

# The cave bear's hibernation: reconstructing the physiology and behaviour of an extinct animal

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## ABSTRACT

When studying an extinct species such as the cave bear (*Ursus spelaeus* ROSENMÜLLER 1794), it is possible to apply a variety of molecular biology techniques such as the study of stable isotopes or mitochondrial DNA (mDNA) to infer patterns of behaviour or physiology that would otherwise remain concealed. Throughout Europe and along time, differences in the isotopic values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of cave bears arise from environmental differences and the Pleistocene climatic evolution. The climate determines the hibernation length, during which the cave bears undergo a particular physiology that can be related to an increase in  $\delta^{15}\text{N}$  during climate cooling. In order to verify whether hibernation affected the isotopic values, we compared cave bears in different ontogenetic stages. The results show that perinatal values reflect the values for mothers during hibernation, while juveniles show differences in maternal investment. A previous study in the literature based on complete mitochondrial DNA sequences of several individuals collected from closely situated caves showed that each cave housed, almost exclusively, a single lineage of haplotypes. This pattern suggests extreme fidelity to the birth site, or homing behaviour, and that cave bears formed stable maternal social groups, at least for the purpose of hibernation. Studies of this type offer unexpected data on the palaeobiology of this extinct animal.

## KEYWORDS

*Ursus spelaeus*; metabolism; stable isotopes; ancient DNA; homing behaviour

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## Introduction

### *Bear hibernation*

If there is one trait that characterises our well-known brown bears (*Ursus arctos* LINNAEUS 1758) and the American (*Ursus americanus* PALLAS 1780) or Asiatic (*Ursus thibetanus* CUVIER 1823) black bears is their ability to overcome winters in hibernation, whether it is in a natural refuge or a refuge that has been dug out by themselves. A bear will then stay in that refuge during the coldest months of the year when food is scarce or simply unavailable. During this time, which can last up to 6 months, the bear does not eat, drink, urinate or defecate (Hellgren 1998). The bear survives by mobilising itself and burning its enormous fat reserves without suffering from atherosclerosis, azotaemia or calcaemia (Arinell et al. 2012; Stenvinkel et al., 2013a). It does not suffer from muscle or bone loss despite its state of immobilisation (Lohuis et al. 2007a; McGee-Lawrence et al. 2008). Its heart rate decreases, but any decrease in body temperature is negligible (Hellgren 1998). The bear metabolism changes to a special state, one that to date is still relatively unknown and is the subject of intensive study via the field of translational medicine (Nelson et al. 1973, 1975) that seeks models in animals that can be applied to humans (Seeger et al. 2011; Arinell et al. 2012; Stenvinkel et al., 2013b).

But, what do we know about the extinct cave bear (*Ursus spelaeus* ROSENMÜLLER 1794) from the Late Pleistocene? Like the brown bear, with whom it is closely related (Knapp et al. 2009), the cave bear hibernated, and did so in the deepest of caves that open into European karstic regions. While brown bear remains are found in these caves, it is not possible to know whether they also hibernated in other types of shelter (for example, caves in granite rock, where their remains would not be preserved due to the acidic nature of the substrate). Notwithstanding, cave bear sites usually contain bone remains from a large number of individuals that, presumably, died during hibernation (Kurtén 1976). Many caves thus functioned as authentic concentration-lagerstätten (konzentrat-lagerstätten), preserving the remains of bears that eventually died during hibernation over periods of hundreds or even thousands of years.

Hibernation is also the period during which the bear mother gives birth to her offspring. Also, in reproduction bears follow a particular system that distinguishes them from other mammals. Many, if not all current ursids, present an embryonic diapause and dormancy in the uterus (delayed implantation) (Spady et al. 2007). Originally, delayed implantation was considered to be a mechanism that allows the birth of offspring to take place in the appropriate season. However, bear gestation is extremely brief and the cubs are born with a very small size and in the middle of winter while the mother is still hibernating.

