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Abstract: The recent development of Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICPMS), notably in the disciplines of earth sciences, now allows the measurement of precise isotope ratios, even under low concentration. Non-traditional isotope systems, such as alkaline earth (Ca, Mg) and transition (Cu, Fe, Zn) metals are now being measured in a variety of biological tissues, including bone and teeth. Although our understanding of the environmental and biological mechanisms behind the fractionation of such elements is still in its infancy, some of these isotopes are suspected to fractionate along the food chain as has been reported in the literature for calcium, magnesium and zinc. Other geochemical methods, such as concentration analyses permit a prior assessment of diagenesis in the fossils to be analysed and such an approach allows recognising that in some circumstances, not only enamel but also dentine or bone can preserve its original biogenic composition. The aims here are to review the current knowledge surrounding these various isotopic tools, address their potential preservation in biological apatite and provide the palaeobiologist a guide on the different toolkits available and discuss their potential applications in vertebrate palaeobiology with a case study involving two mammal assemblages from the Pleistocene of Europe.

Key words: non-traditional isotopes, palaeobiology, calcium, cave bear, Pleistocene.

GEOCHEMICAL tools are a great asset to infer biological characteristics in fossil organisms that otherwise would remain completely unnoticed with morphological

observations alone. Among stable isotopes, carbon (C), oxygen (O) and nitrogen (N) have been and are still widely used in ecology, archaeology and in palaeoecology. From the vertebrate individual organism to the community, it is possible to reconstruct body temperatures and infer thermophysiology, reconstruct ambient palaeoenvironmental conditions and infer habitat use and migrations; and last but not least to reconstruct diet and trophic structures in ancient food webs. Most isotope approaches rely on stable isotope ratios of carbon and oxygen but also on radiogenic strontium preserved in fossilised tooth and bone. Elemental concentrations have also proved useful in many contexts such as dietary inference. For example, elemental ratios of strontium to calcium or barium to calcium allow reconstructing ecosystem structures.

Contrary to light isotope systems, which have a variability beyond 10 ‰, non-traditional isotopes comprising alkaline earths (Ca, Mg) or transition metals (Cu, Fe, Zn), show isotope variability within a maximum range of 2-3‰. Recent analytical progresses from the domains of earth sciences now allow the measurement of such stable isotope systems, which show limited fractionation and require high precision and accuracy of measurements from mass spectrometers. The main basis for all those geochemical applications is already starting to be applied to archaeological studies (see review by Jaouen and Pons, 2016). As long as diagenesis can be assessed, there should be no temporal restrictions for their applications to fossil samples. Here, the reader will be presented briefly with traditional systems used in palaeobiology and for further information will refer to previous reviews in ecology and palaeobiology (e.g. Kelly, 1999; Koch, 2007; Newsome *et al*. 2010). We will then present a summary of the current research on non-traditional isotopes in modern and extinct vertebrates; present the different analytical toolkits available and present new information on a case study using Ca isotopes.

TRADITIONAL ISOTOPES IN PALAEOBIOLOGY

New analytical techniques are now being implemented and allow the measurement of non-traditional isotope ratios. Nevertheless, geochemical approaches to investigate the trophic organization of modern vertebrate ecosystems rely primarily on carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and elemental concentration ratios (Sr/Ca and Ba/Ca). Palaeoenvironmental information can be derived from oxygen ($\delta^{18}O$) and radiogenic strontium ($^{87}Sr/^{86}Sr$) but also sulfur isotopes ($\delta^{34}S$). Because each element is

independently affected by diagenesis and because environmental or physiological processes may add some noise to the observed isotopic variability, it is important to explore new systems that will be used in conjunction with traditional methods. Traditional isotope systems are briefly presented below, underlining their use in vertebrate palaeobiology. For comprehensive reviews on traditional systems, the reader may consult among others Gannes *et al.* (1998), Koch (2007), Newsome *et al.* (2010), Clementz (2012).

C isotopes

The observation that carbon radioisotopic dates were systematically younger than expected when analysing plants such as corn led to the recognition of an isotopic difference between plants with different photosynthetic pathways (Bender, 1968). As a consequence, $\delta^{13}C$ values have been used to address the contribution of C_3 versus C_4 plants in human and animal diets since at least the late '70s (Vogel, 1978; De Niro and Epstein, 1978; van der Merwe, 1982). Such a discovery had some major implications in palaeobiology and the study of ancient climates and environments with the recognition that C_4 plants became predominant in terrestrial ecosystems at the end of the Miocene (Cerling *et al*. 1997). Although carbon isotopes can be measured in various soft tissues, palaeontological studies focus on mineral tissues such as teeth or bone, where carbon takes part in the carbonate fraction of bioapatite. Therefore, carbon isotopes also permitted to highlight trophic dynamics between mammals and changing environments through deep time (Macfadden *et al*. 1999) and provide specific clues as to diets of extinct hominins (Schoeninger, 2012). Across aquatic environments, carbon isotopes can also account for productivity with the highest $\delta^{13}C$ values measured in animal tissues living in upwelling and near-shore marine habitats and the lowest $\delta^{13}C$ values measured in animals living in marine offshore or estuarine / freshwater habitats (Clementz and Koch, 2001).

N isotopes

Nitrogen is a major constituent of proteins and among vertebrate fossil samples, collagen is exclusively retrieved from bone or dentine but not from tooth enamel. Primary producers provide a diversified isotopic baseline to the rest of the food chain

but among vertebrates, physiological processes induce isotopic fractionation, which is responsible for the strong relation of $\delta^{15}N$ with trophic levels. For this reason, nitrogen isotopes are commonly used to discriminate between herbivores and carnivores in ecological studies (De Niro and Epstein, 1981). Other physiological processes related to nitrogen isotope abundances involve nursing and weaning. For example, $\delta^{15}N$ time series highlighting transition from mother's milk diet to an adult diet have been monitored on dentinal annuli of sectioned marine mammal teeth (Newsome *et al*. 2010). In the 1980s, it was demonstrated that collagen could be preserved in recent fossil bones (Armstrong *et al*. 1993; Stafford *et al*. 1988) and this discovery opened the way for using $\delta^{15}N$ in paleodietary investigations (Ambrose, 1990). However, nitrogen isotopes are rarely preserved beyond 100,000 years (Koch, 2007) hence paleodietary inferences are limited to "recent" fossil faunas.

O isotopes

Another important stable isotope system is oxygen with the measurement of its isotope abundances in bone apatite ($\delta^{18}O_p$) allowing inferring the source of drinking water (Longinelli, 1984). Such results can be used to calculate mean air temperatures and reconstruct past climatic conditions as was done in Cretaceous continental ecosystems (Amiot *et al.* 2011). In addition to palaeoenvironmental inferences, this system opened notable perspectives, for example in reconstructing the thermophysiology of long extinct faunas such as dinosaurs (Barrick and Showers, 1994; Amiot *et al*. 2006) or marine reptiles from the Mesozoic (Bernard *et al*. 2010). Measuring oxygen isotopes also has demonstrated interest with incremental tissues recording long paleoclimatic records such as in fossil elephant tusks or theropod tooth enamel (Koch *et al*. 1989; Goedert *et al.* 2016a).

S isotopes

Sulfur enters the composition of collagen and sulfur isotopes have been measured in bone from a variety of archaeological contexts allowing discriminating between marine and terrestrial sources (Richards *et al.* 2001). Because sulfur may substitute to phosphate as sulfate in bioapatite, its investigation in fossil bone and

teeth offers perspectives of research in the fossil record (Koch, 2007; Goedert *et al*. 2016b).

NON-TRADITIONAL ISOTOPES IN MODERN FAUNAS

Bioessential elements are involved in various metabolic processes and have a recognized vital role in the body. Their concentrations are tightly regulated in blood in order to lie between the deficiency and toxicity thresholds (Balter *et al*. 2013). Despite many caveats (Burton and Price, 2002; Ezzo, 1994), this simple rule has often been forgotten in bone-chemistry studies. As such, the concentration of bioessential elements in fossil bones and teeth can potentially be used for tracking paleo-deficiencies or toxicities, at best (Patterson *et al.* 1987; Rasmussen *et al.* 2008). In addition, the regulation of a given bioessential element is associated with kinetic processes, changes in the molecular configuration of binding molecules, and redox conditions that are generally associated with isotopic fractionation. This has been recognized early for Ca (Skulan *et al.* 1997; Skulan and DePaolo, 1999; Heuser and Eisenhauer, 2010) and isotopic fractionation of Ca remains a promising tool for tracing dietary change from bone and tooth compositions (Reynard *et al.* 2010; Reynard *et al.* 2011; Reynard *et al.* 2013). In this section, however, we will also review the current understanding on fractionation related to biological processes on trace elements (TEs) such as Mg, Fe, Cu and Zn, emphasizing on isotopic ratios rather than solely on concentrations.

Ca isotopes

Calcium isotopes were initially measured in earth materials in the 1970s (Russell *et al*. 1978) but the first studies to provide evidence for isotope variation in biological materials, both modern and fossil, are more recent (Skulan *et al*. 1997; Skulan and DePaolo, 1999). It was first recognized that for a same trophic level, the calcium isotope values for marine organisms were higher than those reported for continental organisms (Skulan *et al*. 1997). This observation is of importance because it becomes necessary to keep comparisons within well-defined assemblages, the initial source of calcium being environmental and possibly originating from food and/or drinking water. In fact, seawater calcium value was originally found to be enriched in heavy isotopes compared to river water and quite uniform because of the long residence time

of calcium in the ocean (Zhu and Macdougall, 1998). The second study led to the recognition that trophic levels could be inferred from calcium isotope variability, both in marine and continental assemblages (Skulan and DePaolo, 1999) and later confirmed in a study focusing on modern and fossil marine mammals (Clementz *et al*. 2003) and more recently on modern and fossil elasmobranchs (Martin *et al*. 2015a). As for continental environments, Chu *et al*. (2006) did not find significant calcium isotope variability in relation to geological and environmental conditions and suggested that calcium isotope variability might be largely controlled by diet. Moreover, Chu *et al*. (2006) reported that milk has a distinctly light isotope composition. In a later report, Reynard *et al*. (2010) did not recognize any trophic level effect in a continental archaeological assemblage but confirmed a physiological effect related to lactation. Heuser *et al*. (2011) also suggested calcium isotope fractionation due to physiological differences between mammals, dinosaurs and reptiles. They also suggested minimal diagenetic overprint on calcium isotope values in Mesozoic continental vertebrates. Melin *et al*. (2014) analysed two modern terrestrial mammalian faunas but did not find significant trophic level effects. However, their study highlighted the necessity to use calcium isotopes in conjunction with other isotope proxys, such as carbon. There is certainly differing physiological effects on calcium isotope fractionation and the recent analyses of different mammal organs challenge previous assumptions on the processes behind calcium isotope fractionation (Tacail *et al*. 2014). It is to be noted that some organisms such as fish and elasmobranchs do fit in the trophic level effect hypothesis but that marine mammals only partly fit in that picture, with peculiar physiological processes possibly at play (Martin *et al*. 2015a).

