New frontiers in calcium stable isotope geochemistry:

Perspectives in present and past vertebrate biology

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Abstract

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

Keywords:

Calcium isotopes,

Vertebrate biology,

Palaeobiology,

Bioarchaeology,

Biomedical research,

Bone loss

Preliminary remarks

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

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

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

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

1. Introduction

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

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

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

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

In this article, we first briefly summarize the current understanding of the Ca isotope cycling in vertebrates before reviewing and discussing the potential and future of Ca isotopes in palaeobiology and biomedical research. Finally, we identify challenges and barriers related to the collaborative research necessary for the adoption of Ca isotope tools by the wider scientific community.

2. The cycling of calcium and its isotopes in vertebrates

The biological processing of Ca by vertebrates induces mass dependent isotope fractionations responsible for maintained and observable heterogeneous distributions of Ca isotopes amongst the different compartments of the organisms. Hence, the distribution and biological functions of Ca in vertebrates are central features of the potential of Ca stable isotopes as tools to study their biology.

2.1. Distribution, functions and regulation of Ca in vertebrates

In vertebrate organisms, the distribution of Ca^{2+} is highly compartmentalized at all anatomical levels (e.g., Del Valle et al., 2011; Doherty et al., 2015; Peterson and Riggs, 2010). Briefly, most body Ca is stored in bone mineral as a major constituent of bioapatite ($Ca_{10}(PO_4)_6(OH)_2$). Mineralized tissues account for ca. 99 % of body Ca in vertebrates, while the remaining fraction is found in non-mineralized or so-called soft tissues. The majority of soft tissue Ca (around 90 %) is found inside cells (mainly in organelles), while extracellular

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

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

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

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

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

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

2.2. Cycling of Ca isotopes in vertebrates

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

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

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

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

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

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

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

(i) as diet directly influences the systematically shifted isotope compositions of mineralized tissues, Ca isotopes can be used to reconstruct dietary behaviours in past vertebrates

(ii) as Ca isotopes are fractionated during bone mineralisation, Ca isotope compositions of blood and urine vary with the balance between bone formation and resorption. Calcium isotopes could be used to monitor variations of bone mineral balance in human subjects suffering from bone disorders.

2.3. Future research

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

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

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

3. Calcium isotopes in palaeobiology and bioarchaeology

Stable isotopes and trace elements enable the development of quantitative approaches to reconstruct ecological, physiological and environmental characteristics that otherwise remain unseen. Unravelling the partitioning of resources notably helps to better understand past and present ecosystem dynamics and biodiversity. Geochemical proxies are useful means of complementing inferences of morphofunctional and microwear analyses of osteological remains (e.g., Martin et al., 2017a). The same applies to the study of past human societies, where such tools are valuable to complement the conclusions of archaeology and anthropology (Jaouen, 2018; Jaouen and Pons, 2016). Following the example of other more established proxies, such as collagen carbon and nitrogen stable isotopes or Sr/Ca and Ba/Ca elemental ratios (Balter and Lécuyer, 2004; Koch, 2007; Newsome et al., 2010; Peek and Clementz, 2012), Ca stable isotopes of fossil bioapatite have a high potential for the study of past vertebrate ecosystems and human communities for three main reasons.

First, as discussed above, the Ca isotope composition of diet primarily determines the composition of vertebrate mineralized tissues. This early finding came along with the hypothesis that such a trophic effect could propagate along food chains. The trophic level index, ranging from primary producer to apex predators and decomposers, describes the position of species within trophic chains and thus allows synthetizing the energetic pathways within ecosystems (Polis and Strong, 1996). Early datasets from both marine and terrestrial environments supported this trophic level effect (Clementz et al., 2003; DePaolo, 2004; Skulan et al., 1997; Skulan and DePaolo, 1999). The biological processing of Ca from a trophic level to another would thus lead to a stepwise decrease of the $\delta^{44/42}$Ca values.

Second, as a major constituent of a stable mineralogical phase (~ 40 wt.% Ca of bioapatite), the post-mortem diagenetic processes tend not to alter skeletal bioapatite Ca and its isotope compositions especially in the denser and less porous tooth enamel (Heuser et al., 2011; Melin et al., 2014; Martin et al., 2017; Martin et al., 2018). The determination of Ca isotope compositions in osteological remains makes it possible to explore deep past ecosystems. So far, studies of vertebrate fossils ecology with Ca isotopes focused on assemblages dating back to Late Devonian (Balter et al., 2019), Triassic to Cretaceous (Hassler et al., 2018; Heuser et al., 2011; Martin et al., 2017b) or Pliocene and Pleistocene (Martin et al., 2018, 2015, Tacail et al., 2019). Contrastingly, the conservation of pristine nitrogen isotope compositions of bone collagen primarily depends on the preservation of the organic phase, the degradation of which rarely allows investigating on trophic relationships beyond Holocene or Late Pleistocene (Koch, 2007). On the other hand, the preservation of biogenic Sr/Ca and Ba/Ca trace element ratios strongly depends on burial conditions (e.g., Martin et al., 2018; Reynard and Balter, 2014).

Finally, the abundance of Ca in osteological remains makes it possible to sample minute amounts of material (down to 20 µg or less). Such sample sizes allow studying precious fossils, without significantly altering their physical and structural integrity. It also motivates the development of high resolution sampling techniques involving mechanical sampling of tooth enamel by micro-drilling (Tacail et al., 2016, 2017) and laser cutting (Li et

al., 2016). *In situ* laser ablation isotope analyses were shown to be possible in tooth enamel (Tacail et al., 2016; Zhang et al., 2019), although these approaches could greatly benefit from the implementation of technological innovations such as the currently developing use of collision-cell multi-collector plasma mass spectrometry (*e.g.,* Lewis et al., 2018, Zhao and Simon 2019), with potential interference removal.

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

3.1. Calcium isotopes and vertebrate ecosystem reconstructions

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

3.1.1. Marine ecosystems

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

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

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

These studies identified features that require further dedicated investigation in order to improve the use of Ca isotopes as an indicator of marine ecosystem structures. For instance, data indicates that trophic ecology primarily determines tooth and bone isotope compositions of marine vertebrates, but mammals and fishes (cartilaginous or ray-finned) do not appear to display the same average isotope shifts from one trophic level to another (Clementz et al., 2003; Martin et al., 2017b, 2015). The ingestion of seawater and processing of Ca via gill-mediated osmoregulation in actinopterygian and elasmobranch species is suspected to buffer the diet-skeleton isotope offset. On the other hand, marine mammal organisms are rather closed systems with respect to seawater. The differences in Ca isotope physiology of fishes and mammals should thus motivate dedicated research in order to decipher the physiological and ecological controls on the Ca isotope compositions of their tissues. A better understanding of the isotope systematics of marine mammals would notably be of importance as their ecology makes them useful sentinels of ecosystem changes (e.g., Moore, 2008).

