collision-cell multi-collector plasma mass spectrometry (e.g., Lewis et al., 2018, Zhao and
318 Simon 2019), with potential interference removal.

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319 After the first assessments of the utility of Ca isotopes for reconstructions of
320 vertebrate ecology, several studies further explored and developed strategies to reconstruct
321 biological, ecological and behavioural characteristics of past vertebrates and human
322 populations.

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324 **3.1. Calcium isotopes and vertebrate ecosystem reconstructions**

325 The sensitivity of skeletal Ca isotope compositions to trophic levels was explored and
326 exploited in a series of modern and fossil ecosystems, both in marine and continental
327 environments.

328

329 **3.1.1. Marine ecosystems**

330 Seawater is the primary source of Ca in most marine ecosystems (~ 420 mg/L,
331 Elderfield and Schultz, 1996). The Ca isotope composition of seawater is particularly
332 enriched in heavy isotopes (Figure 5) and relatively homogeneous at the scale of the oceans
333 (e.g., Fantle and Tipper, 2014).

334 Following the first description of decreasing $\delta^{44/42}\text{Ca}$ values with increasing trophic
335 levels (Skulan et al., 1997), Clementz et al. (2003) reported consistent patterns in bones and
336 teeth of both modern and fossil marine mammals. Species of low trophic levels (feeding on
337 marine vegetation or algae and invertebrates) display ^{44}Ca -enriched compositions when
338 compared to higher trophic level mammals (feeding on fishes and other marine mammals).
339 This pattern was also later reported in elasmobranch species of diverse ecologies (i.e.
340 cartilaginous fishes, namely sharks and rays, see Martin et al., 2015). These findings were
341 finally applied to a fossil marine ecosystem dating back to late Cretaceous (Martin et al.,

342 2017b), composed of samples from actinopterygian and elasmobranch fishes, as well as
1
2 343 Mosasaurid and Plesiosaurid large marine reptiles. This assemblage shortly predates the
3
4 344 end-Cretaceous extinction and sheds light on the vulnerability of this ecosystem, where high
5
6 345 trophic level large reptiles were relying on abundant yet poorly diversified resources. The use
7
8 346 of Ca isotopes also yielded new constraints onto the ecology and trophic level of extinct
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11 347 Devonian conodonts organisms for which no modern analogue is known (Balter et al., 2019).

12
13 348 These studies identified features that require further dedicated investigation in order
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15 349 to improve the use of Ca isotopes as an indicator of marine ecosystem structures. For
16
17 350 instance, data indicates that trophic ecology primarily determines tooth and bone isotope
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19 351 compositions of marine vertebrates, but mammals and fishes (cartilaginous or ray-finned) do
20
21 352 not appear to display the same average isotope shifts from one trophic level to another
22
23 353 (Clementz et al., 2003; Martin et al., 2017b, 2015). The ingestion of seawater and processing
24
25 354 of Ca via gill-mediated osmoregulation in actinopterygian and elasmobranch species is
26
27 355 suspected to buffer the diet-skeleton isotope offset. On the other hand, marine mammal
28
29 356 organisms are rather closed systems with respect to seawater. The differences in Ca isotope
30
31 357 physiology of fishes and mammals should thus motivate dedicated research in order to
32
33 358 decipher the physiological and ecological controls on the Ca isotope compositions of their
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35 359 tissues. A better understanding of the isotope systematics of marine mammals would notably
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37 360 be of importance as their ecology makes them useful sentinels of ecosystem changes (e.g.,
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39 361 Moore, 2008).

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44 363 **3.1.2. Continental ecosystems**

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47 364 The nature and isotope compositions of primary Ca dietary sources of most terrestrial
48
49 365 vertebrate trophic chains are more diversified than in marine ecosystems (see Figure 5).
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51 366 Indeed, in addition to the possible variability of isotope compositions of environmental Ca
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53 367 (e.g., interacting bedrock, soil and water), the plant Ca isotope compositions are highly
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55 368 variable between types, species and organs (e.g., Schmitt, 2016 and Figure 5). Depending
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57 369 on the ecological niches of primary consumers (e.g., in vertebrate herbivores), such
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370 variability can propagate throughout the trophic chain and make the interpretation of Ca
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2 371 isotope compositions more complex for trophic relationships reconstructions.
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4 372 Melin et al. (2014) explored the bone Ca isotope compositions of various mammals
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6 373 including primates from two tropical forested ecosystems. The authors confirmed the
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8 374 decrease of Ca isotope ratios in large carnivores, but found no significant differences
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11 375 between lower trophic levels, notably small faunivores and insectivores. They however
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13 376 suggest an encouraging complementary use of carbon (C) and Ca stable isotopes. Martin et
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15 377 al. (2017a) reported encouraging bone and tooth enamel Ca isotope compositions of two
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17 378 Western European Pleistocene mammal assemblages. The stable isotope compositions of
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19 379 each taxonomic group (up to 6 individuals) are rather clustered and carnivores tend to
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21 380 display lower $\delta^{44/42}\text{Ca}$ values than herbivores. However, the outlying compositions of some
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23 381 species remain difficult to interpret in terms of trophic inferences, suggesting either species-
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25 382 dependent physiological effects or peculiar dietary niches effects. Recently, Martin et al.
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27 383 (2018) reported the results of the systematic study of mammal tooth enamel of modern and
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29 384 fossil Pliocene-Pleistocene ecosystems from East Africa. This study further documents the
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31 385 potential of Ca isotopes as helpful markers for trophic inferences. The combination of C and
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33 386 Ca stable isotopes helps to distinguish between C_3 and C_4 trophic chains structures, in extant
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35 387 and extinct ecosystems. Indeed, the carbon stable isotope compositions of the enamel of
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37 388 mammals ($\delta^{13}\text{C}$) are characteristic of the photosynthetic pathways of the plants at the base of
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39 389 the trophic chain (C_3 or C_4 carbon fixing sugars, c.f. Bender 1971), while Ca isotopes appear
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41 390 to allow discriminating between herbivores and carnivores. This is exemplified by the figure
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43 391 4A, where the carnivores appear depleted in ^{44}Ca isotopes with respect to their respective
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45 392 inferred preys, being either predominantly C_3 herbivores with low $\delta^{13}\text{C}$ values (namely
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47 393 browsers) or a mix between the latter and C_4 herbivores with higher $\delta^{13}\text{C}$ values (namely
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49 394 grazers). Furthermore, when taking into account all available geochemical proxies relevant to
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51 395 feeding and habitat ecology (namely Sr/Ca and Ba/Ca ratios and $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, $\delta^{44/42}\text{Ca}$, see
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53 396 Martin et al., 2018), a principal component analysis (Figure 4B) reveals an inferred
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397 ecosystem structure that is very similar to what $\delta^{13}\text{C}$ and $\delta^{44/42}\text{Ca}$ alone suggest (Figure 4A).
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2 398 In other words, the coupling of $\delta^{13}\text{C}$ and $\delta^{44/42}\text{Ca}$ appears to efficiently summarize the
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4 399 ecosystem structure.
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6 400 Finally, in the Mesozoic fossil record, despite the fact that rather inconclusive results
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8 401 were obtained from dinosaur faunas on large geographical and chronological scales (Heuser
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10 402 et al., 2011), the systematic study of skeletal remains Ca isotope compositions from a fossil
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12 403 assemblage at regional scale brings new constraints to the resource partitioning between
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14 404 mid-Cretaceous predatory dinosaurs and more precisely to the ecology of spinosaurids
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16 405 (Hassler et al., 2018).
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20 406 To conclude, the ongoing exploration at regional scale of the ecological controls over
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22 407 vertebrate Ca isotope compositions in the continental environment is encouraging. Calcium
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24 408 isotopes show a very good potential for the reconstruction of dietary relationships in past
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26 409 vertebrate ecosystems, from Palaeozoic to Quaternary, and allow constraining local
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28 410 ecosystem structures and resource partitioning, especially between herbivores and
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30 411 carnivores. These results motivate the development of new strategies that notably include i)
31
32 412 the combination of $\delta^{44/42}\text{Ca}$ with complementary proxies, such as $\delta^{13}\text{C}$, ii) the study of
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34 413 assemblages composed of taxa with diverse ecologies, iii) at a regional scale, iv) with
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36 414 taxonomic groups comprising multiple individuals. Further research needs to be carried out,
37
38 415 notably because some taxa do not entirely fit within this interpretative framework. This could
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40 416 relate to peculiar physiological adaptations or ecological niches (e.g., Martin et al., 2018,
41
42 417 2017a). For instance, the observed isotopic offsets between bone of most secondary or
43
44 418 tertiary consumers and their vertebrate preys are likely to primarily rely on the partial
45
46 419 consumption of mineralized tissues from the latter. The intake of only 1 wt. % bone in total
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48 420 diet is sufficient to cause a significant trophic effect in Ca isotope compositions (-0.50‰ on
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50 421 the $\delta^{44/42}\text{Ca}$ scale) of consumer's tissues in terrestrial vertebrates (Heuser et al., 2011).
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52 422 Although little is known about muscle and bulk soft tissues isotope compositions, the
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54 423 consumption of energy-rich but Ca-poor soft tissues could result in different isotope offsets
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424 between secondary consumers and their vertebrate preys, making trophic level inferences
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2 425 less straightforward.