Those preliminary studies have shown encouraging perspectives for reconstructing trophic structures from calcium isotopes preserved in osteological remains of modern vertebrates (see references above). Certainly, more work is needed to understand the physiological aspects behind calcium isotope fractionation, but used in conjunction with other proxies, $\delta^{44}Ca$ can reveal new palaeobiologcial information. Here, fractionation processes between the diet and the mineralized tissues of a vertebrate takes place at each step of the trophic chain. There is a progressive decrease of calcium isotope ratios from primary producers to higher trophic levels, whether in continental or marine ecosystems.

Mg isotopes

High precision measurements of magnesium isotopes are relatively recent thanks to the advent of MC-ICP-MS technology (Galy *et al.* 2001). As with calcium, magnesium is a bioessential element involved in both plant (Black *et al*. 2006) and animal metabolism (Nadler and Rude, 1995). Recent analyses in bone and teeth of two African mammalian faunas showed an increase of about 0.25‰ of the $^{26}Mg/^{24}Mg$ ratio at each step of a trophic chain (Martin *et al*. 2014). Further analysis in enamel from a mammalian fauna from Gabon has shown a similar ordering according to trophic level although overlap between dietary categories is present. The possible mechanism explaining such isotopic variability is because feces leave the body ^{26}Mg-depleted relative to diet, a depletion that is balanced by a ^{26}Mg-enriched muscle reservoir (Martin *et al*. 2015b). Used in conjunction with Ba/Ca elemental ratios preserved in enamel, $\delta^{26}Mg$ highlighted a distinction between herbivores and omnivores (Martin *et al*. 2015b). Also, a similar correlation was highlighted between $\delta^{26}Mg$ and $\delta^{66}Zn$ on the same samples (Jaouen and Pons, 2016). Contrary to calcium, there is a potential effect of the substrate because of the isotopic heterogeneity of geological reservoirs (Martin *et al*. 2014). Hence, there is a necessity to analyze faunas from a same provenance. This isotope approach is only recently explored and necessitates the establishment of a large database in modern ecosystems. Conversely in the fossil record, no data have been published yet but when possible other geochemical proxys such as radiogenic strontium, if not modified during diagenesis, should be checked to monitor substrate homogeneity. Furthermore, physiological processes responsible for magnesium isotope fractionation have not been explored yet and spatial variability at the scale of tooth, dentition and body reservoirs remain to be looked at in details. There are also open perspectives for studying magnesium isotope variability in marine ecosystems with Mg being highly concentrated in seawater, it is suspected that, as for marine Ca, homogeneity in the primary source of Mg might provide a clear reading of trophic level effects. Research perspectives are therefore high both for physiology, ecology and palaeobiology.

Cu, Fe and Zn isotopes

Transition metals including copper (Cu), iron (Fe) and zinc (Zn) show isotope variability in vertebrate soft tissues that encompasses isotope variability observed in earth materials (Balter *et al*. 2010; Walczyk and von Blanckenburg, 2005; Jaouen *et al*. 2013). Those elements play essential metabolic roles and have the potential to trace physiological processes. Of relevance to palaeontological questions is the record of such fractionation processes in bioapatite, which could be preserved in fossils. Iron and copper isotope variability in male versus female blood is different (Walczyk and von Blanckenburg, 2005; Jaouen *et al*. 2012) and the fractionation process behind it could be menstruations (Jaouen and Balter, 2014). Jaouen *et al*. (2012) found that such isotopic difference is also documented in archaeological bones. Although an inventory of such fractionation processes in modern mammal blood and bone has yet to be conducted, interesting perspectives for sex determination of our ancient relatives can be thought of. Potentially, inference on sex ratios in community structure or sexual dimorphism in the fossil record could be pinpointed.

Zinc isotopes seem to be of relevance for ecological studies, particularly at inferring trophic levels as recently demonstrated in continental (Jaouen *et al*. 2013; 2016a) and coastal (Jaouen *et al*. 2016b) mammal faunas, although some overlap between dietary categories also exists. As for magnesium isotopes, it is suspected that the substrate also influences the heterogeneity of the results (Jaouen *et al*. 2016a). Nevertheless, in the study involving marine mammals (Jaouen *et al*. 2016b), the use of $\delta^{13}C$ and $\delta^{15}N$ highlight a correlation between nitrogen and zinc isotopes. Also, a similar patterning, *i.e.* an increase of the $\delta^{66}Zn$ ratio up trophic chain, is recorded in both bone and enamel (Jaouen *et al*. 2016a), which offers interesting applications to the fossil record.

Sr isotopes

Strontium incorporated in animal tissue is derived from food and drinking water. Radiogenic ^{87}Sr is a decay product of ^{87}Rb, which is highly variable from one geological substrate to another. Hence, the $^{87}Sr/^{86}Sr$ ratio measured in animal tissues mirrors that of the local geological substrate and many studies, especially in archaeology, use this ratio to explore mobility of past human populations; for a comprehensive review the reader is directed to Bentley (2006). Radiogenic strontium can also be used in palaeontology to discuss the mobility and habitat of Mesozoic

vertebrates (Kocsis *et al.* 2009; Martin *et al*. 2016) or to reconstruct palaeoenvironments of even older faunas such as elasmobranchs of the Permian and Triassic (Fischer *et al.* 2012; 2013).

From a palaeodietary perspective, mass-dependent stable strontium isotope composition has recently received attention with $\delta^{88/86}Sr$ (involving the $^{88}Sr/^{86}Sr$ ratio) showing a decrease from herbivores to carnivores, thus bearing important information for diet reconstruction in archaeology (Knudson *et al*. 2010) and potentially also in palaeontology. Variations of the $^{88}Sr/^{86}Sr$ ratio can be measured both with thermal ionization (Krabbenhöft *et al*. 2009) and multicollector ICP-MS (Knudson *et al*. 2010).

FROM THE SAMPLE TO THE ISOTOPE RATIO

In comparison to light stable isotopes, non-traditional ones are heavier and show smaller isotope fractionation in the range of a few per mils. For these reasons, assessing the variability of alkaline earth metals such as Ca (expressed as $\delta^{44}Ca$), Mg ($\delta^{26}Mg$) and transitions metals such as Cu ($\delta^{65}Cu$), Fe ($\delta^{56}Fe$), Zn ($\delta^{66}Zn$) is relatively new in the domain of earth sciences, thanks to significant instrument improvements (e.g. Albarède and Beard, 2004). The different steps from sampling to analyzing such non-traditional isotopes in biogenic apatite are summarized in Figure 1.

The quantity needed for analysis is dependent 1) on the concentration of the element of interest into the sample material (i.e. bone or tooth) and 2) on the limits of detection of the mass spectrometer. Both Ca and Mg are considered major constituents of bioapatite making up to nearly 40 wt% and several thousands of ppm respectively. Fe and Zn make between 20 and 100 ppm while Cu is the least concentrated of all, present at about 10 ppm levels. Isotope ratios are measured on two types of mass spectrometers, either TIMS for Ca and Fe or ICP-MS for Ca, Mg, Cu, Fe, Zn. One measurement on MC-ICP-MS requires about 2 µg of Ca and less than 0.4 µg of Mg, Cu, Fe and Zn. Therefore, considering both elemental concentrations in the sample and the amenable measure, the needed weight for a sample remains small for Ca and Mg (about 1 milligrams will provide 100 measures of Ca and between 10 and 50 measures of Mg) but is relatively larger for Cu, Fe and Zn (40 milligrams of bioapatite is needed for 1 measure of Cu and 10 measures of Fe and Zn) (Table 1 shows two examples, the first is barely destructive to the sample and corresponds to

the minimal weight allowing to comfortably measure Ca and Mg isotope ratios. In the second case, the weight needed is more destructive and corresponds to a typical uptake necessary to conduct $\delta^{15}N$ analysis of bone collagen. This last choice now allows measuring isotope ratios of Cu, Fe and Zn). The advantage of calcium is its high concentration in bioapatite, which allows minimal quantities of sample to be taken. Using a computer-assisted microdrill, it is possible to obtain the desired weight by leaving holes about 350 µm in diameter. This is particularly interesting when asking permission to sample curated specimens. Moreover, this leaves plenty of space for spatial sampling along accreting tissues, for example across incremental annuli in teeth or otoliths recording mineralization during the life of an animal. At this stage, it is possible to either directly place the samples in Teflon beakers and dissolve them in nitric or hydrochloric acid, or to add a step to remove potential diagenetic carbonates and other associated allochtonous elements through leaching.

Following sampling and dissolution, bone or teeth are subjected to chemical purification in the clean laboratory using acids and ion exchange resins in order to remove the phosphate matrix and any other elements that could potentially lead to destabilizations or interferences in the isotopic measurements. Strontium is present in variable concentration in vertebrate apatite (Peek and Clementz, 2011) and needs to be removed. Because both doubly charged strontium and single charged calcium have so close mass to charge ratios (m/z) for main measured isotopes ($^{84}Sr^{++}$, $^{86}Sr^{++}$ and $^{88}Sr^{++}$ interfering with $^{42}Ca^{+}$, $^{43}Ca^{+}$ and $^{44}Ca^{+}$ respectively), the Sr has to be removed from sample to avoid interferences with calcium during analysis. Here, the goal is to retrieve the element of interest in a separate vial and depending on the protocol, all other elements composing bioapatite may be discarded or kept in separate vials for further chemical purification. At this stage, it is very important to collect close to 100 % of the element (referred as yields); else incomplete collection will cause the sample to become fractionated from its original isotope composition, thus leading to skewed results (Russell and Papanastassiou 1978). Complete collection of a given element is first implemented using elution profiles of standard samples. Each element composing the sample travels with different kinematics through the resin depending on the molarity of the acid used. When the right recipe is established, the element is collected at the end of the column in a Teflon beaker. Details about elution profiles and resin chromatography of vertebrate mineralized tissues are described in the literature for calcium (Tacail *et al*. 2014; Martin *et al*. 2015a), for magnesium (Martin

et al. 2014; 2015b) and for copper, iron and zinc (Balter *et al*. 2010). Yields can be assessed by measuring the presence/absence of signals in split solution collected before and after the expected elution of the targeted element. At the end of the purification step, collected volumes containing the purified element are dried down on a hot plate. Once completely dried, the sample can be retaken in a fixed volume corresponding to the expected concentration for subsequent analysis on the mass spectrometer. According to the instrument of choice, the sample is either kept liquid (MC-ICP-MS) or completely re-dried (TIMS).