3.1.2. Continental ecosystems

The nature and isotope compositions of primary Ca dietary sources of most terrestrial vertebrate trophic chains are more diversified than in marine ecosystems (see Figure 5). Indeed, in addition to the possible variability of isotope compositions of environmental Ca (e.g., interacting bedrock, soil and water), the plant Ca isotope compositions are highly variable between types, species and organs (e.g., Schmitt, 2016 and Figure 5). Depending on the ecological niches of primary consumers (e.g., in vertebrate herbivores), such

variability can propagate throughout the trophic chain and make the interpretation of Ca isotope compositions more complex for trophic relationships reconstructions.

Melin et al. (2014) explored the bone Ca isotope compositions of various mammals including primates from two tropical forested ecosystems. The authors confirmed the decrease of Ca isotope ratios in large carnivores, but found no significant differences between lower trophic levels, notably small faunivores and insectivores. They however suggest an encouraging complementary use of carbon (C) and Ca stable isotopes. Martin et al. (2017a) reported encouraging bone and tooth enamel Ca isotope compositions of two Western European Pleistocene mammal assemblages. The stable isotope compositions of each taxonomic group (up to 6 individuals) are rather clustered and carnivores tend to display lower $\delta^{44/42}$Ca values than herbivores. However, the outlying compositions of some species remain difficult to interpret in terms of trophic inferences, suggesting either species-dependent physiological effects or peculiar dietary niches effects. Recently, Martin et al. (2018) reported the results of the systematic study of mammal tooth enamel of modern and fossil Pliocene-Pleistocene ecosystems from East Africa. This study further documents the potential of Ca isotopes as helpful markers for trophic inferences. The combination of C and Ca stable isotopes helps to distinguish between C_3 and C_4 trophic chains structures, in extant and extinct ecosystems. Indeed, the carbon stable isotope compositions of the enamel of mammals (δ^{13}C) are characteristic of the photosynthetic pathways of the plants at the base of the trophic chain (C_3 or C_4 carbon fixing sugars, c.f. Bender 1971), while Ca isotopes appear to allow discriminating between herbivores and carnivores. This is exemplified by the figure 4A, where the carnivores appear depleted in ^{44}Ca isotopes with respect to their respective inferred preys, being either predominantly C_3 herbivores with low δ^{13}C values (namely browsers) or a mix between the latter and C_4 herbivores with higher δ^{13}C values (namely grazers). Furthermore, when taking into account all available geochemical proxies relevant to feeding and habitat ecology (namely Sr/Ca and Ba/Ca ratios and δ^{13}C, δ^{18}O, $\delta^{44/42}$Ca, see Martin et al., 2018), a principal component analysis (Figure 4B) reveals an inferred

ecosystem structure that is very similar to what $\delta^{13}C$ and $\delta^{44/42}Ca$ alone suggest (Figure 4A). In other words, the coupling of $\delta^{13}C$ and $\delta^{44/42}Ca$ appears to efficiently summarize the ecosystem structure.

Finally, in the Mesozoic fossil record, despite the fact that rather inconclusive results were obtained from dinosaur faunas on large geographical and chronological scales (Heuser et al., 2011), the systematic study of skeletal remains Ca isotope compositions from a fossil assemblage at regional scale brings new constraints to the resource partitioning between mid-Cretaceous predatory dinosaurs and more precisely to the ecology of spinosaurids (Hassler et al., 2018).

To conclude, the ongoing exploration at regional scale of the ecological controls over vertebrate Ca isotope compositions in the continental environment is encouraging. Calcium isotopes show a very good potential for the reconstruction of dietary relationships in past vertebrate ecosystems, from Palaeozoic to Quaternary, and allow constraining local ecosystem structures and resource partitioning, especially between herbivores and carnivores. These results motivate the development of new strategies that notably include i) the combination of $\delta^{44/42}Ca$ with complementary proxies, such as $\delta^{13}C$, ii) the study of assemblages composed of taxa with diverse ecologies, iii) at a regional scale, iv) with taxonomic groups comprising multiple individuals. Further research needs to be carried out, notably because some taxa do not entirely fit within this interpretative framework. This could relate to peculiar physiological adaptations or ecological niches (e.g., Martin et al., 2018, 2017a). For instance, the observed isotopic offsets between bone of most secondary or tertiary consumers and their vertebrate preys are likely to primarily rely on the partial consumption of mineralized tissues from the latter. The intake of only 1 wt. % bone in total diet is sufficient to cause a significant trophic effect in Ca isotope compositions (-0.50‰ on the $\delta^{44/42}Ca$ scale) of consumer's tissues in terrestrial vertebrates (Heuser et al., 2011). Although little is known about muscle and bulk soft tissues isotope compositions, the consumption of energy-rich but Ca-poor soft tissues could result in different isotope offsets

between secondary consumers and their vertebrate preys, making trophic level inferences less straightforward.

3.1.3. Future directions

(i.) The development of statistical approaches for diet reconstruction with Ca isotopes could help to refine and make the most of trophic ecosystem reconstructions (such as Bayesian statistical inferences, see for instance Fernandes et al., 2014). The inter-individual variability of Ca isotope compositions within a given taxonomic group could also relate to the degrees of specialisation for various Ca resources (e.g., Martin et al., 2018). By means of statistical methods, Ca isotopes could allow characterizing "isotope niches" and help to document breadths of ecological niches or the specialisation of taxonomic groups (*e.g.,* Yeakel et al., 2015).

(ii.) Profound changes in the biological management of Ca occurred in the course of vertebrate evolution. A series of major evolutionary steps could potentially be studied with the help of Ca isotopes: the appearance of calcium phosphate mineralized endoskeletons, the ecological and physiological adaptations related to fish-to-tetrapod transitions followed by terrestrialisation, the development of egg-laying and carbonated shells in vertebrates or finally the development of pregnancy and lactation in first mammalians (Bouillon and Suda, 2014; Doherty et al., 2015). Each of these evolutions of vertebrate physiology and ecology likely affected their Ca isotopes cycling, which could in turn be exploited in order to document the evolutionary mechanisms at play.

