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A Neandertal dietary conundrum: new insights provided by tooth enamel Zn isotopes from Gabasa, Spain

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Significance statement

Neandertals’ diets are a topic of continued debate, especially since their disappearance has been frequently attributed to their subsistence strategy. There is no clear consensus on how variable their diets were in time and space. Isotope studies have helped quantify meat consumption in Neandertals, but usually rely on nitrogen isotope analyses of collagen, a protein rarely preserved in samples older than 50 ka. Moreover, collagen-extraction for isotope analyses is rarely successful in Iberian skeletal material. Here, we employ zinc isotope analysis of dental enamel of a Neandertal and associated fauna (Gabasa, Spain), which can be applied to contexts >50 ka. This proxy confirms a high level of carnivory in an Iberian Neandertal.

Abstract

The characterization of Neandertals’ diets has mostly relied on nitrogen isotope analyses of bone and tooth collagen. However, few nitrogen isotope data have been recovered from bones or teeth from Iberia due to poor collagen preservation at Paleolithic sites in the region. Zinc isotopes have been shown to be a reliable method for reconstructing trophic levels in the absence of organic matter preservation. Here we present the results of zinc (Zn), strontium (Sr), carbon (C), and oxygen (O) isotope and trace element ratios analysis measured in dental enamel on a Pleistocene food web in Gabasa, Spain, to characterize the diet and ecology of a Middle Paleolithic Neandertal individual. Based on the extremely low δ^{66}Zn value observed in the Neandertal’s tooth enamel, our results support the interpretation of Neandertals as carnivores as already suggested by δ^{15}N isotope values of specimens from other regions. Further work could help identify if such isotopic peculiarities (lowest δ^{66}Zn and highest δ^{15}N of the food web) are due to a metabolic and/or dietary specificity of the Neandertals.

Introduction:

Over the last thirty years, analyses of nitrogen isotopes in collagen ($\delta^{15}N_{collagen}$) have provided direct evidence for Neandertal diets across Europe and Asia. These studies all indicate a carnivorous (1–12), or at least a meat-heavy diet for European Neandertals. However, one peculiarity of Neandertal $\delta^{15}N_{collagen}$ remains the subject of an ongoing debate. From the one Siberian and eight Western European sites, where both Neandertal and associated fauna have been analyzed, nitrogen isotope ratios in Neandertal collagen are systematically higher than that of other carnivores (3, 6–8, 10, 11, 13, 14). An explanation for such elevated values could be the consumption of herbivores, such as mammoths, which themselves exhibit elevated $\delta^{15}N$ values due to the consumption of plants growing on arid soils (1, 2, 7). While mammoth remains are usually scarce at Neandertal fossil localities, they were nonetheless occasionally consumed, as suggested by remains with cut marks and other human butchery signatures (15). The absence of mammoth remains at Middle Paleolithic sites could be a result of a) Neandertals chose to leave large bone elements at the kill-site, and transport other edible carcass products, mainly meat, back to the habitation site (15) or b) mammoths were not frequently consumed, and the $\delta^{15}N$ peculiarity consequently reflects the consumption of other resources enriched in ^{15}N.

Alongside this $\delta^{15}N$ peculiarity, one major obstacle to our knowledge of Neandertals' subsistence patterns is that the preservation of organic matter limits the application of collagen-bound nitrogen isotope analysis to fossil specimens. Collagen degrades over time at a varying speed depending on climatic and environmental conditions (16). The oldest hominin specimen in which bone protein is preserved is that of Scladina (Belgium), which dates to 90,000 cal BP (17), but most studied specimens are younger than 50,000 cal BP (1–3, 6–8, 10–13, 18). Furthermore, these specimens are only from sites in northwestern and central Europe and Siberia, where climatic conditions favored collagen preservation. As a result, the variability of Neandertals' diet over time and between regions may not accurately reflected by the currently available isotope data. In Iberia, where the latest surviving Neandertals have been discovered (19, 20), collagen was successfully extracted for only one site (21). Therefore, our knowledge of Iberian Neandertal diets mostly relies on zooarcheological and dental calculus data, which show some inconsistencies (21–25). For instance, similar to other Western European sites, zooarcheological studies emphasize the consumption of terrestrial mammals and birds (21). In contrast, analysis of dental calculus for micro-remains and aDNA metagenomic analysis (26–28) highlight the frequent consumption of plants and mushrooms. Indeed, Weyrich et al. (26) even suggest that Neandertals at El Sidrón (Figure 1) rarely consumed meat, but often ate mushrooms, which would also result in elevated $\delta^{15}N$ values (29). The consumption of marine foods is also attested for coastal Neandertals, but its frequency cannot be truly assessed in the absence of isotope studies (21, 23–25, 30). Finally, cannibalism has been documented at two Iberian sites (El Sidrón and Zafarraya) (22, 31) (Figure 1), though such practices appear limited and most likely occurred only during periods of nutritional stress (32). Therefore, it is challenging to confirm the homogeneity of Neandertals' diets across time and space, calling into question a direct link between their subsistence strategy and disappearance.

This study aims to investigate if the novel Zn isotope proxy could help elucidate the dietary behaviors of Neandertals and the source of their $\delta^{15}N$ peculiarity, specifically by studying a Late Pleistocene Iberian food web where the presence of mammoth has not been documented (33). The development of Zn isotope analysis ($^{66}Zn/^{64}Zn$, expressed as $\delta^{66}Zn$) has proven that trophic level information can be retrieved from mammalian tooth enamel ($\delta^{66}Zn_{enamel}$) (34, 35), including fossil samples from Pleistocene food webs where organic matter is typically not preserved (36, 37). Previous studies have demonstrated that $\delta^{66}Zn_{enamel}$ decreases by ca. 0.30 to 0.60 ‰ with each step in archeological and modern food webs (34–38) and that $\delta^{66}Zn$ values associated with breastfeeding are higher than post-weaning associated values (39). While the main source of variation of $\delta^{66}Zn_{enamel}$ values is diet, local geology can also likely influence the isotope ratio of a given animal (36, 39). To date, three modern assemblages from Koobi Fora (Kenya), Kruger Park, and the Western Cape (South Africa)(40), a few animals from a historical site (Rennes, France) (41), and three Late Pleistocene sites (Tam Hay Marklot, Nam Lot and Tam Pà Ling, Laos) (36, 37) are the only terrestrial food webs for which Zn isotope data in teeth and/or bones have been published (Figure S14). In the modern Koobi Fora savannah food web, $\delta^{66}Zn_{enamel}$ differences have been observed between browsers and grazers

(35), but this pattern was not seen in any of the three Pleistocene Asian forest food webs (36, 37). Among modern and historical human populations, historically-documented diets relying on plants are associated with higher δ^{66}Zn values than those that include a substantial quantity of animal products (41–44). Zinc isotopes of ancient hominins have been analyzed only in one Pleistocene *Homo sapiens* individual (37) from Southeast Asia, but not yet in any Neandertal specimen.