Originally, non-traditional isotopic systems were measured on Thermal Ionization Mass Spectrometers (TIMS). Isotopic measurements of Ca, Mg, Cu, Fe and Zn can now be achieved on Multi-Collector Inductively Coupled Plasma Mass Spectrometers (MC-ICPMS), the analytical details and guidelines of which are explained in Albarède *et al*. (2004). Both types of spectrometers differ in the introduction method of the sample, but their geometry remains comparable. In TIMS, a drop of the liquid sample is dried on a filament composed of a neutral element such as rhenium or tungsten, which undergoes heating into vacuum. The solid sample eventually becomes ionized and gets accelerated into the mass spectrometer. As for MC-ICP-MS, the sample is diluted in a low concentrated acid (often 0.05 N HNO_3), gets nebulized into a carrier gas, in this case Ar, and is eventually ionized into an argon plasma. At this stage, the sample enters a vacuum space and becomes accelerated into the mass spectrometer. Because plasma-ionized particles have high energy spread, it is necessary to focus the ion beam with an electrostatic analyzer (ESA), which sets the kinetic energy of the ion beam within a narrower energy spread. The beam then proceeds through a magnetic sector, which separates ions according to their mass to charge ratios (m/z). The different isotopes then hit the detector, which is constituted of several faraday cups (hence the name multi-collector) that turn the different ion beams into measurable voltages. Based on the different voltages, isotope ratios are then calculated. This combination of an ESA and a magnetic sector is known as a double focusing geometry, allowing for a better transmission and mass resolution. The TIMS instrument does not need a double focusing geometry because the ion beam provided by thermal ionization has a limited energy spread thanks to the ionization setup method. Therefore, ions are only sorted according to their masses using a magnetic sector.

Laser ablation systems consist in flash-firing a high-energy laser beam onto the sample surface, resulting in a nearly bulk vaporization of solid sample. These setups can now be connected to the MC-ICP-MS. The advantage of this method is that no chemistry is undertaken, allowing for a direct spatial analysis of samples. It is important to remember that flat surfaces are necessary for such analyses and therefore this requires cutting and/or polishing samples, which is obviously destructive and unfeasible for precious specimens. The major drawback remains analytical because ablating a sample implies ionizing a wide variety of elements in the plasma, therefore creating a wealth of interferences that need to be corrected. Calcium isotopes can be measured in this way provided a strict matrix-matching method together with a thorough correction of Sr interferences (Li et al. 2015; Tacail *et al*. 2016) and analytical implementation for laser ablation of Mg, Cu, Fe and Zn isotope ratios in bioapatite is pending.

Isotope fractionation effects in alkaline earths and transition metals are small; in other words, isotope abundance ratios, which can be measured with high precision, show relatively small variation in nature. As raw isotope ratios are difficult to deal with and are pointless as such, we report normalised deviations from a reference material. Moreover, because variations of isotope compositions are small, we multiply it by a magnifying factor, typically 1000. This is all expressed in a single equation, which applies to any traditional or non-traditional isotopic system:

$$\delta^{i/j}X = \left[\frac{\left({}^{i}X \Big/ {}^{j}X \right)_{sample}}{\left({}^{i}X \Big/ {}^{j}X \right)_{reference\ material}} - 1 \right] \times 1000$$

where ${}^{i}X$ and ${}^{j}X$ stand for molar abundances of isotopes i and j of element X respectively and the δ value is expressed in ‰ units. For magnesium, the delta notation is given by $\delta^{x}Mg=[({}^{x}Mg/{}^{24}Mg)_{sample}/({}^{x}Mg/{}^{24}Mg)_{reference} - 1].10^{3}$ where x=26 or 25, and the DSM3 solution for the standard. For copper, the delta notation is given by $\delta^{65}Cu=[({}^{65}Cu/{}^{63}Cu)_{sample}/({}^{65}Cu/{}^{63}Cu)_{reference} - 1].10^{3}$ with the NIST-SRM976 solution for the standard. For iron, the delta notation is given by $\delta^{x}Fe=[({}^{x}Fe/{}^{54}Fe)_{sample}/({}^{x}Fe/{}^{54}Fe)_{reference} - 1].10^{3}$ where x=56 or 57, and the IRMM14 solution for the standard. For zinc, the delta notation is given by $\delta^{x}Zn=[({}^{x}Zn/{}^{64}Zn)_{sample}/({}^{x}Zn/{}^{64}Zn)_{reference} - 1].10^{3}$ where x=66, 67 or 68, and the JMC3-

0749 Lyon for the standard. Different notations are used for Ca where the full isotopic ratio is commonly given in the exponent to express whether ^{40}Ca has been measured or not.

DIAGENESIS OF NON-TRADITIONAL ISOTOPES

Recognizing isotope systems as tracers of biological processes in modern skeletal tissues provides interesting perspectives for their applications in the fossil record. However, delving into the past implies the risk that any original biogenic signal could be overprinted or replaced during diagenesis and the least to be done is to control for its effects. Post mortem modifications of the original chemical composition can be challenging to assess when interpreting isotopic compositions of fossil samples. The intensity of diagenesis depends on the concentration ratio between the diagenetic fluid and the fossil, i.e. the water/rock (W/R) ratio, and the difference of the isotopic composition between the diagenetic fluid and the fossil. Departures from original isotopic composition have been modeled using simple mass balance for Mg, Ca, Fe, Cu and Zn isotopes, for the continental and marine settings (Fig. 2). The developed model does not involve classical W/R equations, as given in Sharp (2007) for example, because it does not take into account the initial and final stages of bioapatite alteration. It neither takes into account isotopic fractionation between fluid and bioapatite, which have been otherwise not determined. The equation reads:

$$\delta M_{mix} = \frac{xM_{bio} * \delta M_{bio} * [M]_{bio} + (1-x)M_{dia} * \delta M_{dia} * [M]_{dia}}{xM_{bio} * [M]_{bio} + (1-x)M_{dia} * [M]_{dia}}$$

where M stands for the metal, δM and $[M]$ its isotope composition and concentration, respectively, the subscripts *bio* and *dia* for biological and diagenetic fraction, respectively, and x the fraction of altered bioapatite. Departure from original isotopic composition is given by the ratio $\delta M_{mix}/\delta M_{bio}$ for a given value of x. Four consequences emerge from these calculations. The first is that mixing of diagenetic Mg and Ca in the terrestrial setting is unlikely because the Mg and Ca concentrations in rivers are negligible relative to those in bioapatite. Second, the mixing of diagenetic Mg in the marine setting is probably very important because the Mg concentration in seawater is similar to that of bioapatite. Third, the diagenesis of transition metals (Fe,

Cu and Zn) in the terrestrial environment is likely to be an efficient process because their concentrations are lower in bioapatite than in rivers. And fourth, the diagenesis of transition metals in the marine environment is probably unlikely because their concentrations in seawater are under the ppb level, while they are 1000 times higher in bioapatite.

Despite the fact that the Ca isotopic composition of bone and tooth is hardly modifiable by diagenesis, there is a risk that the measured Ca and Mg isotope composition is overprinted due to the presence of secondary calcite in the porosity of the samples. As a rule, the fossil bone and teeth have to be rid of secondary carbonates, and more generally of any secondary mineral phase. This step (leaching) is achieved using diluted acetic acid (0.1 M), that will dissolve secondary carbonates, leaving bioapatite, which will be finally dissolved using more concentrated nitric acid (~30%). It exists many leaching procedures and the reader will find some useful references describing the different protocols in Balter *et al*. (2002a). Transition metals, such as Fe, Cu and Zn can be associated with oxyhydroxids that need to get dissolved using reducing agents such as hydroxylamine.

Once calcite and oxyhydroxides have been leached from the fossil bone or enamel samples, it is necessary to proceed to post-hoc tests to check whether the concentrations and the isotope compositions are related or not and whether the measured elements are derived from a diagenetic or a biogenic pool. Tests of diagenesis on trace elements and their isotopes have recently been reviewed in Reynard and Balter (2014). Tests of diagenesis will have to be constantly implemented. As such, if a sizable pool of a biological metal M is exchanged with a diagenetic component, this would affect the M isotopic composition and concentration and the samples would fall on a $1/M$ versus δM line representing the mixing between the biological and the diagenetic M components. This simple scheme holds if the biological and diagenetic pools are different enough in terms of isotopic composition and concentration to generate a single $1/M$ versus δM mixing line. However, if we emphasize that the diagenetic pool would be quite homogeneous in terms of isotopic composition and concentration, the biological pool can be easily characterized by an important variability. This will generate as many mixing lines as it exists of original isotopic compositions, leading to an overall blurred $1/M$ versus δM relationship.

Anyway, such controls are necessary to ensure we provide isotopic values related to biological fractionation and not diagenesis.

The study of diagenetic influence on the isotope composition of calcium in fossil bone and teeth has not received a lot of attention. This is also the case for magnesium and zinc, which have only been recently recognised to bear some trophic level information in modern samples. Heuser *et al*. (2011) have conducted some tests and discussed the effects of diagenesis on calcium isotopes in phosphatic tissues. They measured similar Ca isotope values for fossil bone, dentine and their surrounding sediment (n = 5) either implying exchange between sediment and skeletal tissue or addition of calcium to the tissue in the form of newly formed minerals. Heuser *et al*. (2011) also emitted the hypothesis that Ca isotope composition could reflect that of the local substrate during the lifetime of the animal.

Other methods exist to control for the diagenetic alteration of bone. For example, it will be necessary to monitor the concentration of phosphorous and calcium. Under stoichiometric condition the ratio Ca/P is close to 2.2 in modern biological apatite (bone or teeth) (Sillen, 1986). Nevertheless, that one element is affected by diagenesis does not necessarily means that others will be.

LATE PLEISTOCENE CASE STUDY WITH Ca ISOTOPES

In order to test for the preservation of biogenic information from calcium isotopes, we measured two faunal assemblages of late Pleistocene age. The importance of analysing a relatively "recent" fossil fauna is that dietary behaviour can be approximated with a certain level of confidence and our new calcium results can be compared with other geochemical proxies of the literature to test for consistencies in palaeobiological interpretations. In this particular case, bone preserves collagen so calcium isotopes can be compared with nitrogen isotopes derived from the same samples. Moreover, enamel lacks collagen but trace elements such as strontium and barium are less prone to alteration and their elemental concentrations can be compared to calcium isotopes derived from the same samples.

Two Late Pleistocene mammal assemblages recovered from cave deposits were compared for their isotope variability in $\delta^{44/42}$Ca. The specimens from Scladina cave, Sclayn, Belgium originate from layer 1A, dated >36.2 kyr and 38.7 ± 1.5 kyr BP (Gilot, 1992). The specimens from Jaurens, Corrèze, France originate from a single

cave dated from 29.7 to 32.6 kyr BP (Guerin *et al*. 1979). The fauna from Sclayn is the same as analysed in previous studies for C, N (Bocherens *et al*. 1997) and for Sr/Ca and Ba/Ca elemental ratios (Balter *et al*. 2001, 2002b). The fauna from Jaurens was selected for this study in the collections of Université Lyon 1.