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

3.2. Calcium isotopes and dietary reconstructions in hominids and human societies

3.2.1. From ecosystems to human societies

Major changes in dietary practices in the course of human evolution, being ecologically and/or culturally driven, likely came along with significant changes of their use of Ca resources (e.g., Eaton and Nelson, 1991). The changing dietary habits of early *Homo* or the development of agriculture and livestock farming in the course of Neolithic mark significant modifications of the use of environmental Ca and were associated with major ecological, physiological, genetic or cultural evolutions (e.g., Aiello and Wheeler, 1995; Hublin et al., 2015; Ségurel and Bon, 2017; Vigne, 2011). The utility of Ca isotopes to track these changes has been however poorly explored. While the previously described ecosystem approach using Ca isotopes could help to interpret the evolution of hominid ecologies, the study of bone Ca isotopes in human societies could improve our understanding of their contrasting dietary habits influenced by varied cultural practices.

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

For instance, dairy products constitute the most ^{44}Ca-depleted end-member of this isotopic landscape (with modern human milk being the lightest), while eggs and marine resources display the most ^{44}Ca-enriched compositions. Following these observations, Chu et al. (2006) proposed that Ca isotopes could be used to reconstruct the consumption of dairy products in past human groups, especially during the processes of Neolithization with domestication of dairy producing animals. Indeed, the consumption of significant proportions of Ca from dairy products should induce a global decrease in $\delta^{44/42}$Ca values of dietary Ca and thus in bone of human consumers. Reynard et al. (2010, 2011) tested the potential of Ca isotopes in faunal and human bones as a biomarker of dairy product consumption in several archaeological populations before and after domestication of dairy producing animals. These authors found however no significant change in the $\delta^{44/42}$Ca offset between fauna and human

of the studied archaeological sites after the domestication of milk producing animals. They suggested that controls other than animal milk consumption were likely at play.

Nevertheless, further research including actualistic calibrations in human osteological remains could help refine our understanding of the controls of dietary practices over bone Ca isotope compositions.

3.2.2. Life history reconstructions in individuals

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

So far, the major life history traits explored with Ca isotopes relate to mother's milk consumption by children. The biologically and culturally driven weaning behaviours are central traits of the evolution of mammals and hominins (Humphrey, 2010; Sellen, 2007; Van Noordwijk et al., 2013) but these practices remain fairly undocumented in the deep past because biomarkers are lacking. As milk was early recognized as a ^{44}Ca-depleted material in comparison with diet, Reynard et al. (2013) first tested this hypothesis by analysing bones of past human populations as a function of age of individuals but no significant trends were observed. This hypothesis was later tested on temporary tooth enamel of modern human individuals with known early life histories (Tacail et al., 2017). Contrary to bone which continuously remodels, tooth enamel forms incrementally. Provided the sampling techniques allow sufficient spatial resolution, the enamel structure permits the reconstruction of the evolution of Ca isotope compositions over time of formation of tooth crowns. This study demonstrates that weaning practices are recorded within human tooth enamel by Ca

isotopes and therefore could allow documenting weaning patterns in past humans and hominids. This method was recently applied to the tooth enamel of Pleistocene early hominins of South Africa (Tacail et al., 2019). The results support that early *Homo* infants were breastfed in significant proportions for longer periods than *A. africanus* and *P. robustus* and opens discussion on the evolution of weaning practices in human lineage.

3.2.3. Future directions

(i) Given the reported systematic variability of Ca dietary sources, and provided this variability is further documented, Ca isotopes could be a successful marker of human dietary practices in relation with human cultures and subsistence modes. A more actualistic approach with a focus on modern and historic human populations with documented and contrasting diets would for instance open the way to new archaeological applications, the same way Zn isotopes were shown to be sensitive to consumption of fish and meat (Jaouen et al., 2018).

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

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

4. Calcium isotopes in biomedical research

Stable Isotope Metallomics is the study of normal and disrupted cycles of regulated and essential metals from the perspective of their natural stable isotope compositions. This field of research has developed in the last two decades following seminal works on Ca, Fe,

Cu or Zn, aided by the development and widespread application of multicollection 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.

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 ^{44}Ca-depleted blood and urine. This recognition sparked a vivid interest for the study of Ca isotopes as a marker of disruptions of BMB.

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.

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

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

Skulan et al. (2007) first published a report of significant variations of $\delta^{44/40}$Ca values in weekly-pooled urine of individuals submitted to bed rest experiments. The authors reported significant negative departure from baseline in $^{44}Ca/^{40}Ca$ ratios during bed rest in the group with no countermeasure to bone loss followed by a return to baseline after the experiment. By contrast, subjects treated with pharmacological or exercise countermeasures to bone loss displayed either no change or positive change of their urine $\delta^{44/40}$Ca values. These results provided a first proof of concept. This work was later augmented by Morgan *et al.* (2012) with a report of changes in urine Ca isotope compositions during a bed rest experiment conducted with a finer chronological sampling. This study demonstrated that the decrease in $\delta^{44/42}$Ca values of urine occur after one week, indicating a loss of bone in agreement with markers of osteolytic activity (NATX) but long before any changes in bone mineral density could be detected by means of X-ray methods. These results were further confirmed in the blood of the same subjects (Channon et al., 2015). The recognition of the isotopic fractionation induced by renal function lead the authors to adjust the mathematical models used to link changes in bone mass and urine Ca isotope compositions (*e.g.,* Heuser and Eisenhauer 2010, Morgan et al., 2012). The observed negative offset in urine $\delta^{44/42}$Ca results from loss of Ca from bone but also from an increase in Ca urinary losses in kidneys (*i.e.* a decrease in Ca reabsorption). Taking this into account, Morgan et al. (2012) calculated the relative bone loss during the bed rest experiment. The estimated relative bone loss

agreed with bone loss predicted on the basis of previous experiments in which bed rest was of a duration long enough to produce changes in BMD resolvable by X-ray densitometry (around 0.25 % of total bone mass, for -0.30‰ decrease of $\delta^{44/42}$Ca values).

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

4.2. Calcium isotopes as biomarkers of metabolic bone diseases

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

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

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

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

4.3. Future directions

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

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

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

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

the Ca cycle and have high incidence in modern societies (Peacock, 2010; Shroff et al., 2013). For instance, the progressive disruption of the kidney function in individuals suffering from chronic kidney diseases is likely to affect the distribution of Ca isotopes. Other disturbances of the Ca homeostasis being either induced by drugs or in some digestive disorders could also be explored with Ca isotopes. Dedicated research could also potentially lead to the development of new biomarkers for such conditions of disrupted Ca homeostasis.