This current study contributes significantly to our understanding of Iberian Neandertal diets by providing information on their trophic ecology for a region where traditional nitrogen isotope analyses are unfeasible due to the poor preservation of organic matter. We use the novel Zn isotopic tool as well as other mobility, ecological and dietary proxies applied on tooth enamel from hominin and animal remains from the cave site Cueva de los Moros 1 (Gabasa – Pyrenees – Spain, Figure 1). The site, excavated in the 1980's, is very well documented (for stratigraphic context, see Montes and Utrilla (45) and Supplementary Information 1). All remains come from the layers e, f, and g of a single stratigraphic layer directly above layer h dated to 143±43 ka. Numerous carnivore remains were recovered along with Neandertal remains (layers e and f), allowing for comparison of the different meat-eating taxa. Co-existing herbivores from three different types of environmental contexts are homogenously represented in layers e, f, and g: mountains (Iberian ibex [*Capra pyrenaica*], chamois [*Rupicapra rupicapra*]), forest (cervids including red deer [*Cervus elaphus*]), and open environments (horses [*Equus caballus*], European wild asses [*Equus hydruntinus*]).

Sample collection

Species, sample, and proxy selection

We analyzed the tooth enamel of 65 samples belonging to 43 different teeth of 12 taxa for $\delta^{66}Zn_{enamel}$ (Figure 3, Table S15), 42 samples belonging to 39 different teeth for $\delta^{13}C_{enamel}$, $\delta^{18}O_{enamel}$ (Table S19), 23 samples belonging to 18 different teeth for $^{87}Sr/^{86}Sr$ (Table S7) and 40 samples for trace element ratios (Table S11). We sampled 12 to 50 mg of tooth enamel (1 to 20 mg for Zn isotopes) of five carnivore taxa (lynx [*Lynx spelaea*], wolf [*Canis lupus*], fox [*Vulpes vulpes*], dhole [*Cuon alpinus*], hyena [*Crocuta spelaea*]), one omnivore (bear [*Ursus spelaeus*]) and at least five herbivores (Iberian ibex [*Capra pyrenaica*], chamois [*Rupicapra rupicapra*], red deer [*Cervus *elaphus*], rabbit [*Oryctolagus cuniculus*], horse [*Equus caballus*], European wild ass [*Equus hydruntinus*]), as well as one of the four Neandertal dental remains recovered from the site. Hyenas (*Crocuta spelaea*) are treated as a separate dietary group (bone-eating carnivores) based on the results of the Koobi Fora and Nam Lot food webs (35, 37).

Sampling strategy related to breastfeeding

The single sampled Neandertal tooth is a first molar (M1; Ga1.Rev.303)(46). Previous studies based on tooth wear and trace elements suggest that Neandertals were likely weaned between the age of 1 to 2.5 years old (47–50), thus possibly overlapping with the crown's formation period for this tooth. To minimize destructive analyses, we sampled an enamel fragment from the lower part of the crown, next to the cervical margin. This part of the tooth probably formed after weaning or at least when maternal milk consumption could have been strongly reduced (51). The specimen was heavily worn, making it difficult to evaluate the enamel formation period of the part that we sampled. Still, we were able to evaluate trophic spacing between species while taking into account the influence of maternal milk consumption, which triggers higher δ^{66}Zn values in human M1 (39). To do so, we analyzed $\delta^{66}Z_{enamel}$ on different types of non-hominin teeth (dp4, C, P2, P3, P4, M1, M2, and M3, See SI Table S4) formed at different times during ontogeny and obtained values associated with breastfeeding and/or solid food consumption for all species analyzed from Gabasa. In addition, we analyzed Sr/Ca and Ba/Ca ratios that also show variation between tooth enamel mineralized in utero and during pre-or post-weaning periods (52).

Sampling strategy related to Neandertal mobility and ecology

Other factors such as diagenesis, geographical origin, environmental context, or metabolism can be a source of Zn isotope variability (35, 36, 39, 53). In recent studies, linear mixing models highlighted a possible link between Zn and Sr isotope ratios in teeth, which has been interpreted as resulting from the influence of the local geology on the Zn isotope ratios of its associated food webs (36, 37, 39). To control for this potential bias, we analyzed strontium isotope ratios of Gabasa tooth enamel (^{87}Sr/^{86}Sr$_{enamel}$) for samples with sufficient material (Supplementary Information 5). We also collected Sr isotope data from rocks, soils, plants, and teeth to assess the regional bedrock isotopic data (^{87}Sr/^{86}Sr$_{bedrock}$) and identify whether some animals could have been derived from areas proximal to

Gabasa with different geology (Supplementary Table S18). We hypothesize that the Neandertal consumed local food sources, whereby its $^{87}Sr/^{86}Sr$ should fall within the ranges observed both for the local bedrock and assumed sympatric animals.

We estimated carbon and oxygen isotope compositions ($\delta^{13}C_{enamel}$ and $\delta^{18}O_{enamel}$, respectively) of the Neandertal and associated fauna. Notably, $\delta^{13}C_{enamel}$ and $\delta^{18}O_{enamel}$ values can offer insights into ecological proxies, such as the environment from which the Neandertal individual obtained its prey (i.e. open arid *vs.* closed mesic habitats), and possibly reveal information about which types of prey were consumed (54–56). Moreover, carbon and oxygen isotope data allow us to compare the food web of Gabasa to other food webs where Neandertals have been discovered. For one horse tooth, five samples were taken serially along the growth axis of the teeth to evaluate potential seasonality and intra-tooth variability over time on Sr and Zn isotope ratios.

Sampling strategy to assess the degree of chemical tooth alteration

Although it has been shown that dental enamel preserves biogenic Zn isotope signatures very well in Pleistocene or even older samples (36, 57), we independently confirmed that Zn isotope variability is not related to diagenetic processes. For this purpose, we analyzed trace elements that are abundant in soils but not in tooth enamel (Fe, Al, Mn) and hence are indicative of post-mortem trace element uptake (Supplementary Information 3). Additionally, we examined the carbonate content of dental enamel to eliminate samples with obvious diagenetic alteration (>10 wt% CO_3) (Supplementary Information 3). To capture the influence of diagenesis on isotope ratios, we also performed measurements on dentin samples from two wolves for Sr, C, and O isotopes and trace elements and one wild ass for Zn isotopes. Finally, four roots of teeth whose enamel was sampled (one chamois, two foxes, and one lynx) were used to attempt collagen extraction (Table S9, S10), using a high extraction-yield protocol adapted for radiocarbon dating (58, 59) in the hope of getting additional trophic level information and possibly permitting high-precision radiocarbon dating.

Results

Datasets, details on the method, and additional discussions are given in detail in the supplementary information (SI).