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6 427 **3.1.3. Future directions**

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8 428 *(i.)* The development of statistical approaches for diet reconstruction with Ca isotopes could
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10 429 help to refine and make the most of trophic ecosystem reconstructions (such as Bayesian
11
12 430 statistical inferences, see for instance Fernandes et al., 2014). The inter-individual variability
13
14 431 of Ca isotope compositions within a given taxonomic group could also relate to the degrees
15
16 432 of specialisation for various Ca resources (e.g., Martin et al., 2018). By means of statistical
17
18 433 methods, Ca isotopes could allow characterizing “isotope niches” and help to document
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20 434 breadths of ecological niches or the specialisation of taxonomic groups (e.g., Yeakel et al.,
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22 435 2015).

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26 436 *(ii.)* Profound changes in the biological management of Ca occurred in the course of
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28 437 vertebrate evolution. A series of major evolutionary steps could potentially be studied with
29
30 438 the help of Ca isotopes: the appearance of calcium phosphate mineralized endoskeletons,
31
32 439 the ecological and physiological adaptations related to fish-to-tetrapod transitions followed by
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34 440 terrestrialisation, the development of egg-laying and carbonated shells in vertebrates or
35
36 441 finally the development of pregnancy and lactation in first mammals (Bouillon and Suda,
37
38 442 2014; Doherty et al., 2015). Each of these evolutions of vertebrate physiology and ecology
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40 443 likely affected their Ca isotopes cycling, which could in turn be exploited in order to document
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42 444 the evolutionary mechanisms at play.

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46 445 *(iii.)* Calcium isotope might be useful in other practical applications such as in food forensics.
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48 446 For instance, combined with other stable isotopes, Ca isotopes could help in tracking the
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50 447 consumption of animal meat and bone meals by farmed animals (e.g., cattle, poultry; see
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52 448 Carrijo et al., 2006).

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58 450 **3.2. Calcium isotopes and dietary reconstructions in hominids and human**
59
60 451 **societies**

452 **3.2.1. From ecosystems to human societies**

1
2 453 Major changes in dietary practices in the course of human evolution, being ecologically
3
4 454 and/or culturally driven, likely came along with significant changes of their use of Ca
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6 455 resources (e.g., Eaton and Nelson, 1991). The changing dietary habits of early *Homo* or the
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8 456 development of agriculture and livestock farming in the course of Neolithic mark significant
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11 457 modifications of the use of environmental Ca and were associated with major ecological,
12
13 458 physiological, genetic or cultural evolutions (e.g., Aiello and Wheeler, 1995; Hublin et al.,
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15 459 2015; Ségurel and Bon, 2017; Vigne, 2011). The utility of Ca isotopes to track these changes
16
17 460 has been however poorly explored. While the previously described ecosystem approach
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19 461 using Ca isotopes could help to interpret the evolution of hominid ecologies, the study of
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21 462 bone Ca isotopes in human societies could improve our understanding of their contrasting
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23 463 dietary habits influenced by varied cultural practices.

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26 464 As shown in Figure 5, the major dietary sources of Ca for human have significantly
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28 465 contrasting isotope compositions. Therefore, the culturally driven practices of consumption of
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30 466 particular Ca dietary sources could lead to distinctive distributions of bone $\delta^{44/42}\text{Ca}$ values in
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32 467 human populations. This could be of use to reconstruct dietary practices of past human
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34 468 societies and characterize their subsistence modes.

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37 469 For instance, dairy products constitute the most ^{44}Ca -depleted end-member of this
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39 470 isotopic landscape (with modern human milk being the lightest), while eggs and marine
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41 471 resources display the most ^{44}Ca -enriched compositions. Following these observations, Chu
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43 472 et al. (2006) proposed that Ca isotopes could be used to reconstruct the consumption of
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45 473 dairy products in past human groups, especially during the processes of Neolithization with
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47 474 domestication of dairy producing animals. Indeed, the consumption of significant proportions
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49 475 of Ca from dairy products should induce a global decrease in $\delta^{44/42}\text{Ca}$ values of dietary Ca
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51 476 and thus in bone of human consumers. Reynard et al. (2010, 2011) tested the potential of Ca
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53 477 isotopes in faunal and human bones as a biomarker of dairy product consumption in several
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55 478 archaeological populations before and after domestication of dairy producing animals. These
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57 479 authors found however no significant change in the $\delta^{44/42}\text{Ca}$ offset between fauna and human
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1
2 480 of the studied archaeological sites after the domestication of milk producing animals. They
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4 481 suggested that controls other than animal milk consumption were likely at play.
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6 482 Nevertheless, further research including actualistic calibrations in human osteological
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8 483 remains could help refine our understanding of the controls of dietary practices over bone Ca
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10 484 isotope compositions.

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12 13 486 **3.2.2. Life history reconstructions in individuals**

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15 487 Throughout their development and life, humans experience major adaptations of their Ca
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17 488 cycle both in terms of nutrition (e.g., birth or weaning in offspring) or Ca homeostasis (e.g.,
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19 489 pregnancy or breastfeeding in females) (Humphrey, 2010; Kovacs and Fuleihan, 2006). The
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21 490 characteristics of these events (e.g., timing, frequency) are crucial and informative traits of
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23 491 the life history of individuals, relating to the ecological strategies of species and to cultural
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25 492 behaviours (Kramer and Otarola-Castillo, 2015; Lee, 2012; Robson and Wood, 2008). Such
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27 493 changes can in turn influence the Ca isotope distributions, which could be exploited if
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29 494 archived in human remains.