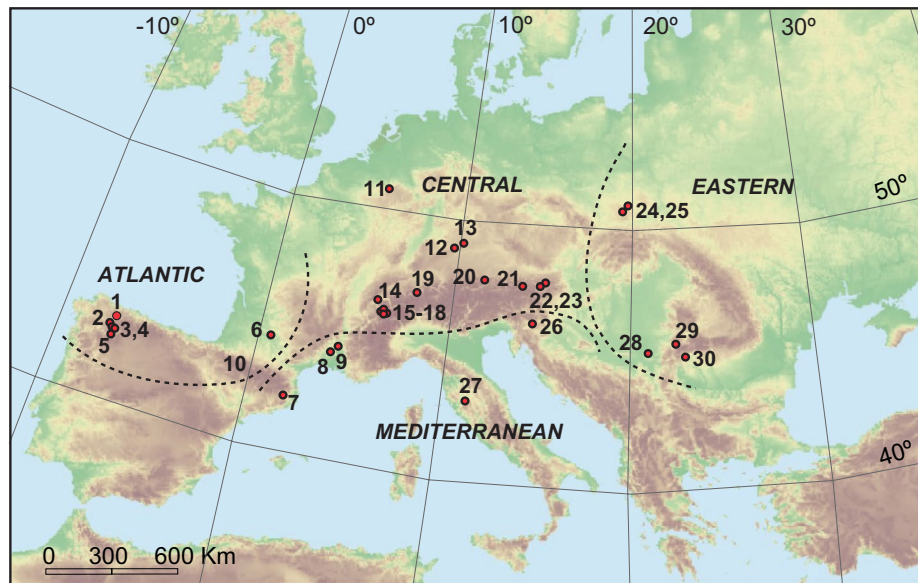
This is because the embryo cannot develop from the fat mobilisation of its hibernating mother (Power and Schulkin 2016). Thus, both traits, hibernation and premature birth of the offspring, are closely related. But, if hibernation is more dependent on climate, seasonality and food availability, with populations today that do not need to hibernate (Nores et al. 2010, Schoen et al. 1987), then premature birth of offspring is a feature that remains unchanged and is maintained even in ursids that inhabit the subtropical areas (where currently seasonality is practically non-existent) and obviates the need to hibernate. For example, the South Asiatic sloth bear (*Melursus ursinus* SHAW 1791) and the South Asiatic sun bear (*Helarctos malayanus* RAFFLES 1821), and even the south American spectacled bear (*Tremarctos ornatus* CUVIER 1825) perform embryonic diapause and have extremely altricial cubs despite a long gestation period (Weinhardt 1988; Johnsingh 2003; Kumar et al. 2014). At the other end of the range is the polar bear (*Ursus maritimus* PHIPPS 1774), which does not hibernate except in the case of a pregnant female which remains in her den for several months until the offspring reach a critical size that allows them to survive outside in an extremely cold and inhospitable environment (Ramsay and Stirling 1988).

Since deferred implantation and premature birth appear to be basal conditions of the ursids (Spady et al. 2007), one can assume that they also apply to the cave bear. The presence of small bones of perinatal individuals is quite common in the sites. A characteristic feature in cave bear ensembles is a U-shaped mortality profile, which corresponds to an accretionary, non-violent accumulation (Kurtén 1957, 1976; Stiner 1998). In most of the sites, young individuals represent up to 70% of the total population (Weinstock 2000). This high mortality in juveniles may be due to different causes, but their lack

of experience in obtaining food for themselves and the difficulties of successfully overwintering may be among the main causes. For the older individuals, mortality would be mainly related to accidents, illness, old age or lack of sufficient fat storages to overcome the winter.

#### Hibernation and bone collagen stable isotopes

Hibernation in ursids requires them to use stored fat and the recycling of urea to maintain their vital body functions, causing significant physiological changes that affect body temperature, heart rate, cell turnover, protein metabolism, etc. (Nelson et al. 1973; Barboza et al. 1997; Hissa 1997; Hellgren 1998). In bears of the present day, some of these changes cause an elevation of the nitrogen isotope signature ( $\delta^{15}\text{N}$ ) in certain tissues such as blood serum (Jenkins et al. 2001) or muscle (Lohuis et al. 2007b). It is possible that in cave bears this higher isotopic signature was recorded, at least occasionally, in bone collagen (which has not been studied in present day bears), since bone tissue also under-goes certain remodelling during the winter period (Donahue et al. 2006; McGee-Lawrence et al. 2008), although this is much less intense than in the active phase (McGee-Lawrence et al. 2015). Therefore, it has been suggested that those bears living during cold pulsations would show more positive  $\delta^{15}\text{N}$  values due to urea recycling or other metabolic effects caused by hibernation (Fernández-Mosquera et al. 2001; Grandal-d'Anglade and Fernández-Mosquera, 2008). We deal with this hypothesis in this paper, since from a biological point of view, it seems likely that the extreme metabolic changes that cave bears suffered during hibernation have been reflected in their body protein, including bone collagen.