Zinc isotope data

Zinc isotope values of Gabasa mammalian tooth enamel range from 0.35 to 1.63 ‰ (n = 65) and are mainly influenced by the dietary group of the animals (Figures 2 and 3) (Kruskal-Wallis χ^2 = 19.496, p-value = 0.0002159 and χ^2 = 18.298, p-value = 0.0003818; samples with elevated Al, Mn, and Fe content are excluded), which are assigned based on those used in other $\delta^{66}Zn_{enamel}$ isotope studies and zooarcheological data (dietary groups: carnivores, bone-eating carnivores, omnivores, and herbivores) (35, 60, 61). Animal values from different archeological layers fall in the same range (Supplementary Information 4). As previously observed for modern, historical, and Late Pleistocene tooth enamel, there is no correlation between Zn isotope values and Zn concentration data (35, 36, 39, 41, 42). This is an excellent indicator to assess the preservation of biogenic Zn isotope signatures (62). On average, large herbivores have higher Zn isotope ratios ($\delta^{66}Zn_{enamel}$ = 1.24 ± 0.17 ‰ 1σ, n = 16) than carnivores ($\delta^{66}Zn_{enamel}$ = 0.92 ± 0.18 ‰ 1σ; n = 16, 0.85± 0.14 ‰ if hyenas and the Neandertal are excluded). The Neandertal tooth exhibits the lowest $\delta^{66}Zn_{enamel}$ value (0.35 ± 0.00 ‰), substantially lower ($\Delta^{66}Zn_{Neandertal\text{-}carnivores}$ = 0.57 ‰) than that of the lowest carnivore's value. Mirroring patterns in the modern Koobi Fora (East Africa) food web, the difference between herbivore and carnivore values is even more pronounced when hyenas are excluded, as bone-consumption is thought to induce higher $\delta^{66}Zn$ values (35). The omnivorous cave bears ($\delta^{66}Zn_{enamel}$ = 1.15 ± 0.13 ‰ 1σ; n = 3) exhibit Zn isotope ratios closer to those of large herbivores and rabbits rather than the carnivores', similar to a pattern often observed in nitrogen isotopes (63–66). The herbivores show the highest $\delta^{66}Zn$ values (0.96 to 1.60 ‰ 1σ; $n_{measurements}$ = 40 from n_{tooth} = 27 teeth) with grazers (European wild ass and horses, $\delta^{66}Zn$ = 1.35 ± 0.18 ‰ 1σ; n_{tooth} = 6) exhibiting the highest values, another similarity to observations in modern food webs (35, 40).

Strontium isotope data

The $^{87}Sr/^{86}Sr_{enamel}$ range from 0.70834 to 0.70916 (n = 24)˙ Strontium and Zn isotope ratios show no correlation, and the Neandertal individual shows an $^{87}Sr/^{86}Sr_{enamel}$ overlapping with those of deer and rabbits (Figure 4A). Strontium isotope ratios from dental enamel and dentin are compatible with the values expected for the region of Gabasa (Supplementary Information 1 and 5). As dentin is likely to be affected by diagenetic alteration and Sr exchange with the soil, these values might reflect soil values and can thus be an indicator of the local bioavailable Sr isotope composition at Gabasa. (Supplementary Information 3).

Carbon and oxygen isotope data

$\delta^{13}C_{enamel}$ data range from -14.13 to -8.35 ‰ (n = 44), and $\delta^{18}O_{enamel}$ data range from -7.46 to 1.64 ‰ (n = 43). Mountain herbivores (chamois and Iberian ibex) have distinct higher $\delta^{13}C_{enamel}$ and $\delta^{18}O_{enamel}$ compared to other herbivores, which might be due to water evaporation on mountain slopes and consumption of plants from more arid contexts (Supplementary Information 6, Figure 2). The Neandertal's $\delta^{13}C_{enamel}$ and $\delta^{18}O_{enamel}$ values overlap with those of cave animals (Figure 4A and B, Figure S12. Five carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) data were possibly impacted by diagenetic carbonate, and those values were excluded for ecological interpretation (mostly rabbits, see Supplementary Information 3 and 6).

Trace element ratios (Ba/Ca and Sr/Ca)

Trace element ratios are mostly discussed in Supplementary Information 3 and 7. The -log(Ba/Ca) ranges from 3.25 to 4.75 (n = 40) and -log(Sr/Ca) from 2.77 to 3.62 (n = 40). The two ratios measured in dental enamel do not correlate. As expected (67), mixed feeders exhibit lower Sr/Ca than grazers, but carnivores (especially hyenas) show Sr/Ca ratios overlapping with those of grazers (Figure S13). We report the highest Sr/Ca and Ba/Ca ratios for mountain and forest animals and the lowest Ba/Ca for the Neandertal individual. Here, these ratios do not correlate with tooth developmental stages (i.e., ontogenetic time of crown mineralization).

Correlations and comparisons between different dietary proxies

Zn isotopes do not correlate with Sr/Ca or Ba/Ca ratios (Figure 4, Supplementary Information 3 and 4). Equids have higher $\delta^{66}Zn$, $\delta^{13}C$, and $^{87}Sr/^{86}Sr$, and lower Ba/Ca than the rest of the herbivores

(Figure 4). When performing a PCA based on five proxies (δ^{66}Zn, δ^{13}C, δ^{18}O, Sr/Ca, and Ba/Ca), the PC1 places the Neandertal among cave animals (hyenas and bears). This first component is mostly based on δ^{13}C, δ^{18}O, and Ba/Ca values. Strangely, it is precisely when cave animals are removed that a positive correlation appears between δ^{13}C and δ^{66}Zn ($R^2 = 0.41$, $p = 0.00009$). This correlation could be due to a combination of a trophic level effect and higher δ^{13}C and δ^{66}Zn values among grazers. No clear trend has been observed between δ^{66}Zn values and the type of tooth sampled (Supplementary Information 4), but it should be noted that this could result from the low sample size per tooth group and different timing of weaning and dental development between species.

Collagen extraction

The collagen extraction performed on the dentin from four teeth was unsuccessful, and not enough collagen was extracted for $\delta^{15}N_{collagen}$ and/or radiocarbon analyses. This failure is not surprising for samples dating back to about 150 ka (62) (Supplementary Information 2).

Discussion

New insights into the diet and ecology of Iberian Pleistocene mammals from Zn isotopes in tooth enamel

Our study shows similar trends to those previously reported in modern and fossil food webs: Herbivores exhibit higher δ^{66}Zn than carnivores ($\Delta^{66}Zn_{herbivores-carnivores}$ = +0.31 ‰ and +0.38 ‰ excluding hyenas). The trophic level depletion is similar to that of the modern food web of Koobi Fora (dental enamel values: +0.40 ‰, hyenas included and +0.44‰ excluding hyenas), Kenya, or the marine mammal food webs of Arctic Canada (+0.32 ‰ on average in bones) (34, 38) but lower than that of the Laotian fossil sites (+0.63 ‰, hyenas excluded). Grazers (horses and European wild ass) tend to show higher δ^{66}Zn than browsers, as observed in the savannah of Koobi Fora (35), but not in the tropical rain forests of Tam Hay Marklot and Nam Lot (36, 37). Hyenas have more elevated $\delta^{66}Zn_{enamel}$ values than other carnivores (Figure 3), as systematically observed in other sites (35, 37, 40). These higher values are likely due to bone consumption (35), although hyenas have extended breastfeeding time than other carnivores (68), and milk would also similarly drive $\delta^{66}Zn_{enamel}$ up. However, high Sr/Ca and low Ba/Ca ratios observed in the different teeth of all hyenas support the interpretation of the signature of solid food consumption and consequently of bones as a likely driving factor behind their elevated $\delta^{66}Zn_{enamel}$ compared to other sympatric carnivores (Figure S13, Table S4). Cave bears exhibit $\delta^{66}Zn_{enamel}$ overlapping with rabbits, deer, and chamois (Figure 3), which confirms the frequent observations based on low $\delta^{15}N_{collagen}$, indicating they were mostly herbivorous (12, 63, 66).