5. Challenges of vertebrate Ca isotope geochemistry

The potential of Ca isotopes is high in the field of vertebrate biology. However the development of new applications and their possible adoption by scientific communities other than Earth scientists faces barriers, and depend on the communities involved.

5.1. Developing the use of Ca isotopes in palaeobiology and bioarchaeology

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

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

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

5.2. Using Ca isotopes from fundamental to applied biomedical research

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

5.2.1. Initiating successful interdisciplinary research projects

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

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

5.2.2. Analytical and methodological challenges

Another challenge is to identify the methodological constraints imposed by the various disciplines involved in the project, and to accommodate these constraints in the study design. For example, the extreme care typically required to maintain sterility and integrity of biological samples for molecular biological assays is generally unnecessary for samples destined for isotope analysis. On the other hand, substances commonly used as preservatives in blood and urine samples can be unacceptable contaminants for isotope analysis. Disciplines also have different requirements for experimental controls. In human experiments involving Ca isotopes, this typically requires sampling and analysis of all dietary and pharmacological Ca sources, as well as multiple pre-experimental sampling of all study subjects in order to establish individual baselines. Finally, isotope geochemistry laboratories are not biomedical laboratories. Ethical and health regulations often require formal

authorization for the handling of human tissues, as well as adaptation of laboratory practices and proper training of isotope scientists, engineers, technicians or students in the hazards posed by these materials. Also, isotope analyses in isotope geochemistry laboratories tend to be slow when compared to routine biomedical analyses, even when machine time is not limited. For Ca isotopes to become a widely used clinical tool, sample throughputs must be increased by several orders of magnitude, while ensuring a sufficient precision and accuracy of the isotope analysis. This is technically feasible (with approaches such as automated sample preparation, spiking methods, collision-cell instruments, e.g., Romaniello et al., 2015, Lewis et al., 2018) but will not happen without being spurred by demand.

5.2.3. Developing a medical biomarker

In general, the development of medical biomarkers is codified and standardized, for instance in terms of clinical trials methodology and statistics or ethical and legal aspects. These are constraints for which isotope geochemists, and their host laboratories and institutions, are neither prepared nor able to address on their own. The first challenge is thus to recruit the assistance of medical scientists in the regulatory and administrative aspects of study design.

In addition to aforementioned challenges, we suggest that another lies in the differences in technical and scientific cultures in which stable isotope geochemists and medical scientists have evolved. For example, stable isotope geochemists tend to direct their research toward a fundamental understanding of processes while medical scientists and hospital doctors may feel more constrained by the need for practical and immediate tools to achieve clinical goals. This urgency is something geochemists do not often encounter on their home turf. As a result, because geochemists tend to assume that practical application proceeds from theoretical understanding, they could tend to underestimate the current clinical utility of their techniques and undersell them.

Finally, medical science is immersed in a biological paradigm that views life as an interaction between complex organic macromolecules (including genes) at the cellular level.

Metal stable isotope analysis, focused as it is on inorganic chemistry, does not mesh well with this paradigm. Apart from the practical clinical tools they can provide, isotope chemists also are in a position to advance a long overdue appreciation of the central role purely inorganic processes play in biology. It is possible to say a great deal about the state of an organism by looking at the distribution of simple ions and isotopes, without reference to cells or macromolecules, in the same way that it is possible to say a great deal about an environmental or geological system without reference to the details of its component parts.

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Captions:

Figure 1: Number of publications per year reporting new research on Ca isotopes in vertebrates (Single column)

Figure 2: Typical Ca cycle in vertebrate organisms. The Ca fluxes for which Ca isotope fractionation is documented, possible or not suspected are indicated with orange dashed arrows, black dashed arrows and black solid line respectively. (2-column)

Figure 3: $\delta^{44/42}$Ca of bone of 7 species of vertebrates as a function of their diet $\delta^{44/42}$Ca (in ‰, relative to SRM915a reference material) shows the shared physiological isotope effect. The red dashed line is the average bone-diet offset, the blue full line is the linear regression and the red shaded area delimits its 95% confidence interval. Error bars are 2sd. [1] Skulan and DePaolo, 1999; [2] Chu et al., 2006; [3] Heuser et al., 2016; [4] Tacail et al., 2014; [5] Hirata et al., 2008. (Single column)

Figure 4: Ecological reconstructions of a modern mammal ecosystem (Tsavo National Park, Kenya, dataset from Martin et a., 2018): A. Tooth enamel $\delta^{44/42}$Ca (in ‰, relative to SRM915a reference material) as a function of δ^{13}C (in ‰, relative to VPDB reference material) (Modified from Martin et al., 2018). B. Principal Component Analysis (PCA) of various chemical proxies in tooth enamel (grouped by main ecological groups and Hippos, as described in Martin et al., 2018, displayed with 95% distribution ellipses). The δ^{13}C-$\delta^{44/42}$Ca space summarizes well the ecosystem structure clearly depicted by the PCA, while hippos stand as outliers that remain to be explained. (2-column)

Figure 5: Compilation of Ca isotope compositions (in ‰, relative to SRM915a reference material) of a series of Ca sources in diets of vertebrates including human. This compilation

includes continental river water considered as fresh water (Tipper et al., 2016), marine resources (fish, mollusc and crustaceans) (Skulan et al., 1997; Skulan and DePaolo, 1999), dicotyledon roots, stems and leaves or fruits and monocotyledon including cereals (Christensen et al., 2018; Chu et al., 2006; Farkaš et al., 2011; Gussone and Heuser, 2016; Heuser, 2016; Hindshaw et al., 2013; Holmden and Bélanger, 2010; Huang et al., 2012; Moore et al., 2013; Page et al., 2008; A. Schmitt et al., 2003; Skulan and DePaolo, 1999; Tacail et al., 2014; Wiegand et al., 2005), hen and quails' eggs (Skulan and DePaolo, 1999; Tacail, 2017), herbivore's soft tissues and blood (Heuser, 2016; Morgan et al., 2012; Skulan and DePaolo, 1999; Tacail et al., 2014), animal milk and dairy products (Chu et al., 2006; Gussone and Heuser, 2016; Heuser, 2016; Tacail et al., 2017) and modern human milk (Chu et al., 2006; Tacail et al., 2017). (2-column)