30
31 495 So far, the major life history traits explored with Ca isotopes relate to mother's milk
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33 496 consumption by children. The biologically and culturally driven weaning behaviours are
34
35 497 central traits of the evolution of mammals and hominins (Humphrey, 2010; Sellen, 2007; Van
36
37 498 Noordwijk et al., 2013) but these practices remain fairly undocumented in the deep past
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39 499 because biomarkers are lacking. As milk was early recognized as a ⁴⁴Ca-depleted material in
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41 500 comparison with diet, Reynard et al. (2013) first tested this hypothesis by analysing bones of
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43 501 past human populations as a function of age of individuals but no significant trends were
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45 502 observed. This hypothesis was later tested on temporary tooth enamel of modern human
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47 503 individuals with known early life histories (Tacail et al., 2017). Contrary to bone which
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49 504 continuously remodels, tooth enamel forms incrementally. Provided the sampling techniques
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51 505 allow sufficient spatial resolution, the enamel structure permits the reconstruction of the
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53 506 evolution of Ca isotope compositions over time of formation of tooth crowns. This study
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55 507 demonstrates that weaning practices are recorded within human tooth enamel by Ca
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508 isotopes and therefore could allow documenting weaning patterns in past humans and
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2 509 hominids. This method was recently applied to the tooth enamel of Pleistocene early
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4 510 hominins of South Africa (Tacail et al., 2019). The results support that early *Homo* infants
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6 511 were breastfed in significant proportions for longer periods than *A. africanus* and *P. robustus*
7
8 512 and opens discussion on the evolution of weaning practices in human lineage.

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12 13 514 **3.2.3. Future directions**

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15 515 (i) Given the reported systematic variability of Ca dietary sources, and provided this
16
17 516 variability is further documented, Ca isotopes could be a successful marker of human dietary
18
19 517 practices in relation with human cultures and subsistence modes. A more actualistic
20
21 518 approach with a focus on modern and historic human populations with documented and
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23 519 contrasting diets would for instance open the way to new archaeological applications, the
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25 520 same way Zn isotopes were shown to be sensitive to consumption of fish and meat (Jaouen
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27 521 et al., 2018).

28
29 522 (ii) As biomarkers of weaning practices, Ca isotopes in tooth enamel would benefit from
30
31 523 the investigation of its complementarity with other biomarkers of early life dietary transitions
32
33 524 such as Ba/Ca ratios in dentin or enamel (e.g., Austin et al., 2013) or $\delta^{15}\text{N}$ in bone and dentin
34
35 525 collagen (Tsutaya and Yoneda, 2015).

36
37 526 (iii) Beyond tooth enamel, other incrementally growing tissues could be explored for their
38
39 527 potential as time resolved archives. Dentin, hair or nail could record Ca isotopic variations
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41 528 relating to weaning practices but also to other physiopathological modifications of Ca
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43 529 homeostasis such as puberty as proposed by Li et al, 2016, the same way other isotope
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45 530 systems can be affected (e.g., Ohno et al., 2005; Tsutaya and Yoneda, 2015).

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48 49 532 **4. Calcium isotopes in biomedical research**

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51 533 *Stable Isotope Metallomics* is the study of normal and disrupted cycles of regulated
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53 534 and essential metals from the perspective of their natural stable isotope compositions. This
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55 535 field of research has developed in the last two decades following seminal works on Ca, Fe,
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Cu or Zn, aided by the development and widespread application of multicollecion inductively coupled plasma mass spectrometry (MC-ICPMS) (see reviews such as Albarède et al., 2017; Costas-Rodríguez et al., 2016). This research has served two main purposes: i) to shed new light on the biological processing of these elements, beyond quantitative description of their elemental distributions (*e.g.*, identify pathways or biochemical mechanisms), ii) to develop new markers for rapid and specific diagnosis or prognosis of diseases affecting these cycles.

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In this context, the potential of Ca isotopes is important. Because of the significant fractionation occurring during bone mineralisation, Ca stable isotopes are directly indicative of the short-term variations of net bone mineral balance (BMB), i.e. the mass balance between bone resorption and formation. Individuals with positive BMB should thus display blood and urine Ca isotope compositions enriched in heavy isotopes with respect to neutral BMB. On the other hand, individuals with a negative BMB should display ⁴⁴Ca-depleted blood and urine. This recognition sparked a vivid interest for the study of Ca isotopes as a marker of disruptions of BMB.

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Disruptions of bone mineral balance are widespread in modern societies, mainly because of the high incidence of metabolic bone disease, such as osteoporosis in ageing populations (Becker et al., 2010; Peacock, 2010). Another related issue is the bone loss induced by microgravity, as experienced by crew members in space missions, for which effective countermeasures are still being investigated (Grimm et al., 2016). Clinical tools for predicting and monitoring the evolution of these physiological and pathological conditions resulting from disruptions of Ca metabolism are currently limited to: (i) molecular markers with poor sensitivity or quantitative insight into BMB changes, (ii) X-ray densitometry techniques that only provide a 6-months to yearly resolution of the changes in bone mineral density (*e.g.*, Kuo and Chen, 2017). The potential of Ca isotopes as a quantitative, radiation-free and non-invasive biomarker for the measurement of BMB has been investigated in a series of studies over the last twelve years.

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4.1. The effects of induced bone loss in bed rest studies

564 Several studies presented evidence of variations in human blood and urine Ca
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2 565 isotope compositions caused by bone loss induced by bed rest (Channon et al., 2015;
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4 566 Heuser et al., 2019; Morgan et al., 2012; Skulan et al., 2007) (see Figure 6). Bed rest
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6 567 provokes significant loss of muscle as well as bone mineral mass in response to inactivity,
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8 568 and mimics the effects of microgravity in spaceflight (Pavy-Le Traon et al., 2007). Bed rest
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10 569 experiments are helpful in identifying the physiopathological causes of bone mineral loss,
11
12 570 and also permit the assessment of drug and physical exercise countermeasures to bone
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14 571 mineral loss.

17 572 Skulan et al. (2007) first published a report of significant variations of $\delta^{44/40}\text{Ca}$ values
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19 573 in weekly-pooled urine of individuals submitted to bed rest experiments. The authors
20
21 574 reported significant negative departure from baseline in $^{44}\text{Ca}/^{40}\text{Ca}$ ratios during bed rest in
22
23 575 the group with no countermeasure to bone loss followed by a return to baseline after the
24
25 576 experiment. By contrast, subjects treated with pharmacological or exercise countermeasures
26
27 577 to bone loss displayed either no change or positive change of their urine $\delta^{44/40}\text{Ca}$ values.
28
29 578 These results provided a first proof of concept. This work was later augmented by Morgan *et*
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31 579 *al.* (2012) with a report of changes in urine Ca isotope compositions during a bed rest
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33 580 experiment conducted with a finer chronological sampling. This study demonstrated that the
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35 581 decrease in $\delta^{44/42}\text{Ca}$ values of urine occur after one week, indicating a loss of bone in
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37 582 agreement with markers of osteolytic activity (NATX) but long before any changes in bone
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39 583 mineral density could be detected by means of X-ray methods. These results were further
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41 584 confirmed in the blood of the same subjects (Channon et al., 2015). The recognition of the
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43 585 isotopic fractionation induced by renal function lead the authors to adjust the mathematical
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45 586 models used to link changes in bone mass and urine Ca isotope compositions (*e.g.*, Heuser
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47 587 and Eisenhauer 2010, Morgan et al., 2012). The observed negative offset in urine $\delta^{44/42}\text{Ca}$
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49 588 results from loss of Ca from bone but also from an increase in Ca urinary losses in kidneys
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51 589 (*i.e.* a decrease in Ca reabsorption). Taking this into account, Morgan et al. (2012) calculated
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53 590 the relative bone loss during the bed rest experiment. The estimated relative bone loss
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591 agreed with bone loss predicted on the basis of previous experiments in which bed rest was
592 of a duration long enough to produce changes in BMD resolvable by X-ray densitometry
593 (around 0.25 % of total bone mass, for -0.30‰ decrease of $\delta^{44/42}\text{Ca}$ values).