The $\delta^{66}Zn_{enamel}$ values of the entire food web of Gabasa are the most elevated values ever measured in dental enamel (Mean values about 0.2 ‰ higher than in the modern food web of Koobi Fora and 0.7 ‰ higher than in the Late Pleistocene food webs of Marklot, Nam Lot and Tam Pa Ling (35, 36, 41, 42), Supplementary Information 4, Figure S14). This is probably not due to the geology because limestones, known to show the highest $\delta^{66}Zn$ (69), are present at both Gabasa and the Laotian sites. Gabasa food web's uniqueness comes from its environmental context as it is the coldest terrestrial biotope ever studied for faunal $\delta^{66}Zn$. Pollen data suggest an arid and cold climate (70) with periods characterized by steppe vegetation and some forest patches mainly composed of conifers (45, 70, 71). Carbon and oxygen isotopes exhibit patterns similar to those observed for mammals from various Middle Paleolithic Western European sites (54, 55, 72) and are compatible with mid-mountain mosaic landscape (Supplementary Information 1 and 6). Tree leaves usually exhibit relatively low $\delta^{66}Zn$ and they were probably scarce in Gabasa, as opposed to the forest of Tam Hay Marklot and the fynbos of Western Cape(40), where the lowest $\delta^{66}Zn$ in herbivore teeth were measured.

The diet of a Neandertal at Gabasa

Our results demonstrate that the Neandertal individual from Gabasa shows a Zn isotope signature of a top-level carnivore, similarly to that observed for nitrogen isotopes for other sites with

Neandertal occupation. Carbon and oxygen isotope and trace element data suggest that the individual inhabited the local area around the cave (Supplementary Information 6 and 7). Of all the animal taxa analyzed in Gabasa, the Neandertal specimen easily exhibits the lowest Zn isotope ratio. As the Sr isotope ratio of the Neandertal tooth enamel is similar to those of other animals (Figure 4), the low $\delta^{66}Zn_{enamel}$ value is unlikely the result of a different geographic origin (and consequently different bedrock and isotope baseline) of this individual, but reflect diet instead. Previous work on animal food webs documented a trophic spacing for $\delta^{66}Zn_{enamel}$ of 0.3 to 0.6 ‰ (34–36, 38). In Gabasa, the average difference between carnivores and herbivores is 0.31 ‰, but the Neandertal shows a $\delta^{66}Zn$ value 0.57 ‰ lower than that of the carnivores – 0.50 ‰ if hyenas are excluded– and 0.85 ‰ lower than the herbivores, which would be about three trophic levels higher than that of the herbivores if we consider a trophic spacing of 0.31 ‰. This mirrors dietary reconstructions based on nitrogen isotopes from individuals from other European sites such as Goyet (18) (Belgium), Jonzac (France) (13), or Les Cottés (7) (France), where Neandertals exhibit higher $\delta^{15}N_{collagen}$ values than sympatric carnivores.

Dietary peculiarities other than high-trophic level carnivory either do not explain the low $\delta^{66}Zn_{enamel}$ values or conflict with other observations. Here we consider: 1) a signature of breastfeeding; 2) Specific diets (the consumption of either light Zn-bearing foods or the absence of consumption of heavy Zn-bearing foods); 3) a metabolic origin.

The breastfeeding signature hypothesis can be ruled out because it leads to high $\delta^{66}Zn_{enamel}$. In a previous study, teeth formed during the breastfeeding period of two human populations from very different geographical and archeological contexts exhibit average $\delta^{66}Zn_{enamel}$ values that are 0.3 ‰ higher than the teeth whose dental enamel forms after weaning (39). Furthermore, the low Ba/Ca of the Neandertal dental enamel suggests that it was formed after the breastfeeding period (49, 52, 73) (Table S20). Moreover, the region of the tooth sampled was very close to the tooth's cervical margin because of tooth wear, which usually corresponds to formation ages of 2-3 years old (74). The turnover of Zn in the body occurs over the course of just a few months, and weaning age (or at least an age with substantial consumption of solid food) among Neandertals is assumed to be around 1 year old (49). Even if milk consumption extended to the period from which enamel was sampled from the tooth, it is unlikely that it was a major source of Zn in the diet at this stage. Therefore, the value observed in the Neandertal tooth enamel is likely a reflection of an adult diet. An early weaning age also prevails in recent humans and has also been documented with Zn isotopes in a Late Pleistocene *Homo sapiens* (37), strongly contrasting with the conditions observed among apes, *Australopithecus* (75), and earlier representatives of the genus *Homo* (76). This adaptation results from the necessity for the breastfeeding mother to share the energetic burden of her child's large brain with other adults able to contribute to solid food. It is central to the human pattern of "cooperative breeding" and has critical social and behavioral implications.

Some dietary explanations for the low $\delta^{66}Zn_{enamel}$ of the Neandertal individual involving light Zn bearing foods are also excluded:

a. Adult vs juvenile prey: In principle, if (as in humans (39)) milk consumption leads to higher $\delta^{66}Zn$, preferential consumption of post-weaning juveniles or adults might lead to lower $\delta^{66}Zn$. However, this explanation seems unlikely for two reasons. First, such an isotopic disparity has not been observed in herbivore teeth in relationship to weaning in this study or elsewhere (36), so the age of the prey does not appear to matter. Secondly, Neandertals targeted younger deer and horses (61) (Supplementary Information 1) and while other carnivores targeted adult ibexes. As such, if prey age mattered, Neandertal Zn isotope compositions should be biased towards higher $\delta^{66}Zn$, not lower. Although we cannot rule out the possibility that the formation period of the Neandertal tooth is asynchronous with the site's period of occupancy, there is currently no evidence to support this..

b. Cannibalism or hypercarnivorism: Cannibalism or carnivore meat consumption could be associated with low Zn isotope ratios, but the absence of cut marks on hominin and carnivore bones rules out this hypothesis.

c. Food with unusually low $\delta^{66}Zn$ values: Archaeological evidence for some isotopically light foods that Neandertals might have specifically targeted may be missing (e.g., animal livers (53, 77, 78), birds, mushrooms, fruits, leaves (79–81), insects (82, 83) or aquatic resources (43)). However, $\delta^{66}Zn_{enamel}$ of the consumers of those foods have been measured and are not associated with lower values than that of sympatric carnivores, both in Gabasa (e.g., lynx are known to consume liver, deer consume leaves, while foxes, bears, and rabbits have been known to consume mushrooms) and other food webs (apes and tapirs for insects and fruits (36)).

d. Aquatic foods: The consumption of aquatic resources has been demonstrated for some Iberian Neandertals, though not at Gabasa (23, 25, 30). Still, the consumption of common trout (*Salmo trutta*) has been suggested for a Mousterian site at the Grotte du Noisetier (84), located in the French Pyrenees (Figure 1). The trophic level of this species could potentially explain low $\delta^{66}Zn_{enamel}$ values, but would imply that the main source of Zn in the diet comes from freshwater fish. Trout Zn content is about ten times less than that of liver or muscle from mammals (85), requiring Neandertals to have consumed trout almost exclusively to shift their $\delta^{66}Zn_{enamel}$ to the observed low value. As no fishbone or other evidence for aquatic resource use were found in the Gabasa region, we can disregard this hypothesis as unlikely.

e. Food processing: Elevated $\delta^{15}N$ values among Neandertals have been suggested to result from food processing such as fermentation or cooking. However, Zn fractionates only at a temperature above ~ 900°C (86), which is not reached in open fires, and even if fractionation did occur, it would enrich the Neandertal food in heavy Zn isotopes (86). Biotic fractionation during meat processing is not documented, but could happen within muscle tissues if the reactions were incomplete during fermentation (87) or other curing processes. However, this would only influence the final $\delta^{66}Zn$ of the consumed food if both reagents and products of the curing process would not be contained in the final food product.