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

Figure 1

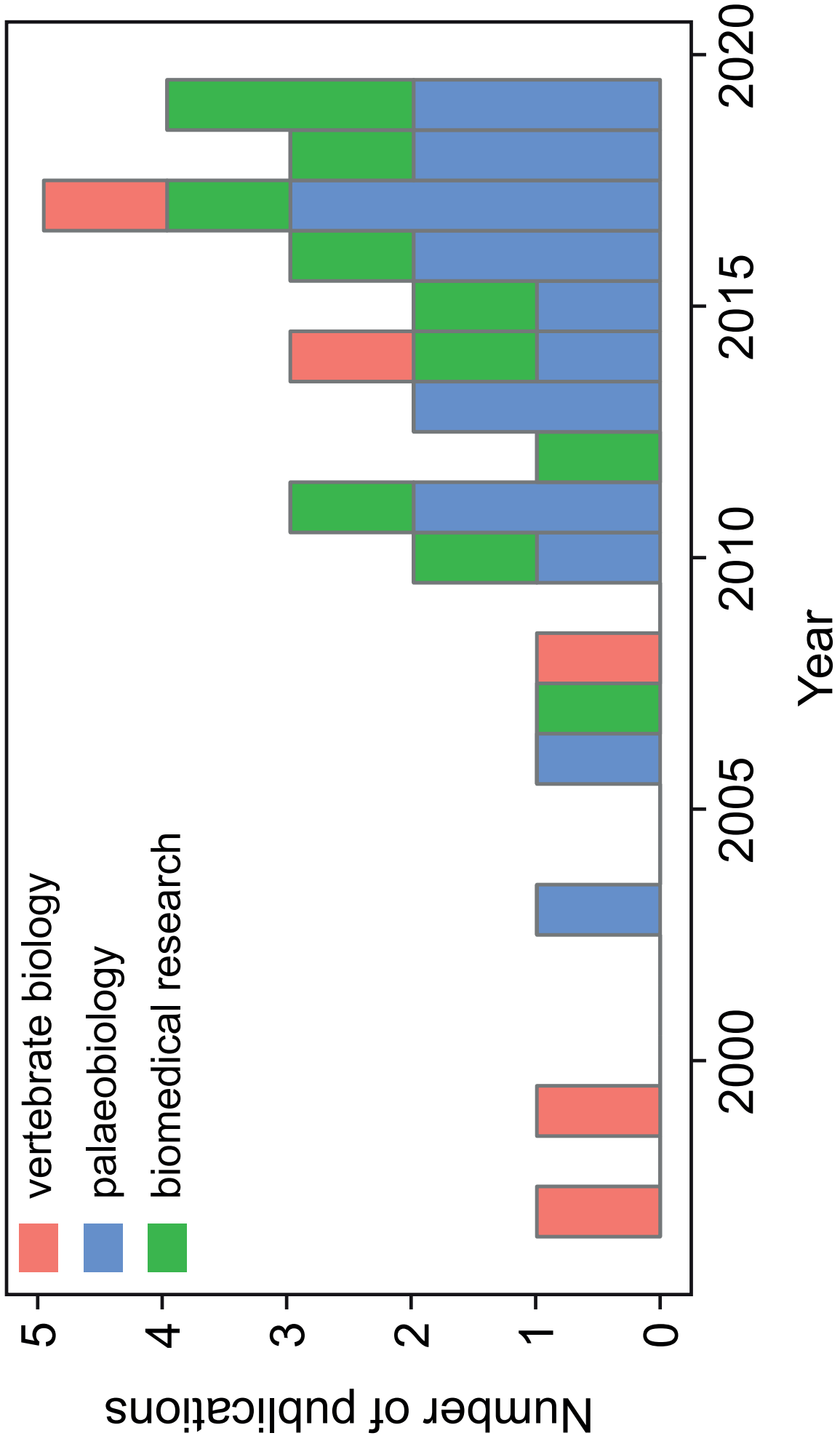
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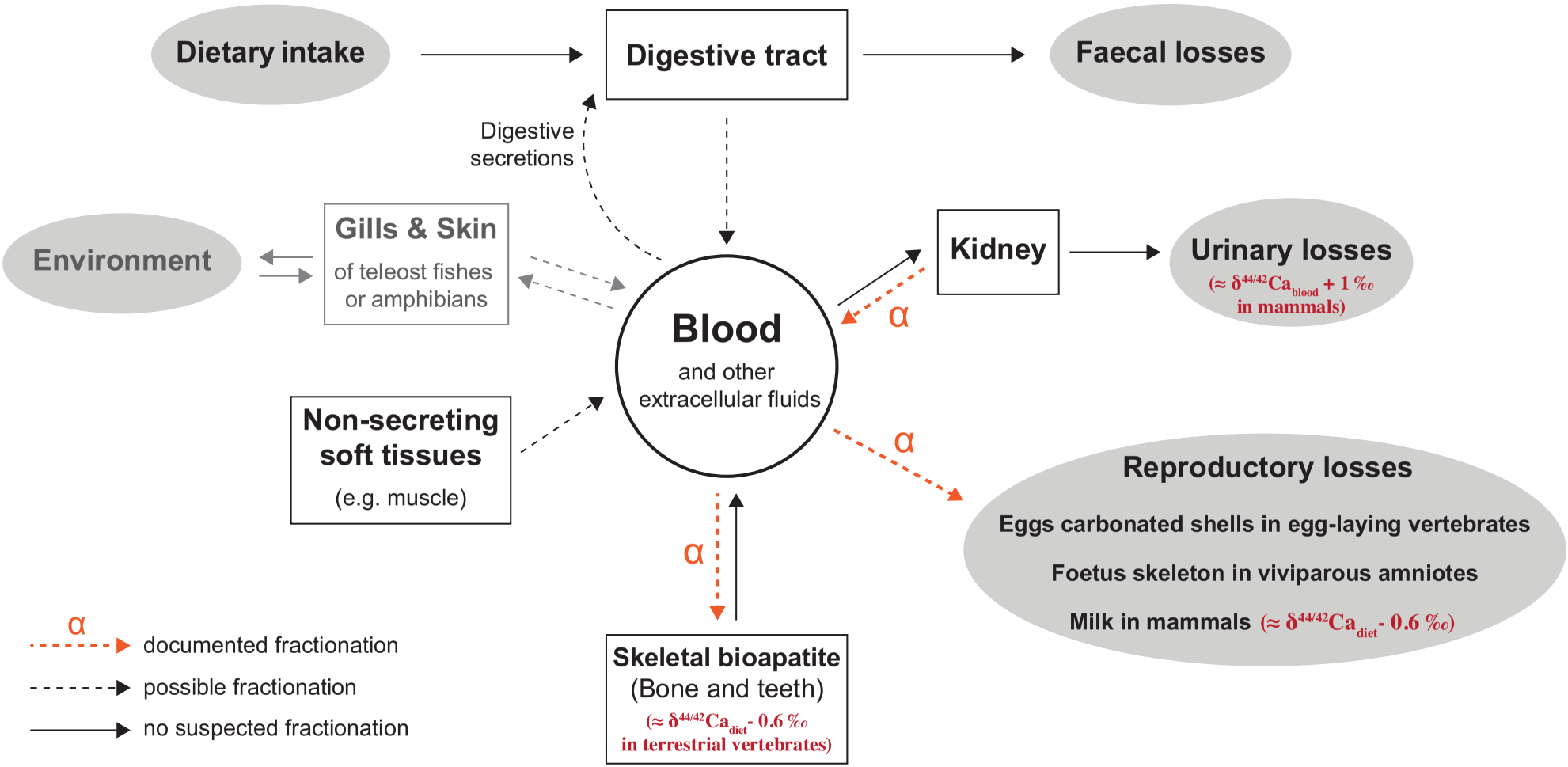
Figure 2