594 More recently, in connection with another bed rest experiment, Heuser et al. (2019)
595 proposed a methodology to deconvolute the combined effects of changes in renal Ca
596 excretion and bone loss. Using both the urine Ca isotope compositions and the measured
597 daily Ca urinary excretion rates, the authors distinguish the contribution of bone loss to the
598 variations in urinary Ca isotope compositions while cancelling out the effects of kidney
599 Rayleigh-type isotope distillation. These results stress the need for the development a more
600 thorough understanding of influence of renal function in the variability of urine Ca isotope
601 compositions while emphasizing the potential of $\delta^{44/42}\text{Ca}$ as a diagnostic biomarker.

603 4.2. Calcium isotopes as biomarkers of metabolic bone diseases

604 Calcium isotopes are sensitive to other bone related disorders. Their efficiency as a
605 biomarker (i.e. sensitivity and specificity) was tested in two published clinical studies so far.

606 Gordon et al. (2014) reported significant ^{44}Ca -depletion of blood serum Ca in subjects
607 affected by active forms of multiple myeloma when compared to subjects with non-active
608 disease. Multiple myeloma is a form of cancer affecting the plasma cells and inducing
609 osteolytic lesions, leading to osteoporosis and bone fracture. While current methods can
610 detect osteolytic lesions only after they have caused significant damage, Ca isotopes could
611 reveal abnormal bone loss, and thus disease activity, long before it can be detected by other
612 means. This preliminary study reported good performances for Ca isotopes as a disease
613 predictor, with significant specificity and sensitivity, regardless of other factors, such as age
614 or gender.

615 Based on those preliminary yet promising first results, Eisenhauer et al. (2019)
616 reported the first clinical trial conducted to assess the potential of Ca isotopes as a diagnosis
617 biomarker for osteoporosis in 80 postmenopausal women. Compared with the outcomes of
618 the gold-standard densitometry diagnosis method, the blood and urine Ca isotope

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619 compositions were significantly ⁴⁴Ca-depleted in the 14 subjects effectively affected by
620 osteoporosis, when compared to subjects without osteoporosis. Calcium isotopes appear to
621 display very strong sensitivity and good specificity to the osteoporosis condition, and are in
622 good agreement with various classical clinical parameters and chemical biomarkers of bone
623 loss. These results suggest that Ca isotopes could be developed into a stand-alone clinical
624 test for osteoporosis.

625

626 **4.3. Future directions**

627 All the work to date has demonstrated that calcium isotopes have characteristics of
628 an efficient isotopic biomarker for bone loss. The clinical applications of Ca isotopes remain
629 in their infancy, but these promising results should stimulate more research to improve
630 diagnostic protocols and develop broad clinical reference databases.

631 (i.) Despite considerable progress, the understanding of how Ca isotopes correlate with
632 epidemiology of metabolic bone diseases could be improved. Continued efforts will be
633 required to advance our understanding of the Ca isotopes cycle in the whole organism both
634 in health and disease. Further characterization of the mechanisms of isotope fractionation at
635 the scale of organs and cells will also greatly benefit to this field of research.

636 (ii.) The reported intra- and inter-individual variability of blood or urine Ca isotope
637 compositions highlights the need for identifying factors of variability other than bone loss.
638 Besides dietary practices varying through time in a given individual and/or from one
639 individual to another, several nutrients and molecules are known to affect Ca homeostasis,
640 and thus potentially intra- and inter-individual variability of their Ca isotope compositions. For
641 instance, dietary Vitamin D has notably been shown to result in changes of urine Ca isotope
642 compositions (Rangarajan et al., 2018). Clearly, strategies need to be implemented in order
643 to take these sources of intra- and inter-individual variability into account and/or cancel them
644 out.

645 (iii.) Other physiopathological conditions affecting Ca homeostasis could be successfully
646 explored with the help of Ca isotopes including diseases or deficiencies that strongly affect

647 the Ca cycle and have high incidence in modern societies (Peacock, 2010; Shroff et al.,
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2 648 2013). For instance, the progressive disruption of the kidney function in individuals suffering
3
4 649 from chronic kidney diseases is likely to affect the distribution of Ca isotopes. Other
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6 650 disturbances of the Ca homeostasis being either induced by drugs or in some digestive
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8 651 disorders could also be explored with Ca isotopes. Dedicated research could also potentially
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11 652 lead to the development of new biomarkers for such conditions of disrupted Ca homeostasis.
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15 654 5. Challenges of vertebrate Ca isotope geochemistry

17 655 The potential of Ca isotopes is high in the field of vertebrate biology. However the
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20 656 development of new applications and their possible adoption by scientific communities other
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22 657 than Earth scientists faces barriers, and depend on the communities involved.
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26 659 5.1. Developing the use of Ca isotopes in palaeobiology and bioarchaeology

28 660 The use of stable isotopes in palaeobiology and bioarchaeology is not new. From the
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31 661 1970's onwards, palaeontologists, paleoanthropologists and archaeologists (as well as
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33 662 modern ecologists and forensic scientists) used *traditional* stable isotopes (C, H, O, N) to
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35 663 assess trophic ecology, habitat use, physiology or migration of past and present vertebrates
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38 664 including humans (Koch, 2007; Newsome et al., 2010).

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40 665 Beyond the currently investigated scientific questions regarding Ca isotopes, the main
41
42 666 leap one could identify lies in the practical and possibly poorly identified differences between
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44 667 these so-called *traditional* stable isotopes and the developing *non-traditional* metal stable
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46 668 isotopes. First, while *traditional* stable isotopes are routinely analysed by means of gas
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49 669 source mass spectrometers, *non-traditional* stable isotopes, including Ca, require the use of
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51 670 different mass spectrometers (mainly MC-ICP-MS but also possible with TIMS) that are so
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53 671 far predominantly hosted by Earth sciences laboratories and require different analytical skills
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55 672 and laboratory set-ups. These analytical challenges can be overcome, as exemplified by the
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58 673 growing use of other isotope systems requiring these analytical set-ups, such as $^{87}\text{Sr}/^{86}\text{Sr}$
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60 674 ratios in archaeology. Clear communication on differences between *traditional* and *non-*

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675 *traditional* stable isotopes is also a key to successful collaborations with communities of
676 palaeobiology and bioarchaeology scientists. For instance, as a major element, Ca allows
677 favourable sampling strategies of osteological remains (small sample sizes, less destructive
678 analyses, high spatial resolution) and grants access to geological periods so far poorly
679 explored with *traditional* stable isotopes. Also, the elements in the focus of *traditional* stable
680 isotope systems do not share the same biological cycles and functions in vertebrate
681 organisms as do metal stable isotopes. While the former constitutes the very structure of a
682 variety of soft tissues building blocks, the more specific functional roles of bio-essential
683 metals allow focussing on new aspects of the biology of vertebrates. The developing use of
684 Ca isotopes by these communities can thus count on pre-existing fruitful exchanges and
685 collaborations between isotope geochemists and specialists of these disciplines but also
686 require a communication effort regarding Ca isotopes and their peculiarities.