Material and analytical methods

The Sclayn assemblage contains 17 specimens all consisting of bone powder representing 7 species: 3 *Bison priscus*, 2 *Crocuta crocuta*, 2 *Equus caballus*, 1 *Mammuthus primigenius*, 2 *Coelodonta antiquitatis*, 1 *Ursus arctos* and 6 *Ursus spelaeus*. The Jaurens assemblage contains 27 enamel specimens and 1 bone specimen (Fig. 3, Figs. S3, S4). The enamel-bone pair belongs to a single species, *Panthera pardus*, but whether it belongs to a single individual cannot be assessed. Species sampled for tooth enamel include 3 *Bison priscus*, 3 *Coelodonta antiquitatis*, 3 *Rangifer tarandus*, 3 *Canis lupus*, 5 *Crocuta crocuta*, 4 *Panthera spelaea* and 4 *Ursus arctos*. About 300 µg of tooth enamel was sampled with a computer-assisted microdrill (New wave Research) under a binocular (Olympus SZ61).

Calcium from fossil samples was purified using a two-step chemical process involving Sr-specific resin followed by a cation-exchange resin (Tacail *et al*. 2014). The purified calcium fraction of each sample was measured for isotope ratios on a Neptune Plus MC-ICP-MS at Laboratoire de Géologie de Lyon (e.g. Martin *et al*. 2015 for details). Delta values were obtained using the standard-sample bracketing method with ICP Ca Lyon as standard (Tacail *et al*. 2014). As MC-ICP-MS technique does not allow the measurement of ^{40}Ca due to interference from ^{40}Ar plasma, isotopic compositions are expressed as delta values calculated using the ^{44}Ca/^{42}Ca isotopic ratio as follows:

$$\delta^{44/42}Ca = \left[\frac{\left({}^{44}Ca/{}_{42}Ca\right)_{sample}}{\left({}^{44}Ca/{}_{42}Ca\right)_{ICP\ Ca\ Lyon}} - 1 \right] \times 1000$$

We report four independent measurements of NIST standard SRM1486 (average = –1.01 ‰, see Table S2 for details) that are in good agreement with previously

published values (–1.04 ± 0.11 ‰, 2SD in Martin *et al*. 2015; –1.03 ± 0.13 ‰, 2SD in Tacail *et al*. 2016). Long-term reproducibility as measured for SRM1486 is 0.12 ‰. When plotted against $\delta^{44/42}$Ca, $\delta^{43/42}$Ca values fall on a line with null y-axis origin and a slope of 0.487 ± 0.022 (2SE) (Fig. S1). Variations of isotope compositions depend thus on mass of isotopes, respecting the mass-dependent fractionation of isotopes that predicts a slope of about 0.5.

Leaching experiments were conducted on two samples from Jaurens to assess their isotope values (Table S2). For each sample, bone or tooth powder was selected, then divided into two batches. The first batch was treated as such with no pretreatment. The second batch was pretreated using 0.1N ultrapure acetic acid to leach carbonates or any diagenetic pollutants from the sample for 30 minutes. This sample was rinsed three times using MQ water and the remaining sample was ran through the same purification technique and analytical procedure as the untreated sample.

Results

In the Sclayn assemblage, calcium isotope values (Table S2, Fig. 4) range from –0.86 to –1.39‰, which is significantly higher than the observed range of values in the Jaurens assemblage from –1.14 to –2.02‰ (Wilcoxon-Mann-Whitney, p = 0.00000077). The lowest values of the Sclayn dataset are represented by *Crocuta crocuta* and *Mammuthus primigenius*. In the Jaurens dataset, the lowest values are represented by the *Crocuta* and the *P. pardus* enamel sample. In both datasets, higher values are represented by *Coelodonta antiquitatis*, *Equus caballus*, *Bison priscus* and *Ursus arctos* and *Ursus spelaeus*. Intermediate values are represented by *Rangifer tarandus*, *Canis lupus* and *Panthera spelaea* in the Jaurens sample. The value for the calcite encrusting a tooth of *Crocuta crocuta* (FSL300610) is the highest of the dataset (–0.28 ‰ ± 0.05, 2SD, n = 3).

The results of the leaching experiment show a slight difference between calcium isotope values obtained for the bone sample (FSL 301081) with the untreated bone sample showing a slightly higher value (–1.38 ‰ ± 0.11, 2SD) than the leached sampled (–1.52 ‰ ± 0.03, 2SD). The leached enamel sample (FSL 300918lea)

compares well with the untreated enamel sample (FSL 300918) with values of –1.48 ‰ ± 0.04, 2SD versus –1.45 ‰ ± 0.06, 2D, respectively.

Concentration analyses for Ca, P, Mg, Sr, Ba and Zn are recorded alongside Ca isotope values in Table S2. Ca/P ratios for the Jaurens samples average 1.98 ± 0.13 2SD.

Are Ca isotope values of biogenic nature?

The range of Ca/P ratios measured in tooth enamel samples from Jaurens and the bone samples from Sclayn respect the stoichiometry of bioapatite (2.02 ± 0.09 2SD) and the values are within the range of expected values for modern bone (Sillen, 1986) as well as for the bone standard SRM1486 measured in the same analytical session (1.95). Such results indicate that if any changes occurred during diagenesis, they affected equally calcium and phosphorous. Here, the average calcium and phosphorous concentrations in tooth enamel are 33.3 % ± 4.4 and 16.3 % ± 4.0 (1SD), respectively. The average calcium and phosphorous concentrations in bones from Sclayn are much lower being 13.4 ± 3.5 % and 6.4 ± 1.7 % (1SD) respectively, indicating important diagenetic-related leaching of these elements. As a comparison, in the modern bone standard SRM1486, the values are of 44.9% and 23.0%. The leaching experiment conducted here (Table S2) on one enamel sample shows that both calcium and phosphorous have been leached away preserving stoichiometry ([Ca] = 20.4 %, [P] = 10.8% for the leached sample (FSL 300918lea), [Ca] = 31.8 %, [P] = 15.9% for the untreated sample (FSL 300918)). In the case of the bone leached here (FSL 300918lea), almost no Ca and P were lost, possibly due to the fact that collagen or other material in the porous structure makes it less soluble. Although the evidence is indirect, these results show that leaching may have been responsible for the observed lower concentrations of calcium and phosphorous into the fossil bones than in fossil tooth enamel samples.

Although stoichiometry is preserved, does leaching have an impact on measured calcium isotope composition? The measured $\delta^{44/42}$Ca difference may not be significant given the larger error obtained on the untreated sample that falls within the range of the leached sample. Nevertheless, it could also be compatible with a small diagenetic overprint in bone from secondary calcite. Indeed, the calcite sample has a ^{44}Ca-enriched calcium isotope composition and diagenetic contamination of

secondary calcite in the pores of fossil bone would tend to lead to heavier isotope compositions. The second sample, which consists of tooth enamel shows both values obtained on untreated and treated samples as undistinguishable (–1.45 ‰ ± 0.06, 2SD vs –1.48 ‰ ± 0.04, 2SD, respectively). We do not observe significant effects of the leaching process on calcium isotope values, although more analyses should be undertaken to confirm this.

Conversely, uptake of diagenetic calcium cannot be confirmed here. One way to look at such diagenetic inclusion is to search for correlations between elements that are absent during the lifetime of an animal and that eventually enter bioapatite during fossilization. Here, we observe no correlation between $\delta^{44/42}$Ca and manganese concentration, which was obviously added into bioapatite after the death of the animal (Fig. S2). Manganese is virtually absent from modern bone as indicated here with SRM1486 with 4 ppm measurable. Manganese is incorporated during fossilization as is often visible in fossils with the characteristic manganese dendrites. Unsurprisingly here, fossil bone is more concentrated in manganese (>600 ppm) than are tooth enamel samples (average = 70 ppm). Other elemental concentrations such as rare earth elements (REE), Uranium or Thorium diagenetic uptakes could not be measured here because the sampled masses were too low to pass detection limits. It is to be noted that other elements used in ecological studies (Sr, Ba, Zn, Mg) were measured for concentration and are within the range of observed values in modern samples (see Table S2).

Further preservation of an original biogenic calcium isotope value is supported by the calcium isotope values that are significantly different from the value measured on the calcite surrounding fossil specimens. The value of calcite (–0.28‰ ± 0.05) is within the range of expected values for carbonates (e.g. Fantle and Tipper, 2014). Given the prominence of secondary calcite surrounding fossil samples in the Jaurens deposit, diagenetic overprinting of calcium isotope values would require a shift of isotope values from fossil samples toward higher values and beyond the range of expected values for modern samples. The fact that mammal tooth or bone values are within the range of values measured in previous studies for modern bone and teeth (Skulan *et al*. 1999; Clementz *et al*. 2003; Reynard *et al*. 2010; Tacail *et al*. 2014; Martin *et al*. 2015) suggests that the $\delta^{44/42}$Ca values recorded in both fossil assemblages reflect a strong biogenic component.

The spread is nearly double in Jaurens (0.88 ‰) than in Scalyn (0.53‰) and it could be argued that bone samples are diagenetically altered toward higher isotope values reflecting that of the substrate. Nevertheless, the observed scatter in calcium isotope values corresponds to the isotope variability recorded in the bone of modern terrestrial mammals, both in modern and archaeological context and are most likely representing biogenic characteristics (Reynard *et al*. 2010; Tacail *et al*. 2014). Whether this is related to dietary or physiological effects is discussed below.

Palaeocological inferences

The analysis of two datasets of similar ages and faunal compositions but of different tissues, the assemblage from Sclayn consisting of bone and the one from Jaurens consisting of tooth enamel; provides some new insights on calcium isotope variability in some recently extinct mammal species. The two localities share three broad ecological categories including herbivores with *C. antiquitatis* and *B. priscus*, omnivores with *U. arctos* and carnivores with *C. crocuta*. The first observation is a systematic difference with higher mean values for bone than for tooth enamel with a $\Delta_{enamel\text{-}bone}$ of –0.39 ‰ for *C. antiquitatis*, of –0.34 ‰ for *B. priscus*, of 0.07 ‰ for *U. arctos* and of –0.60 ‰ for *C. crocuta*. This offset is of –0.47 ‰ if all values are averaged per assemblage (–1.06 ‰ for bone versus –1.53 ‰ for tooth enamel). This $\Delta_{enamel\text{-}bone}$ value is also very close to the Δ value between *P. pardus* bone and tooth enamel within the single assemblage at Jaurens (–0.54 ‰). Previously published offsets are smaller. Heuser *et al*. (2011) found a smaller offset between dentine and tooth enamel of about – 0.20 ‰ for dinosaur and Tacail *et al*. (2014) reported an offset of – 0.30 ‰ between bone and tooth enamel for sheep. Martin *et al*. (2015) reported a positive offset between bone and tooth enamel of + 0.20 ‰ in marine mammals. The enamel-bone spacing observed in the present study provides support that original biogenic information has been retained. Indeed, a better understanding of the fractionation processes between different mineralized tissues requires systematic pairs within a same individual. Given the different residence times of calcium in bone, enamel or dentine reservoirs, further skeletal variability will have to be explored and will have to consider that the variety of bone tissues may also reflect further isotopic differences. Most important here for the purpose of faunal comparison is to keep in

mind that bone and tooth enamel calcium isotope values cannot be readily compared. Comparing fossil assemblages should focus on single tissue analyses.