Dietary intake
Digestive tract
Faecal losses
Digestive secretions
Environment
Gills & Skin
of teleost fishes or amphibians
Kidney
Urinary losses
(≈ δ44/42Ca blood + 1 ‰ in mammals)
Blood
and other extracellular fluids
α
Non-secreting soft tissues
(e.g. muscle)
α
Reproductory losses
Eggs carbonated shells in egg-laying vertebrates
Foetus skeleton in viviparous amniotes
Milk in mammals (≈ δ44/42Ca diet - 0.6 ‰)
α
Skeletal bioapatite
(Bone and teeth)
(≈ δ44/42Ca diet - 0.6 ‰ in terrestrial vertebrates)
α
documented fractionation
possible fractionation
no suspected fractionation

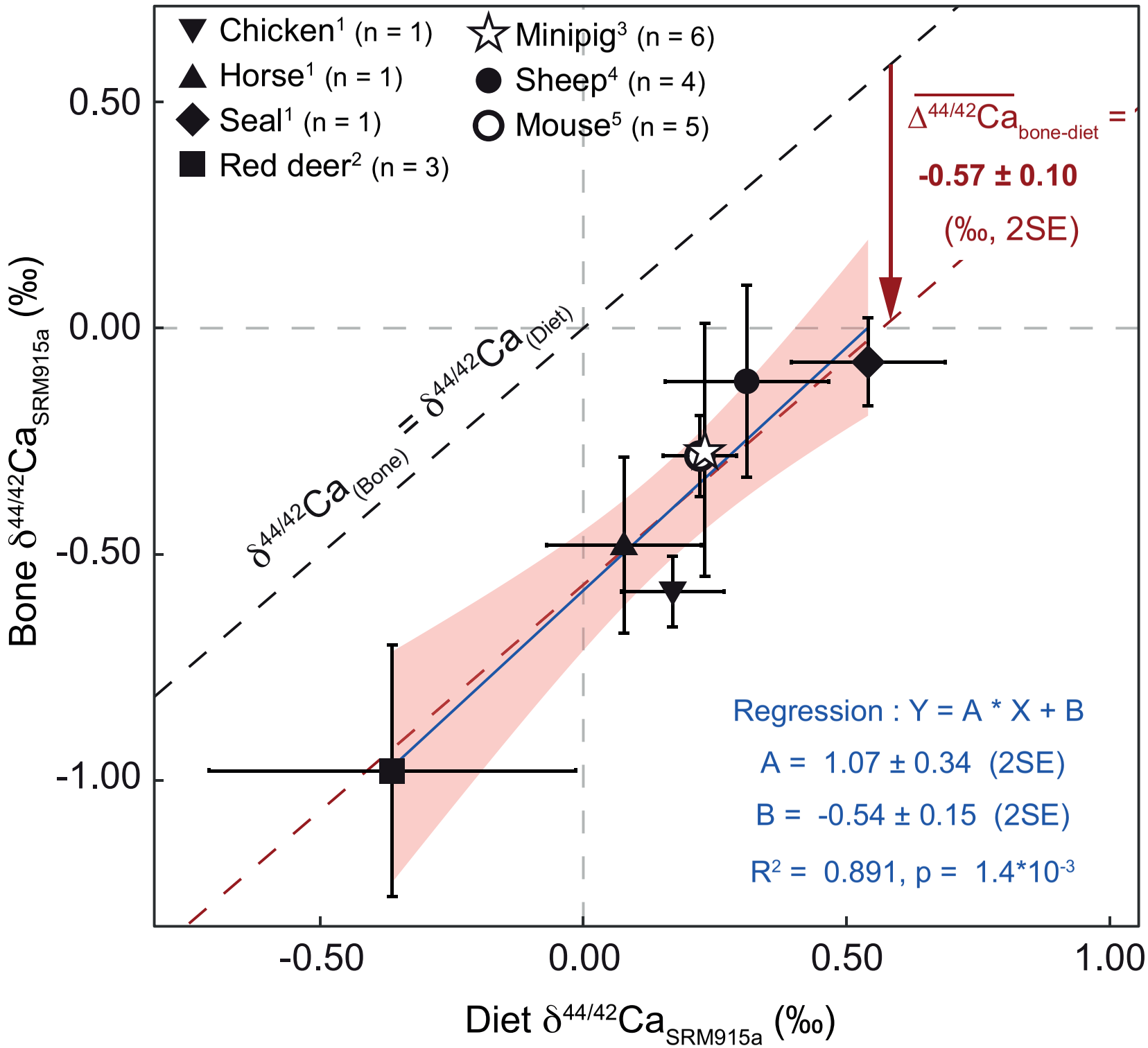

Chicken[1] (n = 1)
Horse[1] (n = 1)
Seal[1] (n = 1)
Red deer[2] (n = 3)
Minipig[3] (n = 6)
Sheep[4] (n = 4)
Mouse[5] (n = 5)
$\overline{\Delta^{44/42}Ca_{bone\text{-}diet}}$ =
-0.57 ± 0.10
(‰, 2SE)
$\delta^{44/42}Ca_{(Bone)} = \delta^{44/42}Ca_{(Diet)}$
Regression : Y = A * X + B
A = 1.07 ± 0.34 (2SE)
B = -0.54 ± 0.15 (2SE)
R^2 = 0.891, p = 1.4*10^{-3}
Bone $\delta^{44/42}Ca_{SRM915a}$ (‰)
Diet $\delta^{44/42}Ca_{SRM915a}$ (‰)
0.50
0.00
-0.50
-1.00
-0.50
0.00
0.50
1.00