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688 **5.2. Using Ca isotopes from fundamental to applied biomedical research**

689 When taking the plunge into biomedical research and aiming at developing diagnosis
690 biomarkers, the challenges are more acute. The natural variations of *traditional* stable
691 isotope ratios are not routinely used by biomedical research scientists or medical doctors in
692 order to understand element cycling, characterize aetiology of diseases, nor as biomarkers,
693 notably because of their limited specificity. A few groups of isotope scientists and biomedical
694 researchers currently investigate the potential of metal stable isotope systems (*e.g.*, Ca, Fe,
695 Cu or Zn, see reviews such as Costas-Rodríguez et al., 2016; Albarède et al., 2017).
696 However, this field of research is currently in its infancy. More generally, all involved
697 communities (stable isotope scientists, biomedical scientists and medical doctors) do not
698 have a shared scientific or technical background and may have different goals. Beyond
699 purely scientific questions, there are potential intellectual and cultural barriers that must be
700 overcome in order to implement the truly interdisciplinary collaboration that biomedical stable
701 isotope research demands.

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703 **5.2.1. Initiating successful interdisciplinary research projects**

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2 704 As recently reported by Sauzéat et al. (2019), interdisciplinary collaboration requires
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4 705 reciprocal curiosity and communication between fields, which in turn requires scientists to
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6 706 understand each other's scientific and technic cultures well enough to identify scientific
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8 707 questions that could be addressed collaboratively. Scientists must endeavour to make their
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10 708 work accessible to people in other fields. For isotope scientists, this implies for instance
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12 709 avoiding unnecessary jargon and preparing clearly written documents, or *vade-mecum*, that
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14 710 explain key concepts of stable isotope metallomics to non-specialists.

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17 711 Also, the fates of such new frontiers research projects are unknown at first and can be
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19 712 seen as risky. It is important to encourage and support the researchers that develop
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21 713 interdisciplinary careers. While the risk is that such profiles are potentially hard to fit in job
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23 714 descriptions, researchers with multiple backgrounds (*e.g.*, medical doctors or biomedical
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25 715 researcher skilled as isotope scientist) pave the way to the future of this research. In this
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27 716 matter, the support of institutions is particularly valuable, when for example implementing
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29 717 interdisciplinary grant schemes and recruitment campaigns.

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35 719 **5.2.2. Analytical and methodological challenges**

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37 720 Another challenge is to identify the methodological constraints imposed by the various
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39 721 disciplines involved in the project, and to accommodate these constraints in the study design.
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41 722 For example, the extreme care typically required to maintain sterility and integrity of
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43 723 biological samples for molecular biological assays is generally unnecessary for samples
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45 724 destined for isotope analysis. On the other hand, substances commonly used as
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47 725 preservatives in blood and urine samples can be unacceptable contaminants for isotope
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49 726 analysis. Disciplines also have different requirements for experimental controls. In human
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51 727 experiments involving Ca isotopes, this typically requires sampling and analysis of all dietary
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53 728 and pharmacological Ca sources, as well as multiple pre-experimental sampling of all study
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55 729 subjects in order to establish individual baselines. Finally, isotope geochemistry laboratories
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57 730 are not biomedical laboratories. Ethical and health regulations often require formal
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1 731 authorization for the handling of human tissues, as well as adaptation of laboratory practices
2 732 and proper training of isotope scientists, engineers, technicians or students in the hazards
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4 733 posed by these materials. Also, isotope analyses in isotope geochemistry laboratories tend
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6 734 to be slow when compared to routine biomedical analyses, even when machine time is not
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8 735 limited. For Ca isotopes to become a widely used clinical tool, sample throughputs must be
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10 736 increased by several orders of magnitude, while ensuring a sufficient precision and accuracy
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12 737 of the isotope analysis. This is technically feasible (with approaches such as automated
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14 738 sample preparation, spiking methods, collision-cell instruments, e.g., Romaniello et al., 2015,
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16 739 Lewis et al., 2018) but will not happen without being spurred by demand.
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22 741 **5.2.3. Developing a medical biomarker**

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24 742 In general, the development of medical biomarkers is codified and standardized, for
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26 743 instance in terms of clinical trials methodology and statistics or ethical and legal aspects.
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28 744 These are constraints for which isotope geochemists, and their host laboratories and
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30 745 institutions, are neither prepared nor able to address on their own. The first challenge is thus
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32 746 to recruit the assistance of medical scientists in the regulatory and administrative aspects of
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34 747 study design.
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37 748 In addition to aforementioned challenges, we suggest that another lies in the differences
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39 749 in technical and scientific cultures in which stable isotope geochemists and medical scientists
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41 750 have evolved. For example, stable isotope geochemists tend to direct their research toward a
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43 751 fundamental understanding of processes while medical scientists and hospital doctors may
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45 752 feel more constrained by the need for practical and immediate tools to achieve clinical goals.
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47 753 This urgency is something geochemists do not often encounter on their home turf. As a
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49 754 result, because geochemists tend to assume that practical application proceeds from
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51 755 theoretical understanding, they could tend to underestimate the current clinical utility of their
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53 756 techniques and undersell them.
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57 757 Finally, medical science is immersed in a biological paradigm that views life as an
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59 758 interaction between complex organic macromolecules (including genes) at the cellular level.
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759 Metal stable isotope analysis, focused as it is on inorganic chemistry, does not mesh well
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2 760 with this paradigm. Apart from the practical clinical tools they can provide, isotope chemists
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4 761 also are in a position to advance a long overdue appreciation of the central role purely
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6 762 inorganic processes play in biology. It is possible to say a great deal about the state of an
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8 763 organism by looking at the distribution of simple ions and isotopes, without reference to cells
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10 764 or macromolecules, in the same way that it is possible to say a great deal about an
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12 765 environmental or geological system without reference to the details of its component parts.
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15 766