The present environmental context is continental and although initial studies recognized a trophic ordering of calcium isotopes values in continental ecosystems (Skulan and DePaolo, 1997; Skulan *et al*. 1999), a more recent study found that this might be less straightforward (Melin *et al*. 2014). That a trophic level effect has been recognized in the marine environment appears settled, although the offset between trophic levels is not large (Skulan *et al*. 1999; Clementz *et al*. 2003; Martin *et al*. 2015). Reasons for this limited trophic level effect have been discussed in the light of seawater as a buffering medium but this study also found that marine mammals do not fully fit in this picture (Martin *et al*. 2015). Clearly, physiological aspects need to be explored to fully understand its impact, together with diet on the observed values. Here, whether for the bone or tooth enamel dataset, calcium isotope values show low dispersion by taxonomic group. To the exception of mammoths and reindeers, all herbivores possess the highest values in the dataset. Carnivores have systematically lower values although this is less significant for *Panthera spelaea* and *Canis lupus*, which show some overlap with the *Bison* group. Ingestion of mineralized tissue such as bone in the diet of *Crocuta crocuta* agrees with the interpretation that a diet composed of mineralized tissues, such as that of *Tyrannosaurus rex*, could explain the low isotope values (Heuser *et al*. 2011). But the present low isotope values recovered for *P. pardus*, which is not known to specifically feed on bone, remains puzzling. This requires further datapoints, as admittedly here, only two samples of *P. pardus* were available for analysis. An obvious outlier is represented by *Rangifer tarandus*, which presents values in the range of the carnivore group. Given that second molars sampled for *R. tarandus* may form early, a nursing effect may be suspected with a milk-based diet potentially explaining the low values (see Chu *et al*. 2006) observed here.

Comparing our calcium isotope data with other geochemical proxies of interest in ecology provides additional insights on the mechanisms behind calcium isotope fractionation. First of all, nitrogen isotopes extracted from bone collagen allow for trophic inferences and the present dataset from Sclayn provides an opportunity to compare $\delta^{15}N$ values from the same samples analysed for calcium isotopes. The two *Crocuta crocuta* samples, representing the only carnivores of the dataset are clearly separated from other mammals with the highest $\delta^{15}N$ and lowest $\delta^{44/42}Ca$ values, which would provide a sound argument for a dietary imprint of

calcium isotopes (Fig. 5A). Interestingly, the single mammoth datapoint also falls within the range of values for *Crocuta crocuta*. Previous studies based on nitrogen isotopes have underlined the discrepancy between mammoth $\delta^{15}N$ values and those of other herbivores highlighting that the mammoth fed on highly ^{15}N-enriched plants (Schwartz-Narbonne *et al*. 2015) or practicing coprophagy (Clementz *et al.* 2009; van Geel *et al.* 2015) making it isotopically looking like a carnivore. The present calcium isotope values could also be linked to specific plant consumption and although more datapoints are needed to confirm this, it is known that $\delta^{44/42}Ca$ is variable in plants, including across different organs (see compilation in Fantle and Tipper, 2014). Comparing the Sr/Ca ratios with $\delta^{44/42}Ca$ values in the Jaurens sample reveals an ordering that follows the biopurification of strontium (Balter, 2004) (see Fig. 5B). Here, *Crocuta crocuta* and *Panthera pardus* are the most biopurified samples, which could be interpreted as taxa occupying the highest trophic position in the ecosystem. This pattern follows their lowest calcium isotope values in the Jaurens dataset and for this reason, it is tempting to interpret calcium isotope data as a proxy for trophic level. Here, in the Sr/Ca versus $\delta^{44/42}Ca$ space, other apex carnivores such as wolves and lions do overlap with bisons and reindeers, which implies that other factors are at play in the isotope fractionation of calcium. However, rhinoceroses are closely located in a putative herbivore space. Further work is needed to decipher the role of different food sources, seasonality or physiological effects such as milking or weaning on the record of calcium isotopes in tooth enamel. In contrast to marine ecosystems, the source of Ca in continental environments is probably extremely diverse for vertebrate organisms (e.g. variability in a single plant from roots to leaves) and could explain the large variability and overlap observed here.

As demonstrated above, used in conjunction with other proxies, calcium isotopes can provide some insights on the diet of extinct taxa. Relevant to our assemblage is the inferred diet of *Ursus arctos* and *Ursus spelaeus*, which remains a matter of debate (e.g. Robu *et al*. 2013) although recent studies have underlined mostly plant-based consumption for *U. spelaeus* (e.g. Krajcarz *et al*. 2016). Although at Sclayn, *U. arctos* is represented by a single individual in our dataset, we do not find a significant difference between its calcium isotope value and those of *U. spelaeus*.

The Jaurens tooth enamel dataset where Sr/Ca and $\delta^{44/42}Ca$ values are plotted show that *U. arctos* falls in mid-range values together with bisons, reindeers and part of the wolves and lions (Fig. 5). If we consider those values to represent trophic

levels, then it could be interpreted that *U. arctos* from Jaurens were omnivorous or herbivorous in agreement with modern observations (Robu *et al*. 2013). As for the Sclayn bone dataset, *U. spelaeus* also falls with other herbivores within the $\delta^{15}N$ versus $\delta^{44/42}Ca$ space. The sole *U. arctos* datapoint is positioned slightly outside the herbivore group in what could correspond to the omnivore group but admittedly, a single datapoint is not enough here to draw conclusions on its dietary habits and also because the carnivores are clearly underrepresented. The absence of a clear calcium isotope difference between *U. arctos* and *U. spelaeus* is preliminary here and it should be stressed out that such comparison involves two assemblages of different locations and slightly different ages as well as comparing enamel versus bone datasets. Also, as discussed above, it cannot be ruled out that diet is not the only factor on calcium isotope fractionation and deciphering dietary versus physiological influence on calcium isotope fractionation requires some extensive research efforts. Several studies based on nitrogen isotopes from bone collagen conclude to a herbivorous status for *U. spelaeus* (Krajcarz *et al*. 2016). Moreover, similar studies from a single locality recover *U. spelaeus* with significantly lower $\delta^{15}N$ values relative to *U. arctos*, implying a distinct herbivorous versus an omnivorous diet for these two taxa (Bocherens *et al*. 2011). Nevertheless, omnivory for *U. spelaeus* has been proposed on the basis of nitrogen and carbon isotopes (Richards *et al*. 2008; Robu *et al*. 2013) and on the basis of microwear (Peigné *et al*. 2009). Dietary heterogeneity at the scale of the population but also in relation to season is to be expected and opens perspectives of research notably with the help of other proxies, including the geochemical proxies presented in this work.

CONCLUSIONS

Traditional isotope approaches to vertebrate palaeobiology offered and are still offering major insights into the ecology and evolution of extinct faunas. Important information on palaeoenvironments, resource use and physiology can be derived from oxygen, carbon, and sufur isotopes. Indeed, the major limitation on paleodietary inference is linked to the preservation of collagen, which is a prerequisite for the measurement of nitrogen isotopes. On the other hand, preservation potential of non-traditional isotopes seems high in biomineralised tissues. Moreover, fractionation appears to be controlled by physiological processes and there is increasing evidence

that some of these non-traditional isotope systems (e.g. Mg, Ca, Zn) may represent useful paleodietary tracers. The palaeoecogical inferences drawn from calcium isotopes are still in their infancy and among the subjects to be addressed, there remains variability linked to physiology and diet. Calcium offers interesting perspectives because it requires only a small quantity of sample (less than half a milligram) and therefore allows for the development of precise time series and the study of seasonal variations at the scale of tissue increments. Moreover, where collagen has completely degraded, calcium (and perhaps magnesium) will furnish promising aspects on future research due to their relatively high concentrations and preservation potentials in mineralized tissues.

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FIGURE CAPTIONS

FIG. 1. Illustration of the various steps from fossil sampling to isotope ratio mass spectrometry. A, sampling a few milligrams of fossil apatite using a drill bit mounted on a microdrilling device or a dremel tool; B, sample digestion with acid in a Teflon beaker; C, the dissolved sample is subjected to ion-exchange chromatography in order to be purified for an element of interest such as Ca, Mg, Cu, Fe or Zn; D, MC-ICP-MS analysis consists in ionizing the purified sample into a plasma; the different elements will be accelerated into a vacuum and will pass through an electrostatic analyzer that focuses ions according to their kinetic energy, then through a magnetic sector that will sort the isotopes according to their ion mass / ion charge ratios (m/z); E, Data acquisition and processing. Each mass will hit a different detector, which will convert isotopic abundance into a measurable voltage, allowing calculation of isotope ratios and delta values.

FIG. 2. Departure from the original isotope composition (%) as a function of the water/rock ratio (W/R, %), which is assumed to be representative of the fraction of altered bioapatite. In this simple mass balance, no assumption is made concerning diffusion processes. Values used for the calculation are given in supplementary table 4.

FIG. 3. *Ursus arctos* molar from Jaurens, France sampled in this study. The top picture is before sampling. The arrow in the bottom picture shows the microdrill perforation after sampling highlighting that a minimal amount of sample is needed for calcium isotope measurements. Scale bar represents 1 cm.

FIG. 4. Fossil mammal $\delta^{44/42}$Ca (in ‰ relative to ICP Ca Lyon standard) variability in two Late Pleistocene assemblages. A, the Sclayn (Belgium) dataset exclusively includes bone samples whereas B, the Jaurens (France) dataset includes mostly enamel samples as well as a single bone sample. To the exception of the felids, all silhouettes are taken from www.phylopic.org (Public Domain licence).

FIG. 5. Fossil mammal $\delta^{44/42}$Ca (in ‰ relative to ICP Ca Lyon standard) variability in two Late Pleistocene assemblages A, plotted against δ^{15}N (‰ atmospheric N_2) for the bone dataset of Sclayn (Belgium) (Bocherens et al., 1997; Balter et al., 2002b) and B, plotted against log (Sr/Ca) for the enamel dataset of Jaurens.

Table 1. Indicative concentrations of Ca, Mg, Cu, Fe and Zn in bioapatite and the quantity of bioapatite needed for one isotopic measurement on MC-ICP-MS.

SUPPLEMENTARY DATA

Fig. S1. Triple isotope plot ($\delta^{44/42}$Ca versus $\delta^{43/42}$Ca in ‰) of all data analysed in this study. Regression line is Y = 0.487 (± 0.022) X – 0.024 (± 0.030) with R^2 = 0.974. This is in agreement with mass dependent fractionation slope of 0.507 according to exponential law.