**Figure 1.** Location of the cave bear deposits (*Ursus spelaeus/ingressus*) included in this work and geographical regions delimited in this study. 1, Santos; 2, Eirós; 3, Liñares; 4, A Ceza; 5, Rebolal; 6, Font de Gaume; 7, Ermitons; 8, Mialet; 9, Chauvet; 10, Olaskoa (unknown exact location); 11, Goyet; 12, Höhle Fels; 13, Geissenklösterle; 14, Rochedane; 15, Baume aux Pirotas; 16, Grotte de l'Ubena; 17, Grotte de la Toffière; 18, Grotte des Plaints; 19, Bärenloch; 20, Tischoferhöhle; 21, Liegloch; 22, Gamsulzen; 23, Ramesch; 24, Nietoperzowa; 25, Perspektywiczna; 26, Divje Babe; 27, Chiostraccio; 28, Oase; 29, Cioclovina; 30, Mulierii; 31, Medvezhiya (Urals, out of map range).

## Material and methods

We approach the bear ecology during hibernation, and also bear reproduction and growth, using techniques of molecular palae-ontology. Specifically, we use stable isotopes of C and N in bone collagen, which is usually preserved in favourable conditions that exist in cave deposits. Collagen is a protein that is synthesised from amino acids that come from the diet or synthesised through various metabolic pathways from other proteins in the body. In each reaction of these processes and that routes that are often long, an isotopic fractionation occurs, i.e. a preferential mobilisation of the heavy or light isotope, as the case may be, which causes a difference in the proportion between the two isotopes in the final product. For this reason, the isotopic study is used in order to know the ecology of individuals, as it reflects their diet type, metabolic fractionation and the environmental conditions in which they lived (Gannes et al. 1998; Wolf et al. 2009; Boecklen et al. 2011).

To get an overview of the evolution of  $\delta^{15}\text{N}$  over time, we analyse isotopic data from adult cave bears ( $n = 130$ ) and ungulates ( $n = 58$ , of which 30 are red deer *Cervus elaphus* LINNAEUS 1758 and 28 are horses, *Equus ferus* LINNAEUS 1758) with direct radiocarbon dating from the literature. The deposits (31 for cave bears and 20 for ungulates), including their chronological and isotopic data and the bibliographical source are detailed in Tables S1 and S2 (online supplementary material). Eleven of the samples are hitherto unpublished material, the methodology and detailed results of which are shown in Table S3 (online supplementary material). Figure 1 shows the location of the cave bear deposits that are included in this work.

Radiocarbon ages were calibrated in order to frame isotopic faunal data on the climatic curve. There are several available calibration curves, each of which yields slightly different ages. We used the CalPal Online programme (Danzeglocke et al. 2013), which uses the CalPal\_2007\_HULU curve (Weninger and Jöris 2008). The period covered is delimited by the maximum date achieved by the calibrated  $^{14}\text{C}$  dates and the most recent cave bears, thus covering an interval that is approximately within the range of 28,000 to 56,000 years before the present day. However, the main problem of including only individuals with direct dating is that the number of included cases is drastically reduced, especially with respect to herbivores. Moreover, in many cases the very uncertainty of the dating itself, having wide margins of error, does not allow direct correspondence between relatively short climatic fluctuations and the lives of individuals. It is also possible that the marked fluctuations in temperatures have not always had a synchronous reflection in the N pool of soils, which may show some resilience to changes that can sometimes occur over periods of hundreds of years (Shaver et al. 2000). Therefore, smooth out the climatic curve and isotopic values using the LOWESS statistic (Cleveland and Devlin 1988) in order to better observe the general trend in both the climatic curve and the isotopic values of fauna.