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30 776

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1157 **Captions:**

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41159 Figure 1: Number of publications per year reporting new research on Ca isotopes in
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61160 vertebrates (Single column)
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11162 Figure 2: Typical Ca cycle in vertebrate organisms. The Ca fluxes for which Ca isotope
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131163 fractionation is documented, possible or not suspected are indicated with orange dashed
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151164 arrows, black dashed arrows and black solid line respectively. (2-column)
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201166 Figure 3: $\delta^{44/42}\text{Ca}$ of bone of 7 species of vertebrates as a function of their diet $\delta^{44/42}\text{Ca}$ (in ‰,
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221167 relative to SRM915a reference material) shows the shared physiological isotope effect. The
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241168 red dashed line is the average bone-diet offset, the blue full line is the linear regression and
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261169 the red shaded area delimits its 95% confidence interval. Error bars are 2sd. ¹ Skulan and
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291170 DePaolo, 1999; ² Chu et al., 2006; ³ Heuser et al., 2016; ⁴ Tacail et al., 2014; ⁵ Hirata et al.,
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311171 2008. (Single column)
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351173 Figure 4: Ecological reconstructions of a modern mammal ecosystem (Tsavo National Park,
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381174 Kenya, dataset from Martin et al., 2018): A. Tooth enamel $\delta^{44/42}\text{Ca}$ (in ‰, relative to SRM915a
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401175 reference material) as a function of $\delta^{13}\text{C}$ (in ‰, relative to VPDB reference material)
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421176 (Modified from Martin et al., 2018). B. Principal Component Analysis (PCA) of various
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451177 chemical proxies in tooth enamel (grouped by main ecological groups and Hippos, as
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471178 described in Martin et al., 2018, displayed with 95% distribution ellipses). The $\delta^{13}\text{C}$ - $\delta^{44/42}\text{Ca}$
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491179 space summarizes well the ecosystem structure clearly depicted by the PCA, while hippos
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521180 stand as outliers that remain to be explained. (2-column)
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541181
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561182 Figure 5: Compilation of Ca isotope compositions (in ‰, relative to SRM915a reference
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581183 material) of a series of Ca sources in diets of vertebrates including human. This compilation
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1184 includes continental river water considered as fresh water (Tipper et al., 2016), marine
1185 resources (fish, mollusc and crustaceans) (Skulan et al., 1997; Skulan and DePaolo, 1999),
1186 dicotyledon roots, stems and leaves or fruits and monocotyledon including cereals
1187 (Christensen et al., 2018; Chu et al., 2006; Farkaš et al., 2011; Gussone and Heuser, 2016;
1188 Heuser, 2016; Hindshaw et al., 2013; Holmden and Bélanger, 2010; Huang et al., 2012;
1189 Moore et al., 2013; Page et al., 2008; A. Schmitt et al., 2003; Skulan and DePaolo, 1999;
1190 Tacail et al., 2014; Wiegand et al., 2005), hen and quails' eggs (Skulan and DePaolo, 1999;
1191 Tacail, 2017), herbivore's soft tissues and blood (Heuser, 2016; Morgan et al., 2012; Skulan
1192 and DePaolo, 1999; Tacail et al., 2014), animal milk and dairy products (Chu et al., 2006;
1193 Gussone and Heuser, 2016; Heuser, 2016; Tacail et al., 2017) and modern human milk (Chu
1194 et al., 2006; Tacail et al., 2017). (2-column)

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1196 Figure 6: Bedrest experiments induce bone loss and a decrease of urine and blood $\delta^{44/42}\text{Ca}$
1197 values. Changes in Ca isotope compositions ($\Delta^{44/42}\text{Ca}$) of blood or urine at each date of
1198 experiment are calculated as the difference with average baseline (pre-bedrest) composition
1199 in all subjects of three studies: (A.) $\Delta^{44/42}\text{Ca}$ in urine of 4 individuals over 17 weeks of bedrest
1200 (Skulan et al., 2007), (B.) $\Delta^{44/42}\text{Ca}$ in urine of 6 individuals over 21 days of bedrest (Heuser et
1201 al., 2019), (C.) $\Delta^{44/42}\text{Ca}$ in urine (top) and blood (bottom) of 12 individuals over 30 days of
1202 bedrest (Morgan et al., 2012, Channon et al., 2015). Graphs display boxplots of $\Delta^{44/42}\text{Ca}$
1203 values, indicating 5, 25, 50, 75 and 95% quantiles. Red diamonds and red dashed lines show
1204 the evolution of average $\Delta^{44/42}\text{Ca}$ values with time. (2-column)