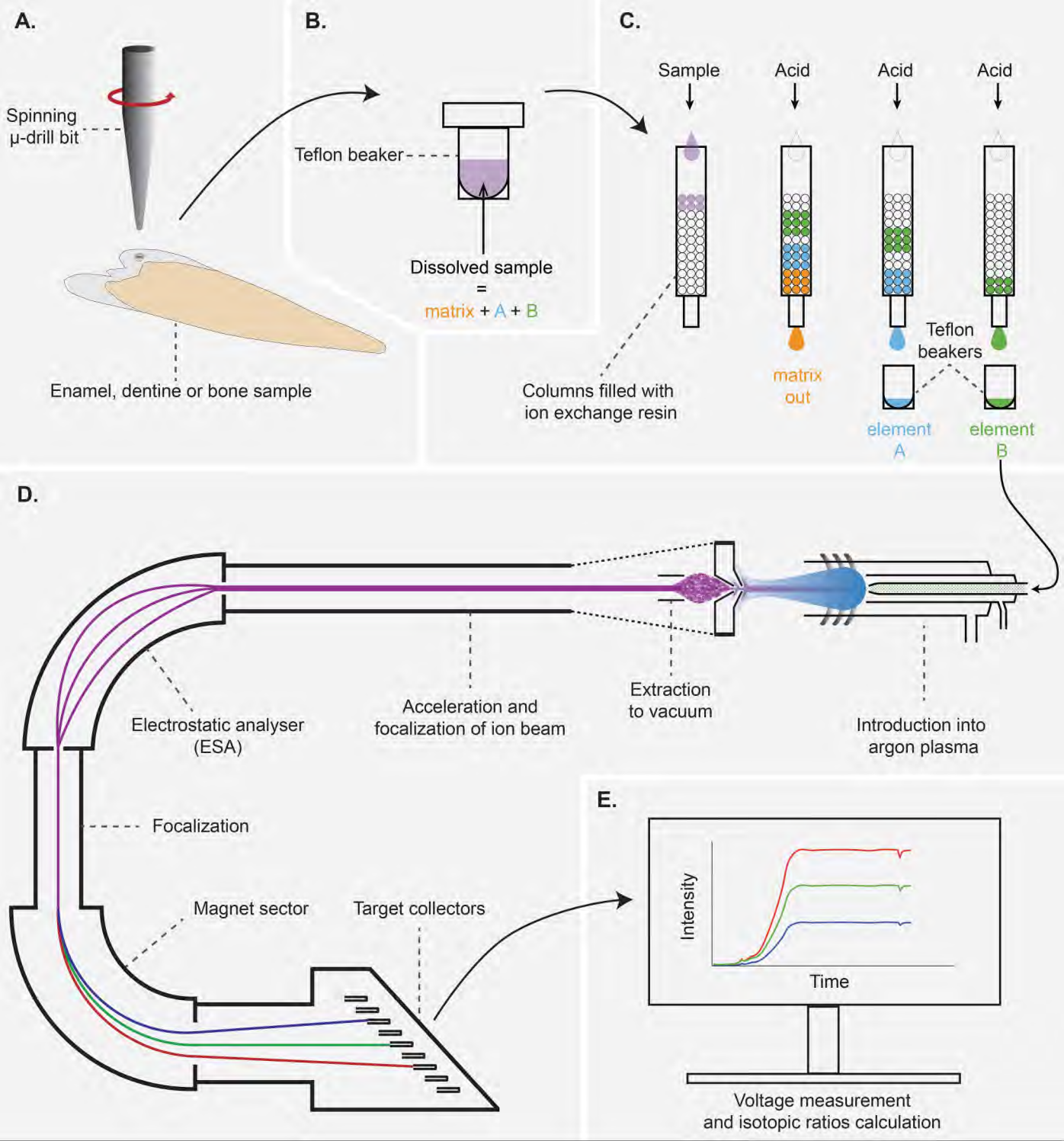
Fig. S2. Manganese plotted against $\delta^{44/42}$Ca values for bone and enamel samples measured in this study.

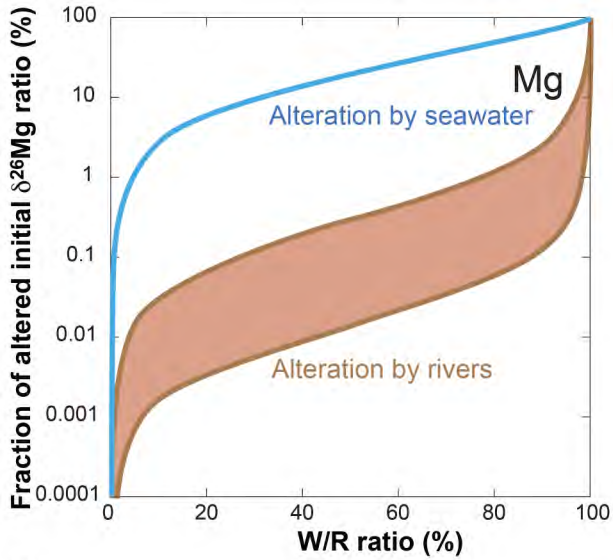
Fig. S3. Plate with all samples from Jaurens analysed in this study. For each sample, a picture before and after sampling is presented.

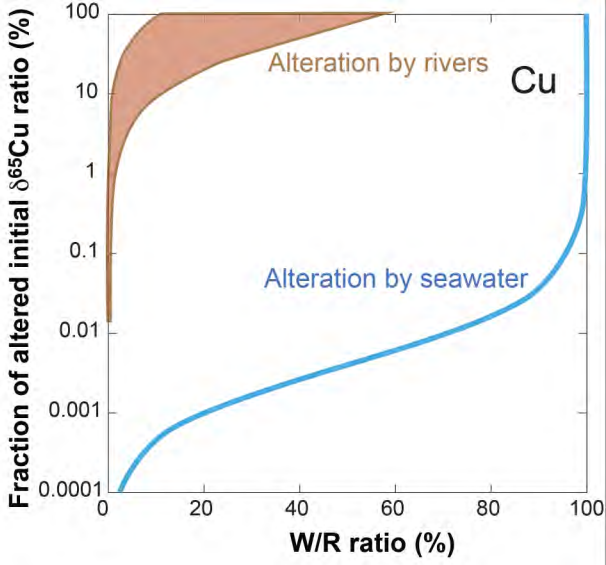
Fig. S4. Same as SuppFig.3 for the rest of the samples from Jaurens.

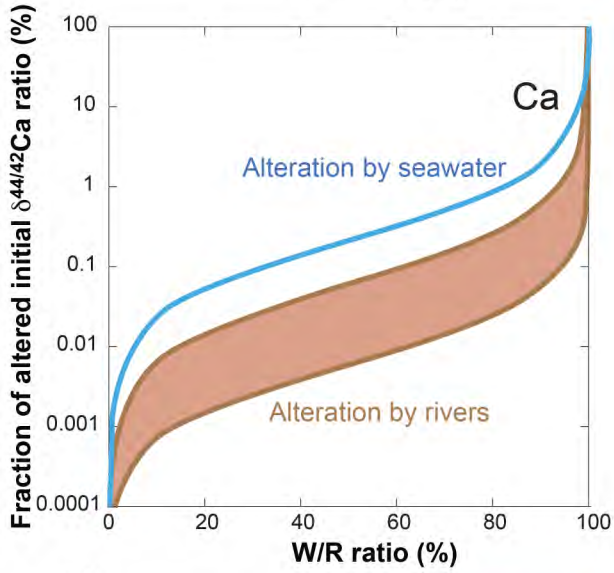
Table S1. Data from the literature used in the model of the water/rock ratio presented in Fig. 3.

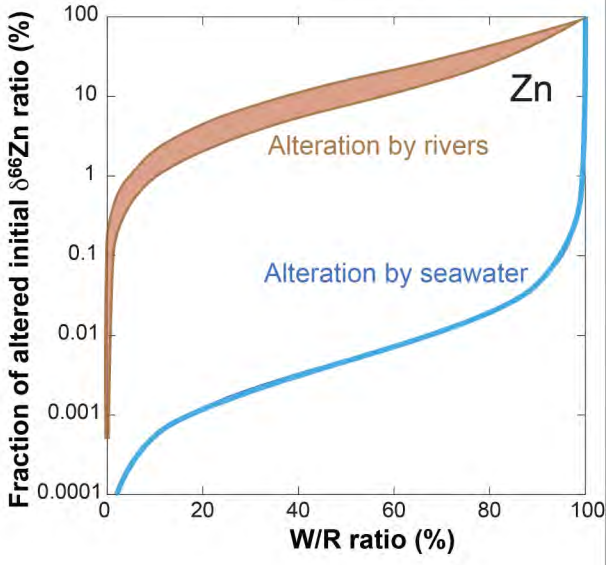
Table S2. Calcium isotope values (expressed as $\delta^{44/42}$Ca and $\delta^{43/42}$Ca (in ‰) relative to standard ICP Ca-Lyon) measured on two Pleistocene assemblages as well as elemental concentrations for some major and trace elements. (1) δ^{13}C and δ^{15}N values from Bochoerens et al. 1997. Tooth type: capital letters refer to the upper tooth row.

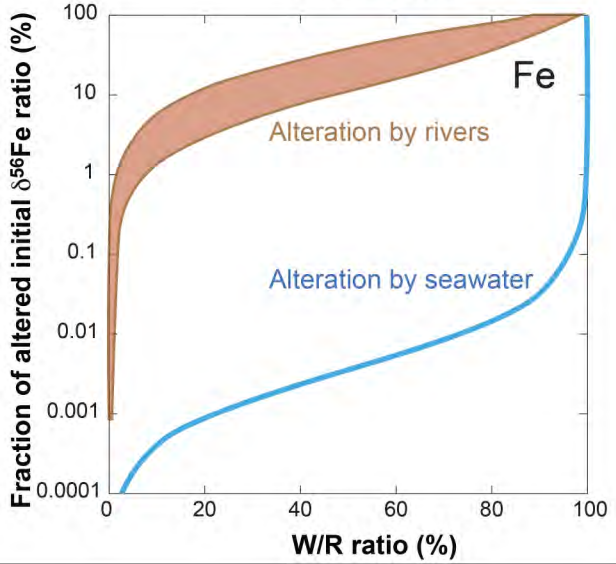

A.
Spinning
µ-drill bit
Enamel, dentine or bone sample
B.
Teflon beaker
Dissolved sample
=
matrix + A + B
C.
Sample
Acid
Acid
Acid
Columns filled with
ion exchange resin
matrix
out
Teflon
beakers
element
A
element
B
D.
Electrostatic analyser
(ESA)
Acceleration and
focalization of ion beam
Extraction
to vacuum
Introduction into
argon plasma
Focalization
Magnet sector
Target collectors
E.
Intensity
Time
Voltage measurement
and isotopic ratios calculation