A combination of dietary practices might explain the low $\delta^{66}Zn_{enamel}$ value of the Neandertal. Zinc isotopes are highly fractionated in mammal tissues (77, 78, 88, 89), and different mammal species have different isotope compositions. Thus, a low $\delta^{66}Zn$ value might reflect consumption of muscles and liver (low $\delta^{66}Zn$) from deer and rabbits (lower $\delta^{66}Zn$ than other herbivores, Figure 3, also supported by $\delta^{13}C$ and $\delta^{18}O$ for which Neandertals exhibit values close to these animals (Figure 2, 4, S10)), while excluding bones and blood (high $\delta^{66}Zn$). Although cut marks suggest Neandertals hunted deer and horses (61, 90), rabbits are the most abundant taxon of the site (Figure S2), and some rabbit bones show cut marks (Figure S3). Disarticulation of rabbits requires minimal use of tools (61), so they could have been heavily consumed without physical evidence. Temporal asynchrony between cave deposits and tooth growth might explain a discrepancy between zooarchaeological and isotopic dietary conclusions if the deposits represent a period when Neandertals preferentially ate horses and deer, whereas the portion of the tooth corresponds to a period when rabbits were preferentially eaten. Higher $\delta^{66}Zn$ among other sympatric carnivores hunting the same species as Neandertal (Table S3) (61, 91) might partially reflect bone consumption: hyenas tend to have elevated $\delta^{66}Zn_{enamel}$ values, likely due to bone consumption (35, 37). Red foxes and wolves gnaw on bones (although they ingest much less bone than hyenas (92)), while lynxes partially digest the bones of rabbits, which make up 80-100% of their typical diets (91). In addition, all these carnivores may consume substantial blood, possibly helping to explain higher $\delta^{66}Zn$ values than the Neandertal.

Metabolic specificity (different diet-tissue isotopic offsets) for Neandertals compared to other animals might also explain the unusually low $\delta^{66}Zn$ value and possibly the higher $\delta^{15}N_{collagen}$ values that have been measured elsewhere. It is indeed striking how similar the patterns between $\delta^{66}Zn_{enamel}$ and $\delta^{15}N_{collagen}$ mirror each other: in both proxies, Neandertals typically exhibit isotope ratios that would appear to imply a higher trophic level than sympatric carnivores (1, 3–5, 7, 11, 63). Although such distinct compositions have been interpreted to reflect aquatic food resources for some Paleolithic humans (11), compound-specific isotope analysis of amino acids shows that anatomically modern humans from Buran Kaya (Crimea) relied on terrestrial resources, even though they exhibit the highest $\delta^{15}N_{collagen}$ known for the Paleolithic (93). These authors suggest preferential consumption of mammoths (absent from Gabasa), but a metabolic origin might yield similarly high $\delta^{15}N_{collagen}$ values. We acknowledge that some studies show minimal offsets in $\delta^{15}N_{collagen}$ between humans and sympatric herbivores (94). However, the trophic spacing between the Paleolithic and Neolithic periods did not decrease to the extent expected for a transition from hunter-gatherer to a more cereal-based diet (95). Furthermore, for Zn isotopes, a medieval population with expected high meat and marine fish consumption shows strikingly lower $\delta^{66}Zn_{enamel}$ values than for sympatric herbivores (0.6 ‰ lower) and for a dog, cat, and pig (0.3 ‰ lower), while $\delta^{15}N_{collagen}$ values were strikingly higher than herbivores (4 ‰) and dog, cat, and pig (1 ‰) (41). These observations closely resemble patterns for Neandertals at Gabasa (for Zn isotopes) and elsewhere (for N isotopes), and suggest a metabolic effect. Controlled feeding experiments on animals with an omnivorous diet are necessary to assess this further. The reason for this metabolic difference remains unknown, but it should be noted that the Neandertal is the most carnivorous species among primates, and the only meat-eater not from the order *Carnivora* within the Gabasa faunal assemblage. Therefore, Neandertals may have had some metabolic peculiarities compared to other carnivores and primates regarding adaptations of their gastrointestinal tract and their dietary preferences (96).

Concluding remarks.

Zinc isotope analysis of tooth enamel can successfully characterize past animal and human diets in the absence of collagen preservation for $\delta^{15}N$ analysis. Whichever method is used to characterize Neandertals' diets ($\delta^{66}Zn_{enamel}$ and $\delta^{15}N_{collagen}$), isotope values represent an extreme in carnivore trophic levels, and Iberian Neandertals appear to be no exception. Meat consumption is supported by both the zooarcheological data at Gabasa and $\delta^{66}Zn_{enamel}$ analysis. Furthermore, the low Zn isotope ratio observed in the single measured Gabasa Neandertal specimen suggests that this individual might have had a distinct diet compared to other carnivores (possibly avoiding the consumption of bones and blood) and/or may not have fractionated Zn isotopes like other sympatric carnivores. Our study represents the first successful analysis of Zn isotopes from a Neandertal tooth, and further work should focus on understanding whether such unusual compositions result from specific diets or metabolic fractionation in these extinct hominins.

Material and Methods

Material

Details on the context of Gabasa cave deposits, geology, dating, zooarcheology, paleoenvironment and sampling strategy are available in the Supplementary Information. The material used in this study is associated with the permit "Resolution" by the Gobierno de Aragon on the February 10th 2016, and August 31st 2018.