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Figure 1

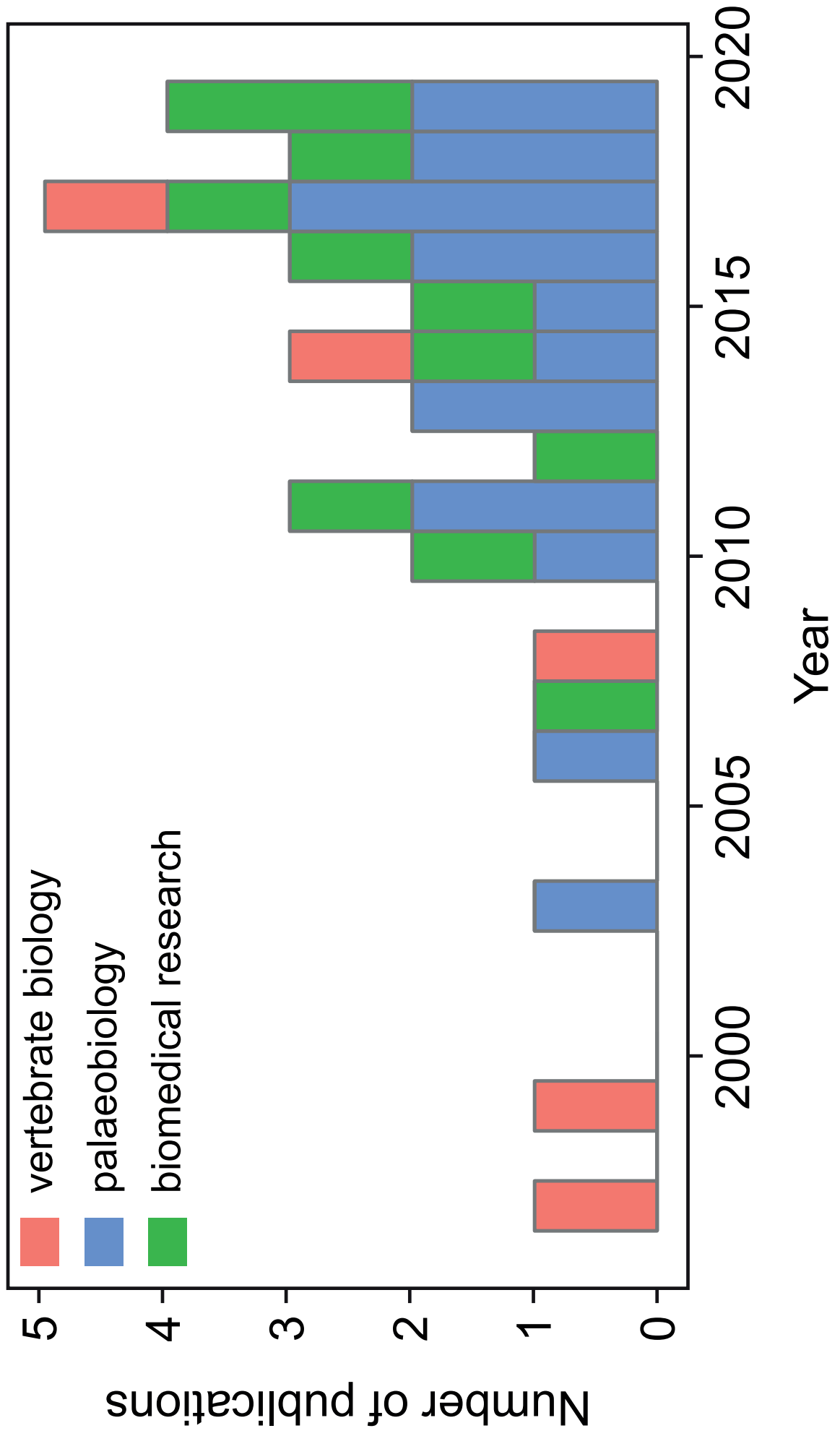


Figure 2

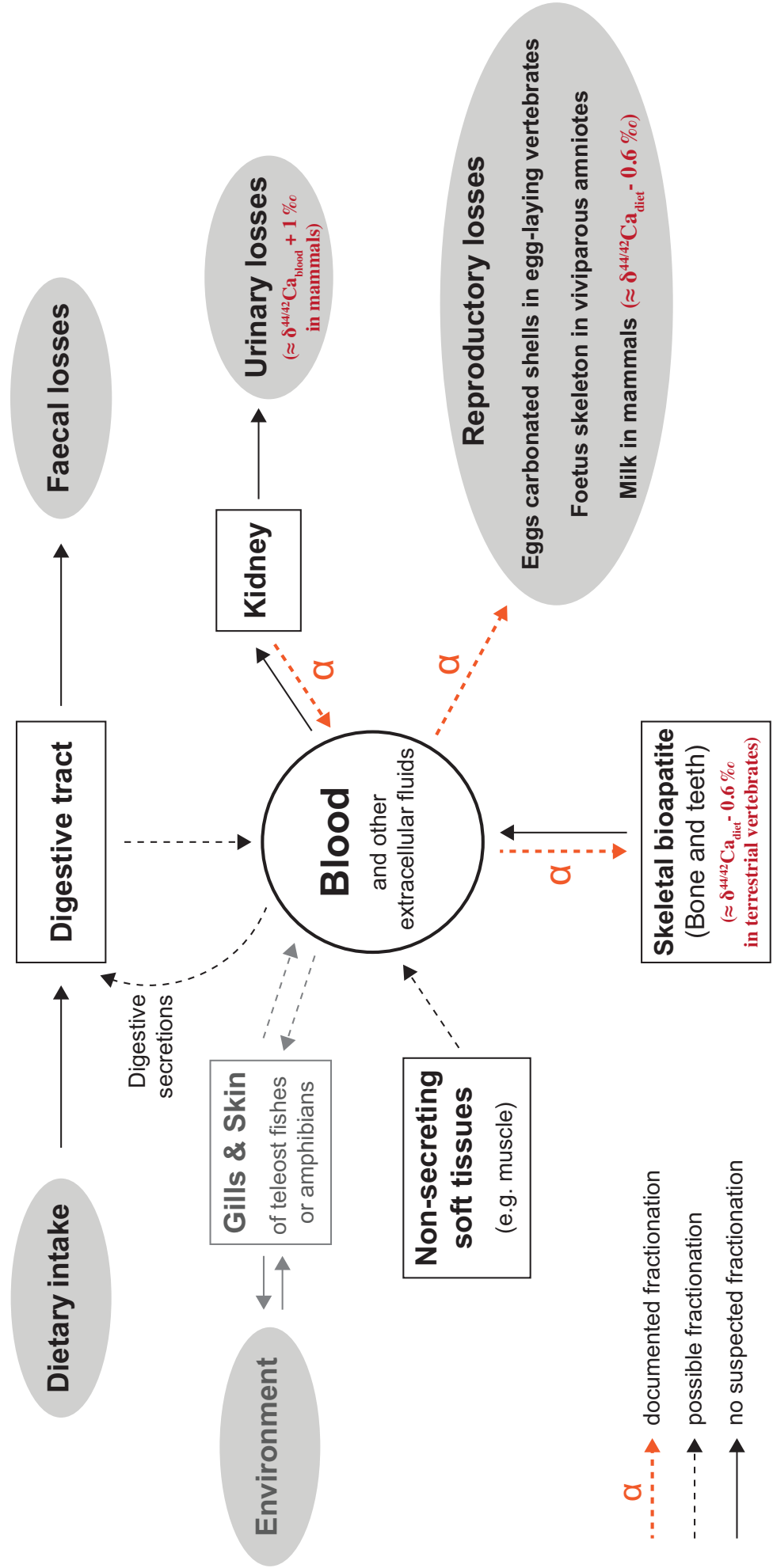