Mg
Alteration by seawater
Alteration by rivers
Fraction of altered initial δ26Mg ratio (%)
W/R ratio (%)
100
10
1
0.1
0.01
0.001
0.0001
0
20
40
60
80
100


Cu
Alteration by rivers
Alteration by seawater
Fraction of altered initial δ65Cu ratio (%)
W/R ratio (%)
100
10
1
0.1
0.01
0.001
0.0001
0
20
40
60
80
100


Ca
Alteration by seawater
Alteration by rivers
Fraction of altered initial δ44/42Ca ratio (%)
W/R ratio (%)
100
10
1
0.1
0.01
0.001
0.0001
0
20
40
60
80
100


Zn
Alteration by rivers
Alteration by seawater
Fraction of altered initial δ66Zn ratio (%)
W/R ratio (%)
100
10
1
0.1
0.01
0.001
0.0001
0
20
40
60
80
100


Fe
Alteration by rivers
Alteration by seawater
Fraction of altered initial δ56Fe ratio (%)
W/R ratio (%)
100
10
1
0.1
0.01
0.001
0.0001
0
20
40
60
80
100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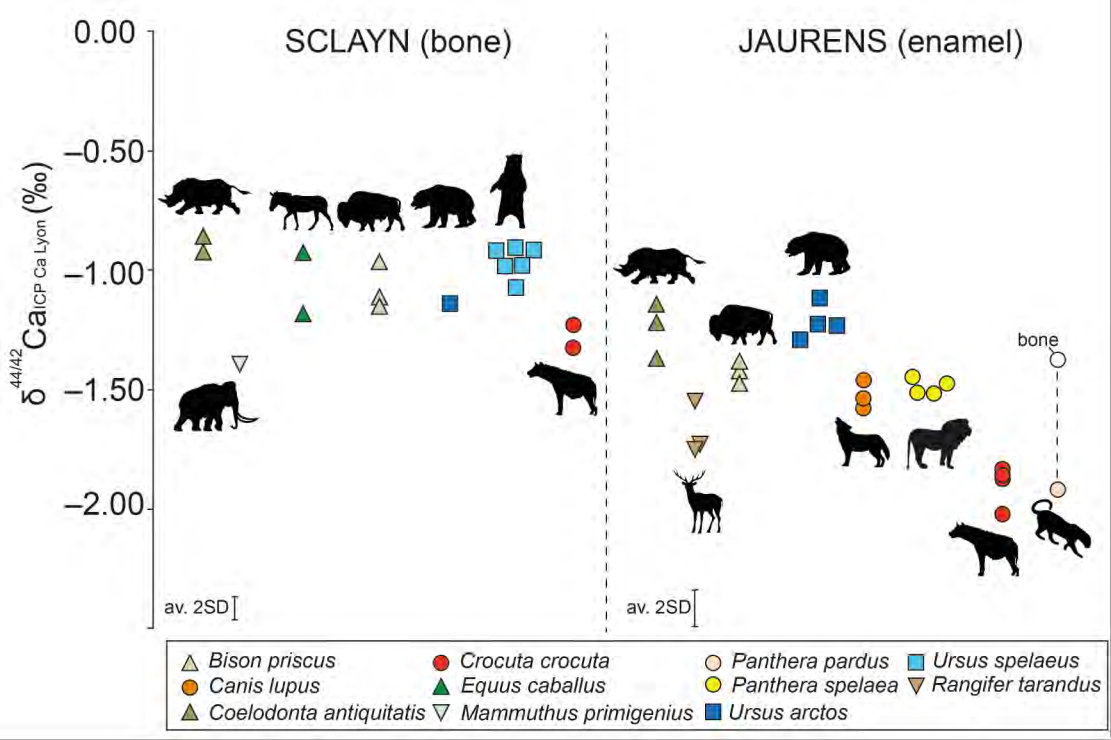
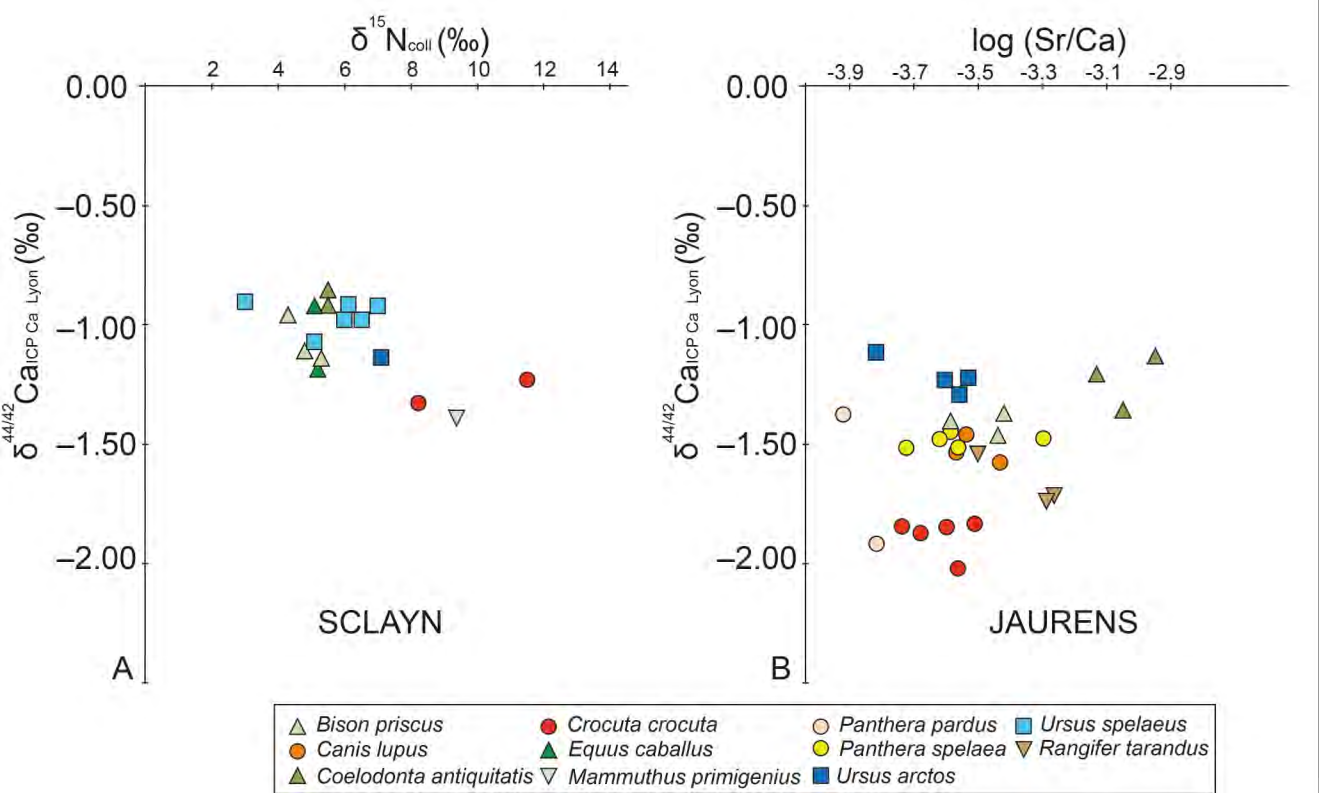

SCLAYN (bone)
JAURENS (enamel)
0.00
−0.50
−1.00
−1.50
−2.00
δ44/42CaICP Ca Lyon (‰)
av. 2SD
av. 2SD
bone
Bison priscus
Canis lupus
Coelodonta antiquitatis
Crocuta crocuta
Equus caballus
Mammuthus primigenius
Panthera pardus
Panthera spelaea
Ursus arctos
Ursus spelaeus
Rangifer tarandus


δ15Ncoll (‰)
2
4
6
8
10
12
14
0.00
−0.50
−1.00
−1.50
−2.00
δ44/42CaICP Ca Lyon (‰)
SCLAYN
A
log (Sr/Ca)
-3.9
-3.7
-3.5
-3.3
-3.1
-2.9
JAURENS
B
Bison priscus
Canis lupus
Coelodonta antiquitatis
Crocuta crocuta
Equus caballus
Mammuthus primigenius
Panthera pardus
Panthera spelaea
Ursus arctos
Ursus spelaeus
Rangifer tarandus

	concentration in bioapatite	amount required for 1 measure on ICPMS	**case 1** mini sampling **1**millig of bioapatite n measures	**case 2** equiv. bone collagen sampling **250**millig of bioapatite n measures
Ca	40%	2µg	100	25000
Mg	2000 to 10000ppm	0.2µg	10 to 50	400 to 12500
Cu	10ppm	0.4µg	not measurable	40
Fe	100ppm	0.4µg	not measurable	400
Zn	100ppm	0.4µg	not measurable	400

	Bone		Rivers		Seawater		
	μg/g	‰	μg/g	‰	μg/g	‰	
Mg	5000	~-1	1 _ 20	-0.3 _ -1.5	1300	-2 _ 0	Tipper et al., 2006
Ca	380000	~-1	15 _ 150	+0.9 _ +2	400	~+2	Tipper et al., 2016
Fe	100	-1 _ 0	10 _ 40	-1 _ +0.3	0.003	-2 _ +0.3	John and Adkins, 2010; Bergquist & Boyle, 2006
Cu	10	-1 _ 0	1 _ 10	+0.1 _ +1.3	0.001	+0.5 _ +1.4	Vance et al, 2008
Zn	100	0 _ +2	10 _ 20	+0.1 _ +0.3	0.005	-0.1 _ +0.2	Bermin et al., 2006

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Table S1. Data from the literature used in the model of the water/rock ratio presented in Fig. 2.