Methods

Tooth enamel fragments and powders were sampled at the Department of Human Evolution of the Max Planck Institute for Evolutionary Anthropology (MPI-EVA, Leipzig, Germany) using a drill with a diamond saw and at the Géosciences Environnement Toulouse of the Observatoire Midi Pyrénées (GET/OMP, Toulouse, France) using a MicroMill. For Zn isotope analyses, 1 to 20 mg enamel fragments were dissolved in 1mL 1N HCl. Samples were cleaned with double-distilled H_2O in an ultrasonic bath and dried at 50°C. Zinc was then extracted by column chromatography using the modified protocol of Moynier et al. (2006) described by Jaouen et al (2016b) (34, 97). The $\delta^{66}Zn_{enamel}$ analyses were conducted on three different MC ICP MS instruments: a Nu 500 (ENS Lyon, France), a Neptune (MPI-EVA, Leipzig, Germany) and a Neptune Plus (GET/OMP, Toulouse, France) using a Cu doping technique (see Supplementary Information 4). The $\delta^{66}Zn_{enamel}$ values were corrected to take into account the offset (+0.27 ‰) existing between our in-house standard, bracketing standard (AA-MPI) and the JMC-Lyon solution. Zinc isotope data are thus reported toward the JMC-Lyon values, and this correction does not impact the relative offsets or our interpretations. Strontium isotope analyses were conducted on 5 to 20 mg of enamel powder, after performing a purification step based on a modified protocol of Deniel and Pin (2001) (98) described by Richards et al (2008) (13), using a Triton plus TIMS and a Neptune Plus at GET/OMP Toulouse. O and C isotope data were obtained from untreated tooth enamel powder (<100 µg) using the "cold trap" method with a Thermo Scientific Delta V IRMS in the Vonhof laboratory at the Max Planck Institute for Chemistry (MPIC, Mainz, Germany) after Vonhof et al. (2020) (99). The reference materials NIST SRM 1400 (for Zn), NIST SRM 1486 (for Sr), IAEA-603, NBS18, NIST SRM 120c and three internal house standards - a carbonate standard (VICS) and a tooth enamel structural carbonate (AG-Lox) (for C and O) and a dentin sample (AZE) for Zn were analyzed in the same runs as the samples and gave consistent results for each lab and compared to previous studies (Supplementary Tables S3 to S5). Trace element analyses were conducted at the GET using an ICap (ThermoFischer) Triple Quadrupole (TQ-ICP-MS). Finally, collagen was extracted from four tooth roots for $\delta^{15}N$ analyses using the protocol of Talamo and Richards (2011) (58). All trace elements and isotope results are available in the supplementary data.

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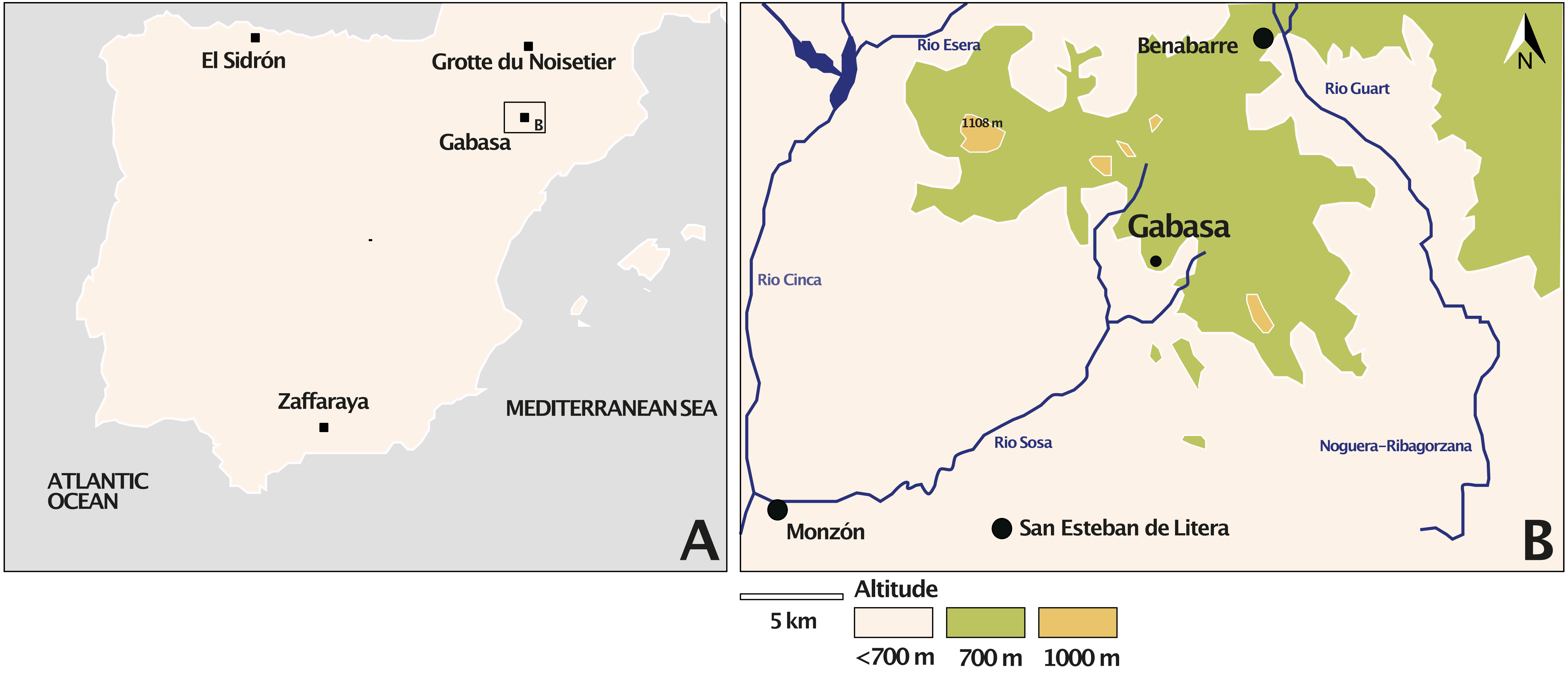
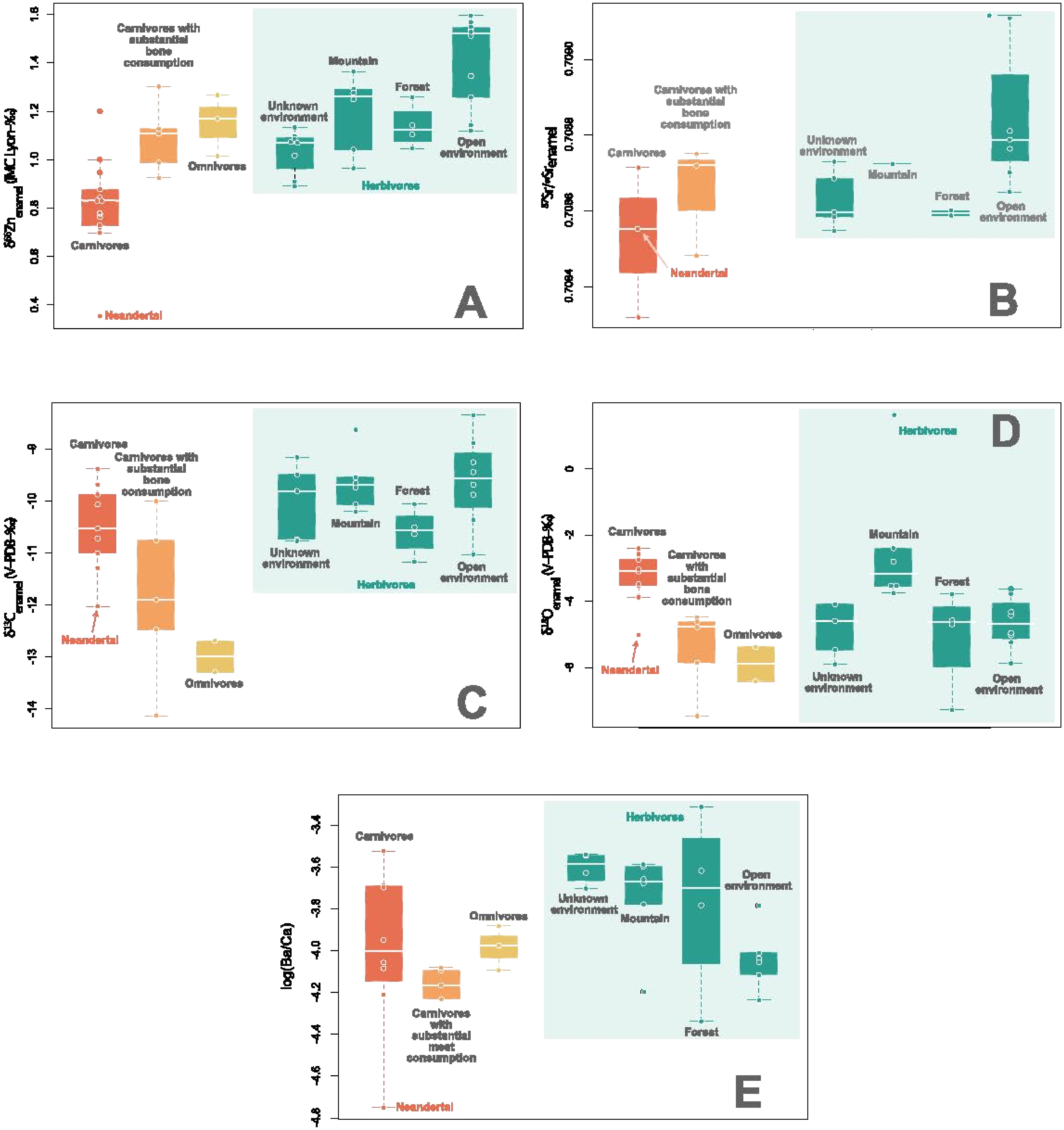
Figure 1. A. Location of the Gabasa site as well as other Neandertal sites mentioned in the text. B. Detailed map of the Gabasa region. San Estaban de Litera and Benabarre are nearby modern cities.