To see the evolution of isotopic values with age, we use the isotopic data of Pérez Rama et al. (2011a, 2011b) that were taken from subadult bears in the Eirós Cave and which includes per-inatal individuals.

All data analyses were performed using the PAST (Paleontological Statistics software) version 3.18 (Hammer et al. 2001).

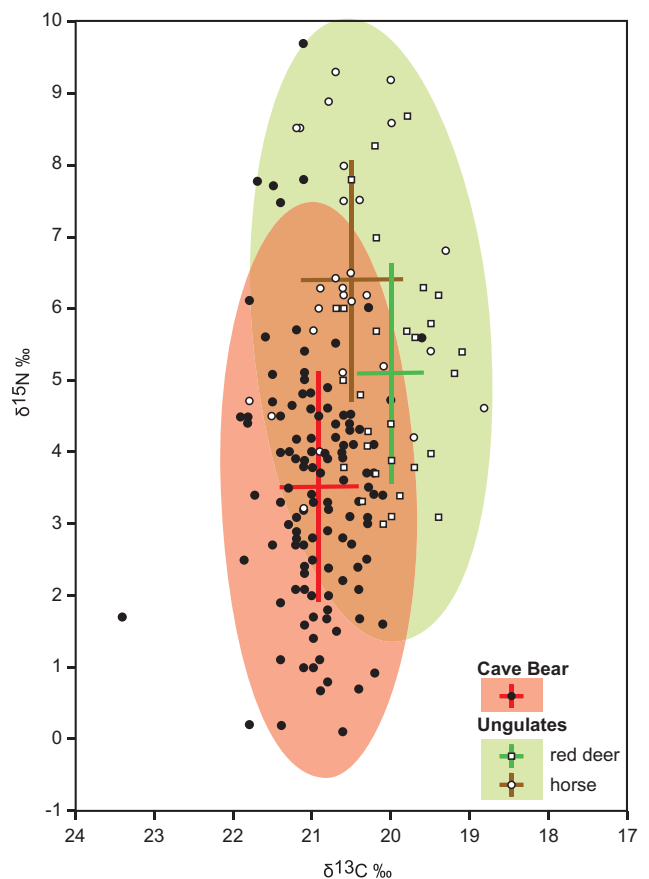
Finally, although it is not a commonly used tool to study the ethology of extinct animals, ancient mitochondrial DNA does, in this case, provide us with valuable information about the denning behaviour of cave bears.