curation number	lab name	locality	taxon	tissue	tooth type	$\delta^{44/42}$Ca (‰)	2SD	$\delta^{43/42}$Ca (‰)	2SD	n	Mg (ppm)	Ca (%)	P (%)	Ca/P	Sr (ppm)	Ba (ppm)	Zn (ppm)	Mn (ppm)	δ^{13}C (‰) (1)	δ^{15}N (‰) (1)	C/N	
Sc83.282	sc3	Sclayn, Belgium	*Bison priscus*	bone	-	-1.15	0.01	-0.60	0.03	3	303	16.7	7.9	2.1	124	22	141	111	-19.9	5.3		3.1
Sc89.94	sc10	Sclayn, Belgium	*Bison priscus*	bone	-	-1.12	0.01	-0.57	0.05	3	231	11.8	5.9	2.0	81	291	146	2466	-20.5	4.8		3.2
Sc86.21	sc14	Sclayn, Belgium	*Bison priscus*	bone	-	-0.97	0.06	-0.47	0.09	3	177	13.5	6.5	2.1	86	8	98	29	-20.5	4.3		3.2
Sc83.93	sc1	Sclayn, Belgium	*Crocuta crocuta*	bone	-	-1.33	0.03	-0.65	0.07	3	502	17.5	8.0	2.2	99	49	49	93	-19.7	8.2		3.2
Sc83.93b	sc9	Sclayn, Belgium	*Crocuta crocuta*	bone	-	-1.23	0.05	-0.59	0.07	3	317	16.1	7.6	2.1	90	48	84	582	-19.4	11.5		3.2
Sc89.135	sc5	Sclayn, Belgium	*Equus caballus*	bone	-	-0.93	0.01	-0.49	0.06	3	409	14.1	6.9	2.0	97	65	85	45	-21.7	5.1		3.1
Sc87.140	sc6	Sclayn, Belgium	*Equus caballus*	bone	-	-1.18	0.06	-0.62	0.09	3	160	7.9	3.8	2.1	53	69	76	198	-21.7	5.2		3.2
Sc85.121	sc17	Sclayn, Belgium	*Mammuthus primigenius*	bone	-	-1.39	0.10	-0.70	0.08	3	619	17.4	8.4	2.1	123	34	54	139	-21.5	9.4		3.2
Sc81.205	sc7	Sclayn, Belgium	*Coelodonta antiquitatis*	bone	-	-0.93	0.06	-0.47	0.07	3	203	9.8	4.5	2.2	86	36	88	259	-20.6	5.5		3.2
Sc82.210	sc8	Sclayn, Belgium	*Coelodonta antiquitatis*	bone	-	-0.86	0.03	-0.46	0.04	3	271	15.5	7.4	2.1	111	59	165	786	-20.9	5.5		3.2
Sc85.94	sc16	Sclayn, Belgium	*Ursus arctos*	bone	-	-1.14	0.08	-0.59	0.16	3	302	15.3	8.2	1.9	97	9	50	3	-20.2	7.1		3.2
Sc82.131	sc4	Sclayn, Belgium	*Ursus spelaeus*	bone	-	-0.91	0.12	-0.47	0.15	3	227	11.8	5.3	2.2	86	129	140	107	-21.8	3		3.2
Sc86.136	sc11	Sclayn, Belgium	*Ursus spelaeus*	bone	-	-0.92	0.04	-0.49	0.09	3	75	4.4	2.1	2.1	30	29	88	45	-22	6.1		3.2
Sc83.63b	sc12	Sclayn, Belgium	*Ursus spelaeus*	bone	-	-0.92	0.07	-0.46	0.09	3	379	11.4	5.3	2.1	72	125	117	218	-22.5	7		3.2
Sc83.295	sc13	Sclayn, Belgium	*Ursus spelaeus*	bone	-	-0.98	0.02	-0.45	0.07	3	221	15.6	7.4	2.1	96	9	72	2	-23	6.5		3.2
Sc87.103	sc15	Sclayn, Belgium	*Ursus spelaeus*	bone	-	-1.07	0.02	-0.54	0.04	3	283	14.5	6.9	2.1	79	59	135	271	-21.8	5.1		3.2
Sc87.171	sc18	Sclayn, Belgium	*Ursus spelaeus*	bone	-	-0.98	0.04	-0.52	0.10	3	368	14.6	6.9	2.1	85	9	115	11	-22.2	6		3.2
UCBL-FSL 452157	452157	Jaurens, France	*Bison priscus*	enamel	m3	-1.42	0.08	-0.73	0.16	3	1686	17.7	9.1	1.9	107	88	208	23	-	-	-	
UCBL-FSL 452144	452144	Jaurens, France	*Bison priscus*	enamel	m3	-1.47	0.05	-0.75	0.06	3	1274	26.3	13.7	1.9	173	107	98	17	-	-	-	
UCBL-FSL 452150	452150	Jaurens, France	*Bison priscus*	enamel	m3	-1.38	0.04	-0.72	0.03	3	1620	34.0	17.7	1.9	138	90	162	108	-	-	-	
UCBL-FSL 300448	300448	Jaurens, France	*Canis lupus*	enamel	p3	-1.46	0.09	-0.74	0.03	3	1857	33.8	15.7	2.1	133	23	145	16	-	-	-	
UCBL-FSL 300432	300432	Jaurens, France	*Canis lupus*	enamel	c or C	-1.57	0.09	-0.86	0.14	3	1219	30.4	14.8	2.1	126	16	82	15	-	-	-	
UCBL-FSL 300433	300433	Jaurens, France	*Canis lupus*	enamel	c or C	-1.54	0.08	-0.75	0.05	3	1296	38.6	19.8	2.0	102	13	39	5	-	-	-	
UCBL-FSL 309010	309010	Jaurens, France	*Coelodonta antiquitatis*	enamel	P	-1.14	0.13	-0.60	0.16	4	1129	32.5	17.1	1.9	420	37	146	159	-	-	-	
UCBL-FSL 396015	396015	Jaurens, France	*Coelodonta antiquitatis*	enamel	m	-1.22	0.08	-0.59	0.23	4	1019	33.3	17.6	1.9	296	20	135	55	-	-	-	
UCBL-FSL 396055	396055	Jaurens, France	*Coelodonta antiquitatis*	enamel	m	-1.37	0.12	-0.67	0.22	3	1411	32.0	17.2	1.9	328	30	45	24	-	-	-	
UCBL-FSL 300601	300601	Jaurens, France	*Crocuta crocuta*	enamel	c or C	-1.84	0.10	-0.91	0.13	4	1688	32.4	16.2	2.0	83	105	148	65	-	-	-	
UCBL-FSL 300599	300599	Jaurens, France	*Crocuta crocuta*	enamel	c or C	-1.85	0.08	-0.92	0.03	3	1696	35.2	17.8	2.0	107	37	151	32	-	-	-	
UCBL-FSL 300610	300610	Jaurens, France	*Crocuta crocuta*	enamel	P3	-1.87	0.09	-0.93	0.07	3	958	37.1	18.6	2.0	81	34	319	181	-	-	-	
UCBL-FSL 300583	300583	Jaurens, France	*Crocuta crocuta*	enamel	p3	-1.83	0.09	-0.86	0.10	3	1664	32.0	15.8	2.0	117	32	75	44	-	-	-	
UCBL-FSL 300573	300573	Jaurens, France	*Crocuta crocuta*	enamel	P3	-2.02	0.04	-1.04	0.03	3	1760	33.4	17.2	1.9	108	55	299	29	-	-	-	
UCBL-FSL 301061	301061	Jaurens, France	*Panthera pardus*	enamel	P3	-1.92	0.02	-0.95	0.10	3	1962	34.1	16.4	2.1	55	28	109	171	-	-	-	
UCBL-FSL 301081	301081lea	Jaurens, France	*Panthera pardus*	leached bone	-	-1.52	0.03	-0.75	0.15	3	212	26.5	13.7	1.9	23	17	695	356	-	-	-	
UCBL-FSL 301081	301081	Jaurens, France	*Panthera pardus*	bone	-	-1.38	0.11	-0.75	0.09	3	360	23.8	11.4	2.1	49	34	857	624	-	-	-	
UCBL-FSL 300918	300918lea	Jaurens, France	*Panthera spelaea*	leached enamel	c (r)	-1.48	0.04	-0.73	0.08	3	917	20.4	10.8	1.9	33	9	90	16	-	-	-	
UCBL-FSL 300918	300918	Jaurens, France	*Panthera spelaea*	enamel	c (r)	-1.45	0.06	-0.72	0.20	3	2243	31.8	15.9	2.0	88	23	230	60	-	-	-	
UCBL-FSL 300830	300830	Jaurens, France	*Panthera spelaea*	enamel	m1 (r)	-1.51	0.05	-0.75	0.02	3	2465	33.4	16.3	2.0	104	36	96	27	-	-	-	
UCBL-FSL 300825	300825	Jaurens, France	*Panthera spelaea*	enamel	c (l)	-1.48	0.05	-0.80	0.08	3	2121	32.6	16.4	2.0	176	65	166	150	-	-	-	
UCBL-FSL 300995	300995	Jaurens, France	*Panthera spelaea*	enamel	p4	-1.52	0.11	-0.78	0.03	3	1371	34.5	16.7	2.1	90	31	104	-	-	-	-	
UCBL-FSL 451263	451263	Jaurens, France	*Rangifer tarandus*	enamel	M2 (r)	-1.55	0.09	-0.75	0.14	3	1567	33.5	16.9	2.0	103	77	117	28	-	-	-	
UCBL-FSL 451271	451271	Jaurens, France	*Rangifer tarandus*	enamel	M2 (r)	-1.73	0.07	-0.87	0.13	3	2220	33.9	17.0	2.0	200	86	149	23	-	-	-	
UCBL-FSL 451267	451267	Jaurens, France	*Rangifer tarandus*	enamel	M2 (r)	-1.75	0.09	-0.86	0.04	3	1003	45.5	23.8	1.9	287	160	165	26	-	-	-	
UCBL-FSL 301210	301210	Jaurens, France	*Ursus arctos*	enamel	P4	-1.29	0.05	-0.63	0.11	3	1678	36.4	18.3	2.0	110	59	111	367	-	-	-	
UCBL-FSL 301193	301193	Jaurens, France	*Ursus arctos*	enamel	m2	-1.23	0.15	-0.58	0.12	4	1156	33.0	16.4	2.0	96	25	69	66	-	-	-	
UCBL-FSL 301190	301190	Jaurens, France	*Ursus arctos*	enamel	M2	-1.23	0.08	-0.60	0.13	4	1654	34.3	17.8	1.9	110	22	114	123	-	-	-	
UCBL-FSL 301217	301217	Jaurens, France	*Ursus arctos*	enamel	m2 (r)	-1.12	0.11	-0.56	0.09	3	1691	33.3	16.2	2.1	55	26	80	19	-	-	-	
UCBL-FSL 300610	300610cc	Jaurens, France	calcite crust	sediment	-	-0.28	0.05	-0.13	0.13	3	364	34.5	0.0	-	12	10	6	49	-	-	-	
NIST SRM1486	SRM1486	-	standard	bone	-	-1.07	0.02	-0.58	0.01	2	-	-	-	-	-	-	-	-	-	-	-	
NIST SRM1486	SRM1486 0616 #6	-	standard	bone	-	-0.98	0.14	-0.52	0.26	4	-	-	-	-	-	-	-	-	-	-	-	
NIST SRM1486	SRM1486 0616 #3	-	standard	bone	-	-1.02	0.10	-0.57	0.08	6	-	-	-	-	-	-	-	-	-	-	-	
NIST SRM1486	SRM1486 0516 #3	-	standard	bone	-	-0.97	0.07	-0.50	0.09	3	-	-	-	-	-	-	-	-	-	-	-	

Table 2. Calcium isotope values (expressed as δ44/42Ca and δ43/42Ca (in ‰) relative to standard ICP Ca-Lyon) measured on two Pleistocene assemblages as well as elemental concentrations for some major and trace elements. (1) δ13C and δ15N values and C/N ratios from Bochoerens et al. 1997. Tooth type: capital letters refer to the upper tooth row.