Figure 2. Average dental enamel isotope and trace element ratios for the different dietary categories at Gabasa, Spain. A: $\delta^{66}Zn_{enamel}$, B: $^{87}Sr/^{86}Sr$ C: $\delta^{18}O_{enamel}$, D: $\delta^{13}C_{enamel}$, E: Ba/Ca. Omnivores correspond to one single taxon (cave bears) as well as carnivores with significant bone consumption (cave hyenas).

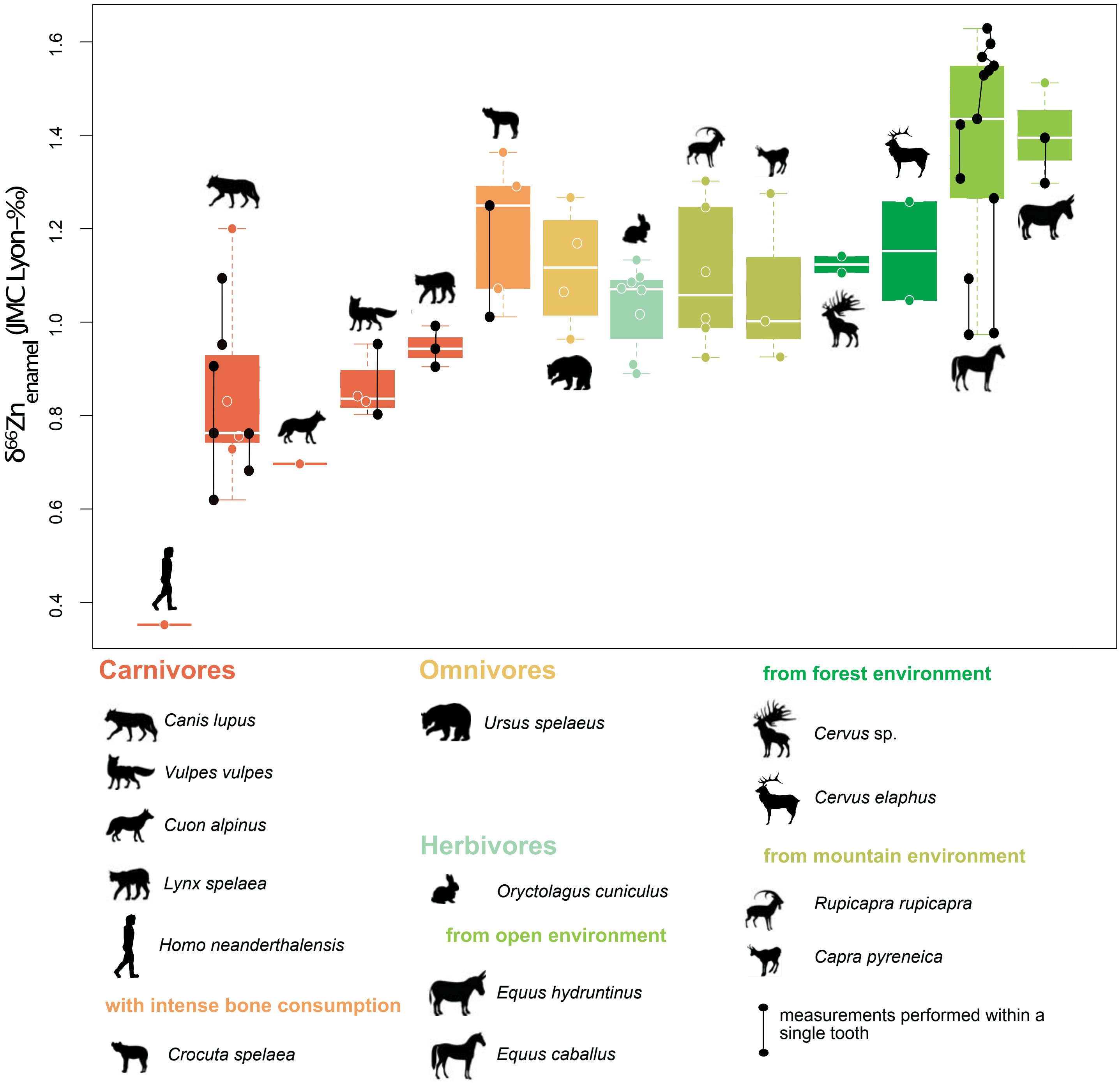
Figure 3. Enamel $\delta^{66}Zn$ values per mammal taxon for different dietary groups from Gabasa, Spain given as box plots. Note that sub-samples taken from one single tooth reflect differences in dietary ^{66}Zn values. See text for detailed discussion about the low Neandertal $\delta^{66}Zn$ value.

Figure 4. Zinc isotope values (δ^{66}Zn, average dental enamel values per tooth) cross plotted versus A) carbon (δ^{13}C), B) oxygen(δ^{18}O) and C) strontium (^{87}Sr/^{86}Sr) isotope composition as well as D) Ba/Ca ratios measured in faunal and Neandertal tooth enamel at Gabasa, Spain. Each point corresponds to a single tooth, except for the horse tooth for which five samples were taken (samples are connected with a line). Error bars correspond to the SD between analytical duplicates for C and O isotopes (0.02 to 0.36 ‰; one sample with higher SD has been removed) and for Zn (analytical duplicates: 0.00 to 0.04 ‰; SD for separate samples taken from a single tooth: 0.04 to 0.21 ‰). SE for strontium isotope ratios are 0.000003 to 0.000010 and are too small to display.