## Results on adult cave bears

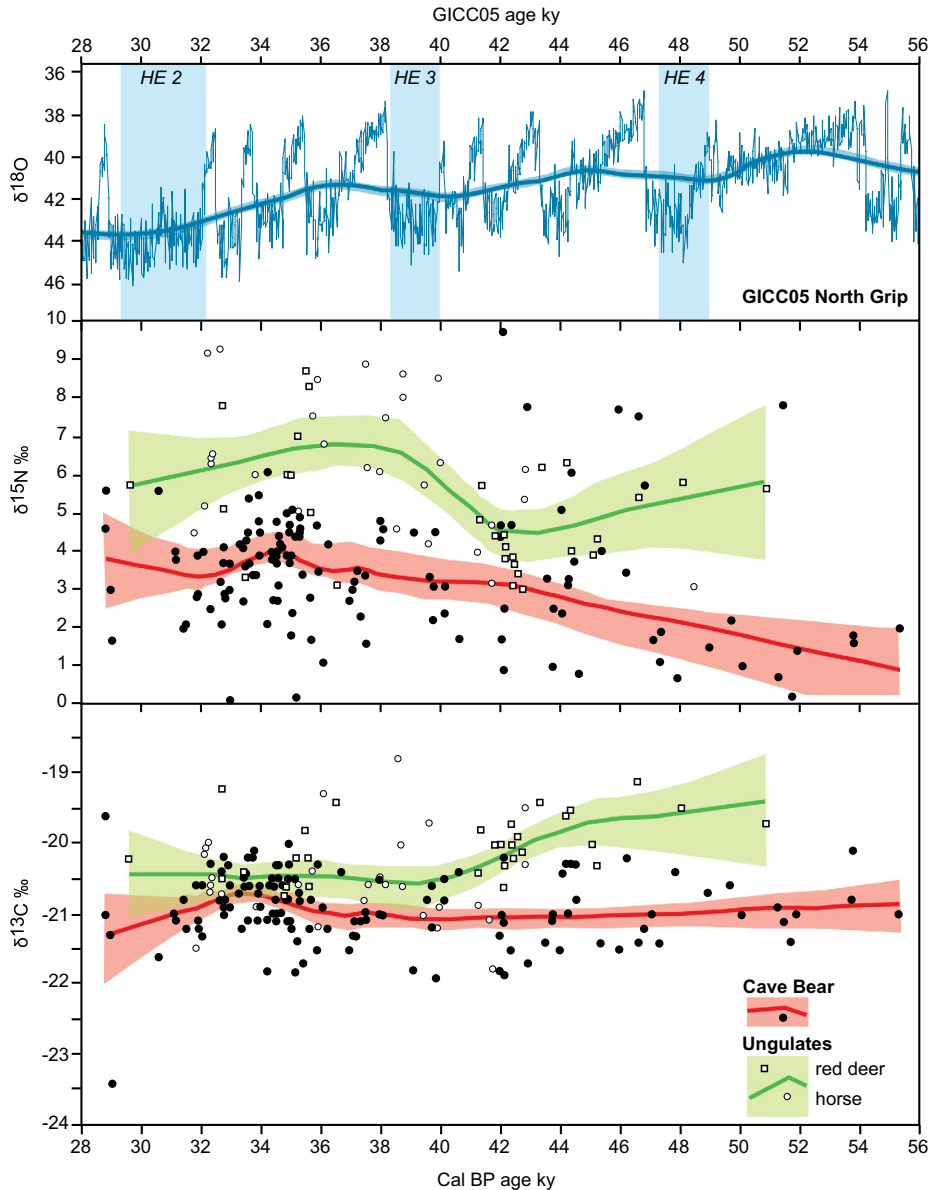
### Variation in isotope signatures compared to herbivore ungulates

When we project the isotopic values of cave bears (Figure 2), there is a great span in the values of  $\delta^{15}\text{N}$  that they present, with a range from 0.1‰ to 9.7‰. The highest values come from the Oase cave in the Carpathians of Eastern Europe (Robu et al. 2018). Such a span in the  $\delta^{15}\text{N}$  values of the cave bear could be considered as indicative of a more carnivorous diet in some examples, if it were not for the fact that the herbivorous ungulates included in the study show a similar variation (or greater, if we exclude some of the Carpathian outliers). In ungulates,  $\delta^{15}\text{N}$  range from 3.0‰ to 9.3‰. Even so, the average values for cave bears (average  $\delta^{15}\text{N} = 3.3\text{‰} \pm 1.6$ ) are clearly lower than those of ungulates (average  $\delta^{15}\text{N} = 5.7\text{‰} \pm 1.7$ ).

In the case of carbon, bears also show more negative mean values (average  $\delta^{13}\text{C} = -20.9\text{‰} \pm 0.5$ ) than ungulates (average  $\delta^{13}\text{C} = -20.3\text{‰} \pm 0.6$ ). In this isotope there is more variability in cave bears (from -23.4‰ to -19.6‰) than in ungulates (from -21.8 to -18.8‰), but this is due to the presence of a single individual (from the Bärenloch high Alpine cave in Switzerland) with an exceptionally negative  $\delta^{13}\text{C}$  value; although it should be noted that the original identification is doubtful (see Table S1 and Blant et al. 2010). If we exclude this outlier, the span in  $\delta^{13}\text{C}$  values is significantly reduced and is lower than that of herbivores.



**Figure 2.** Bivariate plot of bone collagen isotopic values of 130 cave bears and 58 herbivore ungulates (red deer  $n = 30$ , horse  $n = 28$ ). Crosses show mean values and 2 standard deviations for each group. Ellipses represent a 0.95 probability of finding a new sample of the same group on the surface defined by the ellipse.