Figure 3

Figure 4

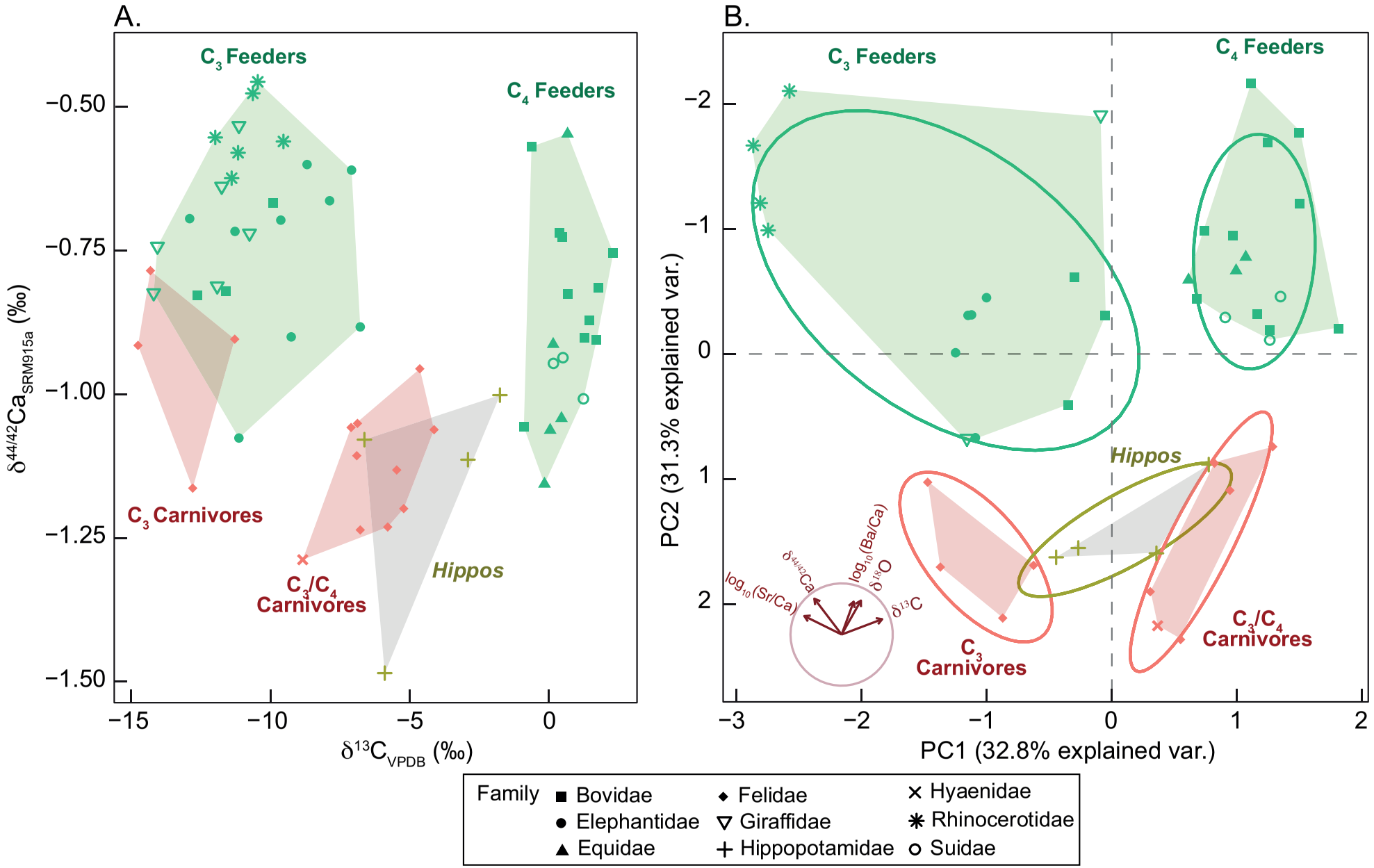

A.
B.
C3 Feeders
C4 Feeders
C3 Carnivores
C3/C4 Carnivores
Hippos
δ44/42CaSRM915a (‰)
δ13CVPDB (‰)
PC2 (31.3% explained var.)
PC1 (32.8% explained var.)
log10(Sr/Ca)
δ44/42Ca
log10(Ba/Ca)
δ18O
δ13C
Family
Bovidae
Elephantidae
Equidae
Felidae
Giraffidae
Hippopotamidae
Hyaenidae
Rhinocerotidae
Suidae