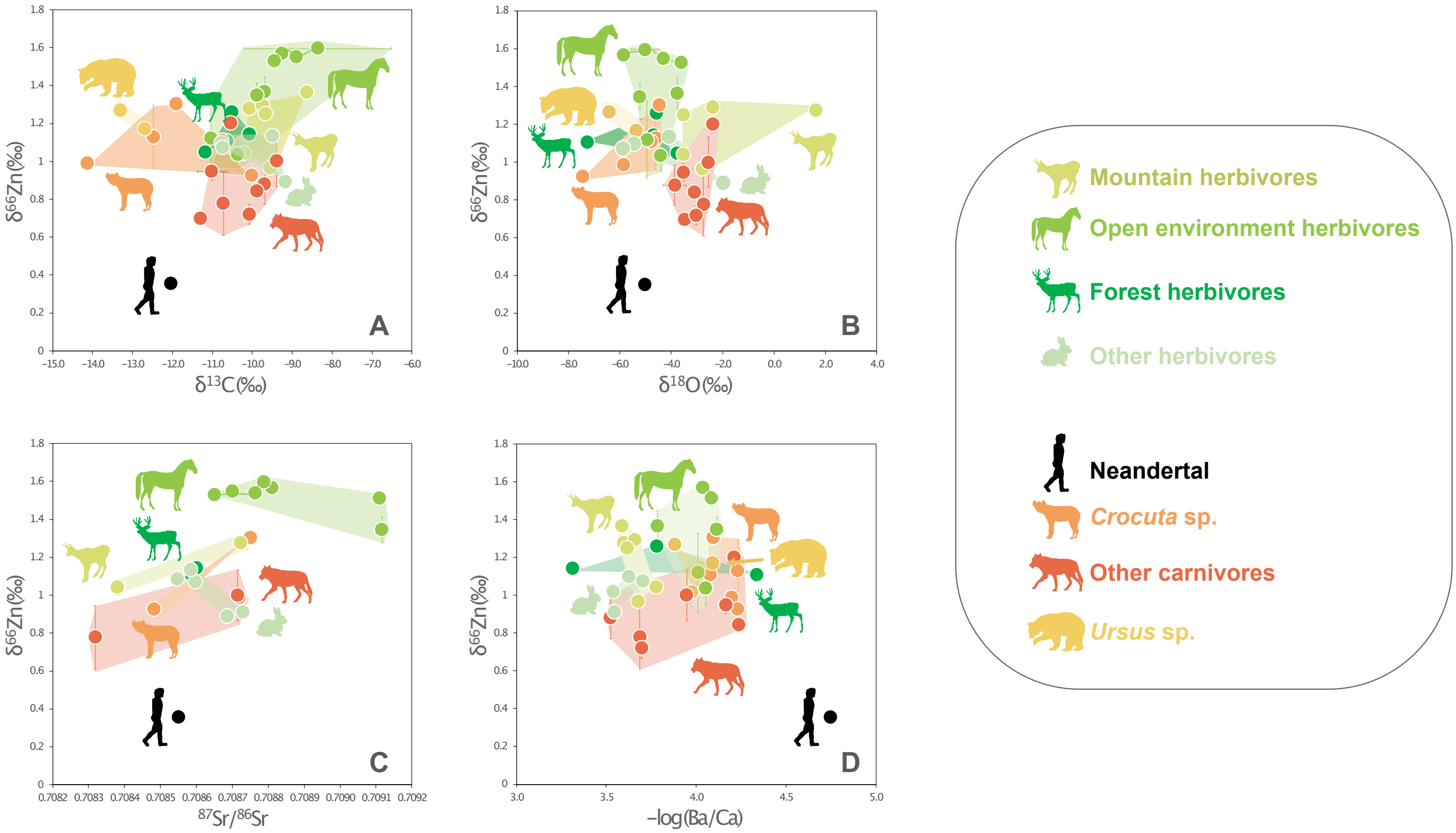

El Sidrón
Grotte du Noisetier
B
Gabasa
Zaffaraya
MEDITERRANEAN SEA
ATLANTIC OCEAN
A
Rio Esera
Benabarre
N
Rio Guart
1108 m
Gabasa
Rio Cinca
Rio Sosa
Noguera–Ribagorzana
Monzón
San Esteban de Litera
B
5 km
Altitude
<700 m
700 m
1000 m



A
$\delta^{66}Zn_{enamel}$ (JMC Lyon-‰)
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Carnivores with substantial bone consumption
Carnivores
Omnivores
Unknown environment
Mountain
Forest
Open environment
Herbivores
Neandertal
B
$^{87}Sr/^{86}Sr_{enamel}$
0.7084
0.7086
0.7088
0.7090
Carnivores
Carnivores with substantial bone consumption
Neandertal
Unknown environment
Mountain
Forest
Open environment
C
$\delta^{13}C_{enamel}$ (V-PDB-‰)
-9
-10
-11
-12
-13
-14
Carnivores
Carnivores with substantial bone consumption
Neandertal
Omnivores
Unknown environment
Mountain
Forest
Open environment
Herbivores
D
$\delta^{18}O_{enamel}$ (V-PDB-‰)
0
-2
-4
-6
Herbivores
Carnivores
Carnivores with substantial bone consumption
Omnivores
Neandertal
Mountain
Forest
Unknown environment
Open environment
E
log(Ba/Ca)
-3.4
-3.6
-3.8
-4.0
-4.2
-4.4
-4.6
-4.8
Carnivores
Herbivores
Omnivores
Unknown environment
Mountain
Open environment
Forest
Carnivores with substantial meat consumption
Neandertal



$\delta^{66}Zn_{enamel}$ (JMC Lyon-‰)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Carnivores
Canis lupus
Vulpes vulpes
Cuon alpinus
Lynx spelaea
Homo neanderthalensis
with intense bone consumption
Crocuta spelaea
Omnivores
Ursus spelaeus
Herbivores
Oryctolagus cuniculus
from open environment
Equus hydruntinus
Equus caballus
from forest environment
Cervus sp.
Cervus elaphus
from mountain environment
Rupicapra rupicapra
Capra pyreneica
measurements performed within a single tooth

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
δ66Zn(‰)
–15.0
–14.0
–13.0
–12.0
–11.0
–10.0
–9.0
–8.0
–7.0
–6.0
δ13C(‰)
A
–10.0
–8.0
–6.0
–4.0
–2.0
0.0
2.0
4.0
δ18O(‰)
B
0.7082
0.7083
0.7084
0.7085
0.7086
0.7087
0.7088
0.7089
0.7090
0.7091
0.7092
87Sr/86Sr
C
3.0
3.5
4.0
4.5
5.0
–log(Ba/Ca)
D
Mountain herbivores
Open environment herbivores
Forest herbivores
Other herbivores
Neandertal
Crocuta sp.
Other carnivores
Ursus sp.