Figure 3

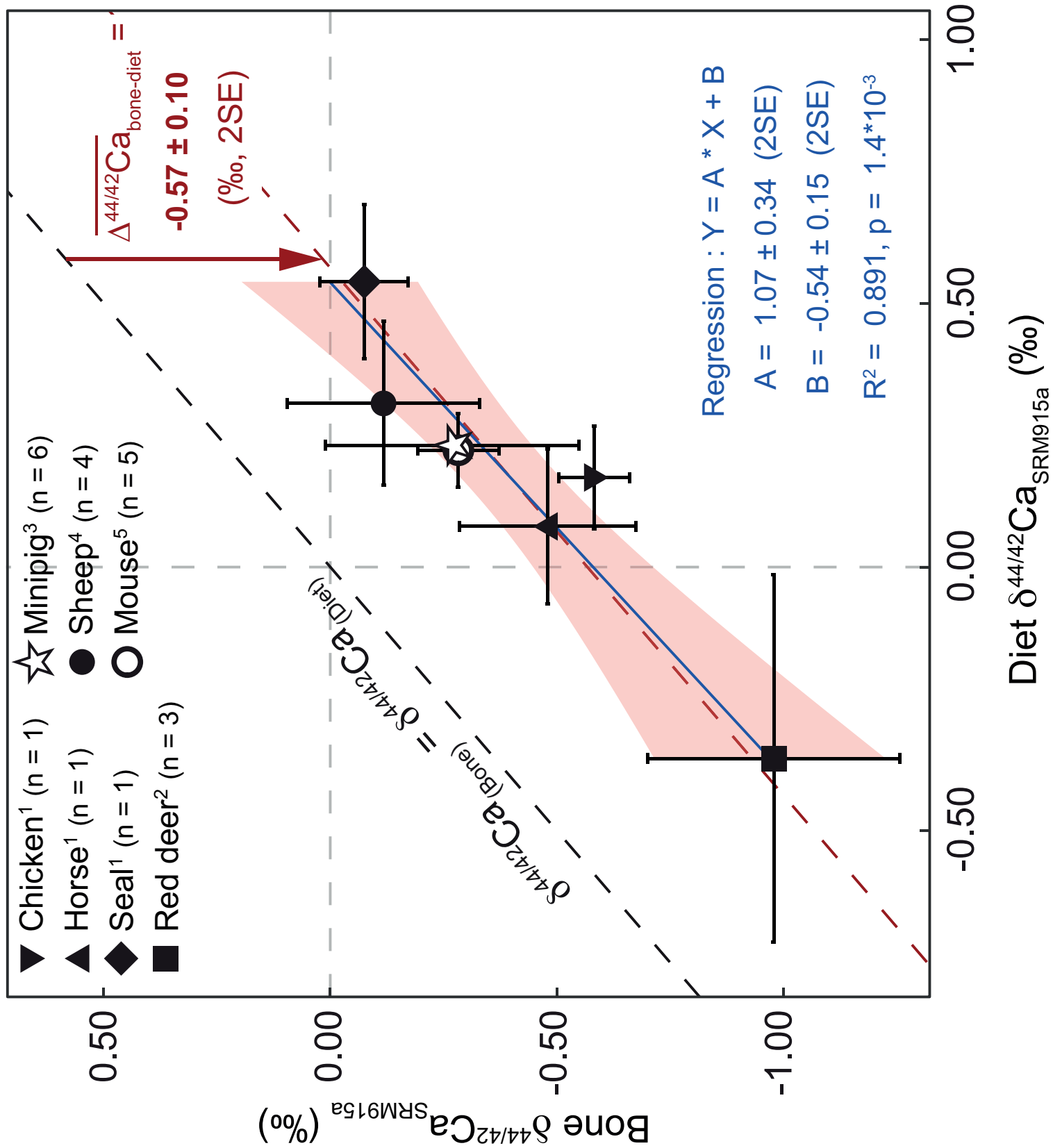


Figure 4

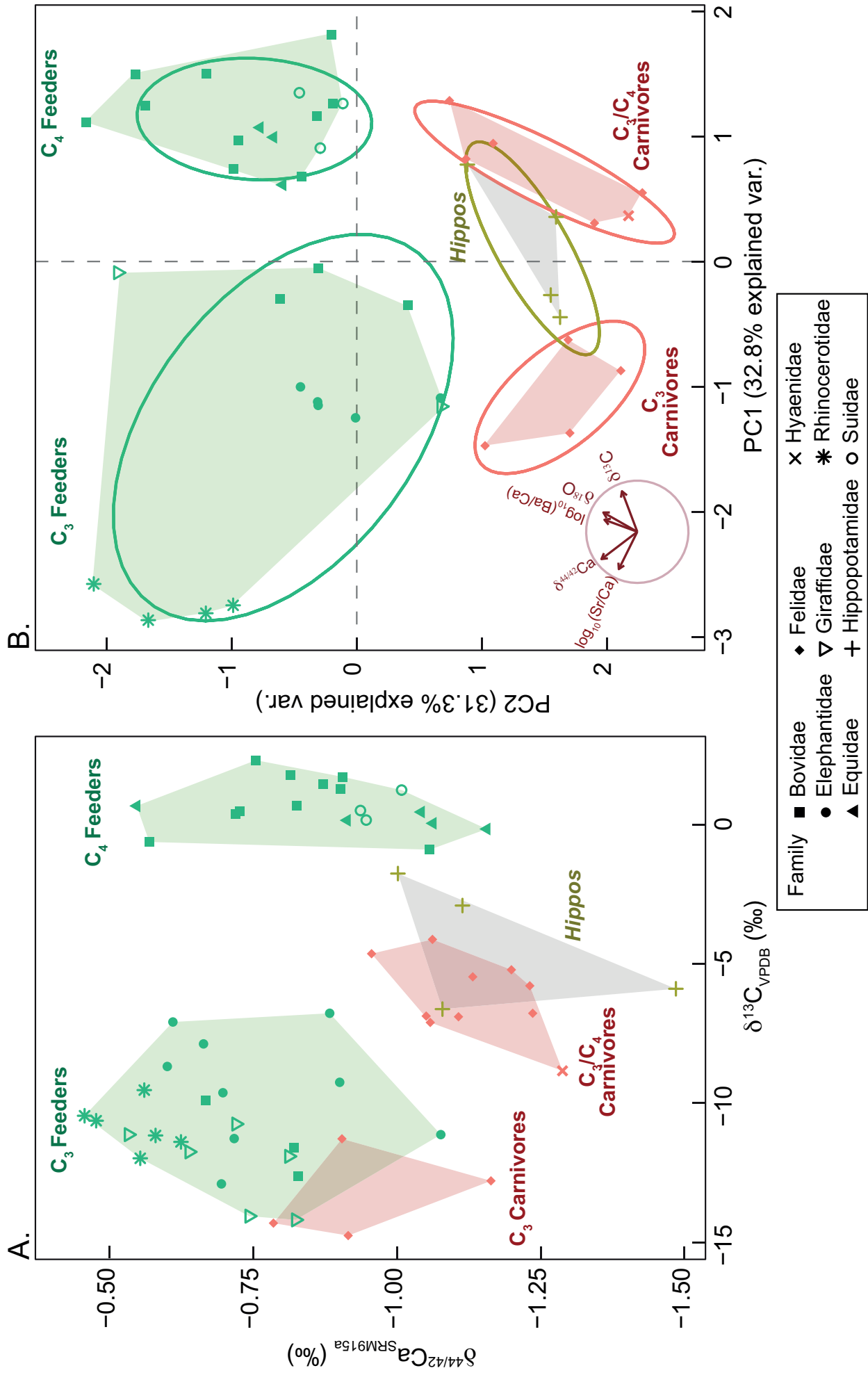


Figure 5

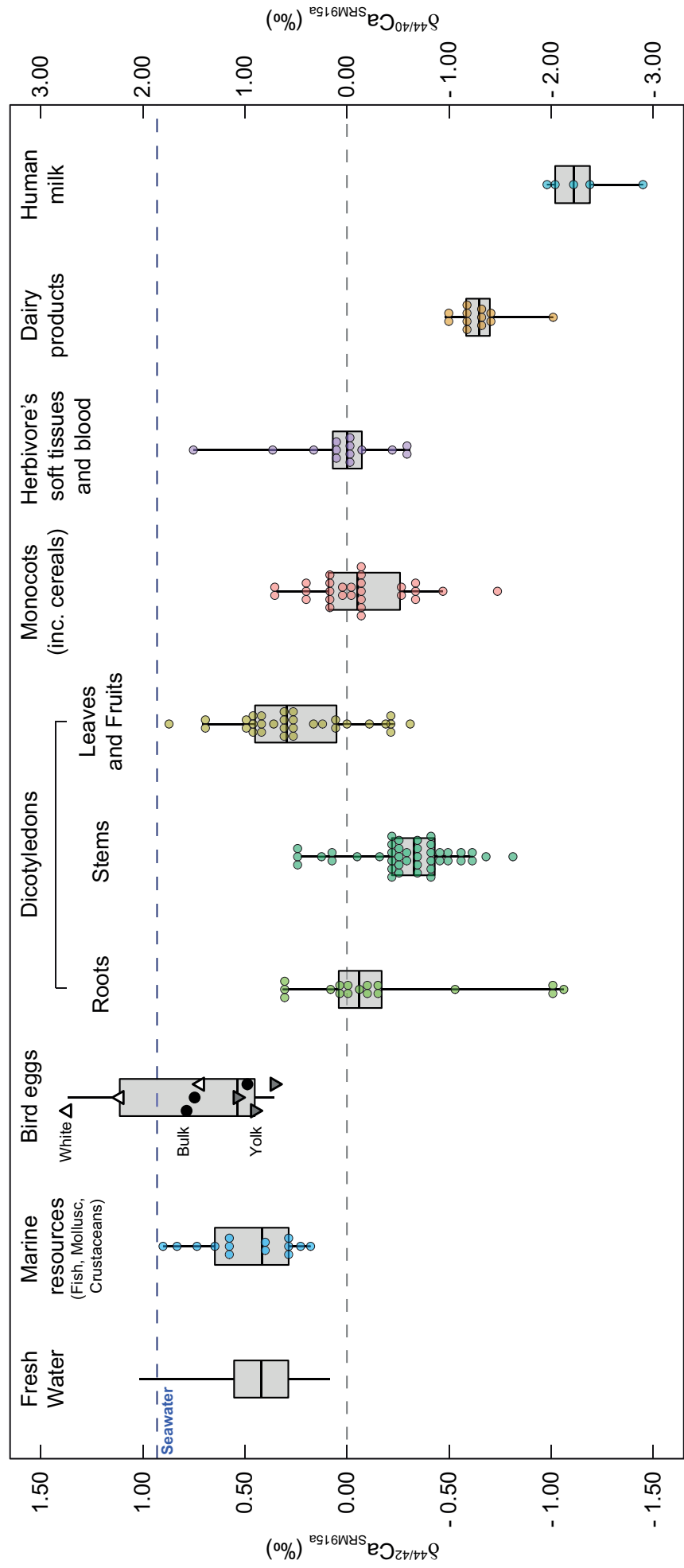


Figure 6