Figure 5

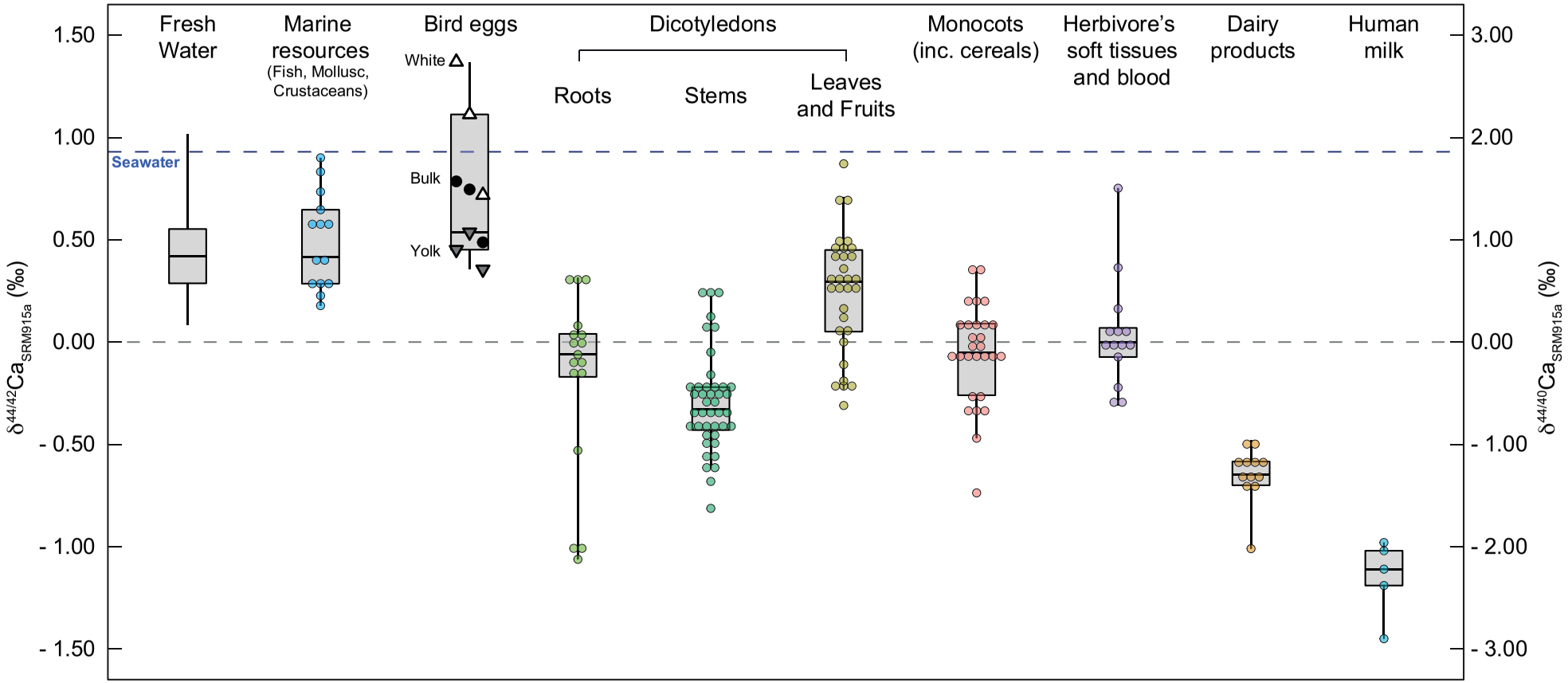
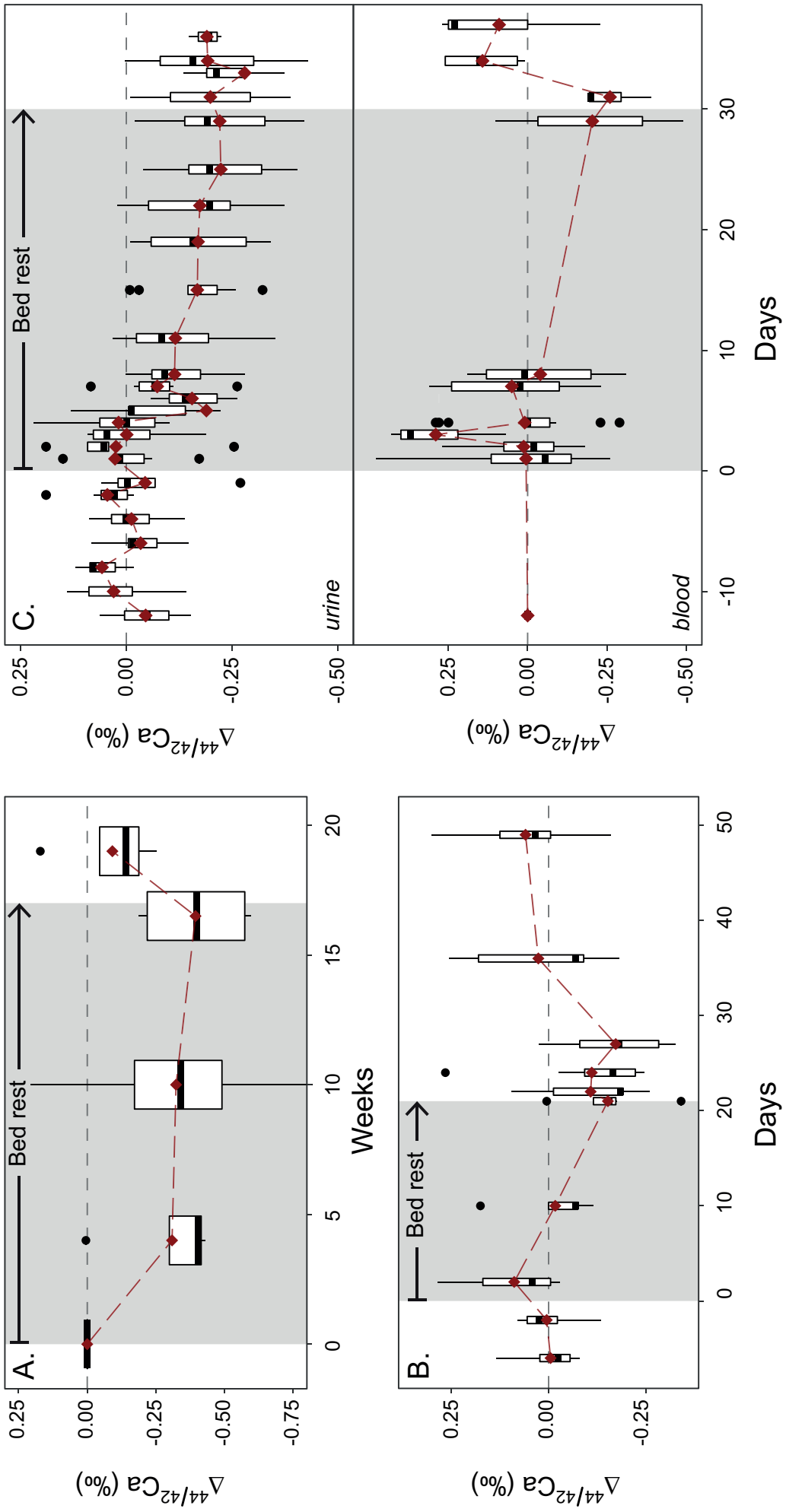

Fresh Water
Marine resources (Fish, Mollusc, Crustaceans)
Bird eggs
White
Bulk
Yolk
Dicotyledons
Roots
Stems
Leaves and Fruits
Monocots (inc. cereals)
Herbivore's soft tissues and blood
Dairy products
Human milk
Seawater
1.50
1.00
0.50
0.00
- 0.50
- 1.00
- 1.50
3.00
2.00
1.00
0.00
- 1.00
- 2.00
- 3.00
δ44/42CaSRM915a (‰)
δ44/40CaSRM915a (‰)

Figure 6


A.
B.
C.
Bed rest
Weeks
Days
urine
blood
$\Delta^{44/42}$Ca (‰)
0.25
0.00
-0.25
-0.50
-0.75
0
5
10
15
20
30
40
50
-10