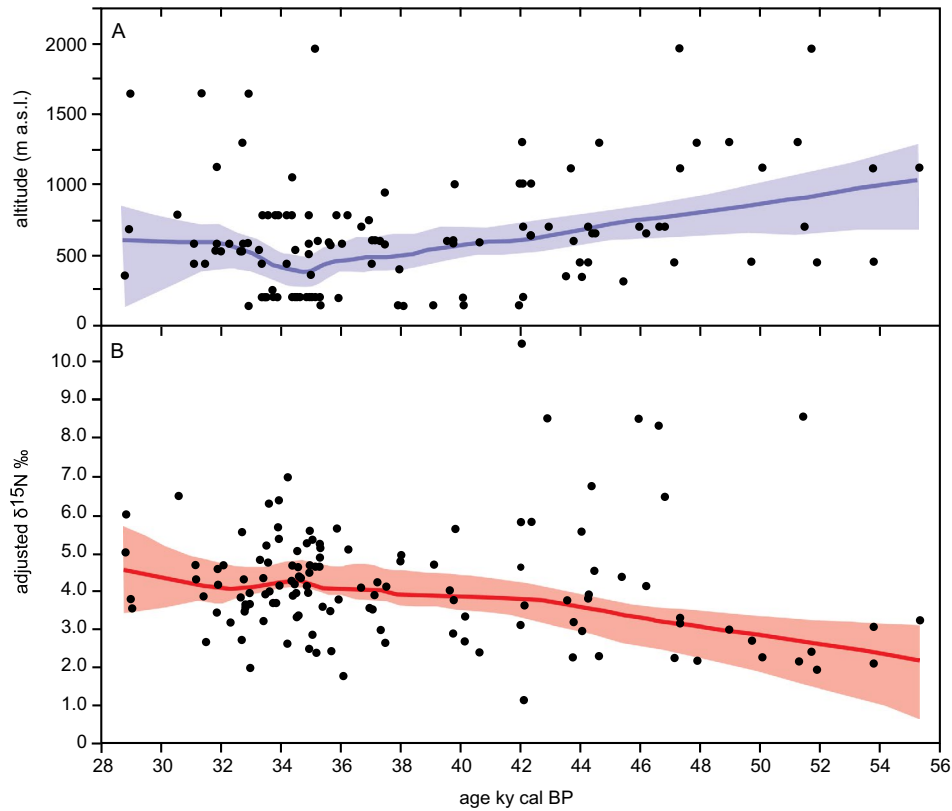
**Figure 3.** (A) Climate fluctuations between 56,000 and 28,000 years BP according to Greenland Ice Core Chronology 2005 (GICC05), based on NorthGRIP ice core  $\delta^{18}\text{O}$  curve (‰ VSMOW) (Svensson et al. 2008). (B) Variation along time of  $\delta^{15}\text{N}$  in cave bears and ungulates. (C) Variation in  $\delta^{13}\text{C}$  in cave bears and ungulates. All three curves are smoothed by means of the algorithm LOWESS (LOCally WEighted Scatterplot Smoothing; Cleveland 1979), with a smoothing factor of 0.5. 95% confidence band is based on 999 random replicates by PAST (Paleontological Statistics software) version 3.18 (Hammer et al. 2001).

In addition to possible differences in the contribution of animal protein in the diet of bears, another possible source of variation of isotopic signals are those signals that already come from the isotopic baseline in soils and plants, which depend on climatic conditions such as temperature, precipitation or insolation (Morecroft and Woodward 1990; Körner et al. 1991; Amundson et al. 2003). However, when studying the diversity of isotopic signals of the cave bear throughout Europe during OIS 3, Krajcarz et al. (2016) did not observe a clear trend in the isotopic values of  $\delta^{15}\text{N}$  with respect to latitude or longitude, although they did observe a clear trend with respect to altitude. Altitude affects  $\delta^{15}\text{N}$  values because the base values of soils and plants are determined by a colder climate and weaker soil development (Amundson et al. 2003). Thus, Alpine deposits show the lowest values in  $\delta^{15}\text{N}$ .

On the contrary, a particular case is the very high  $\delta^{15}\text{N}$  signals of certain sites in the Carpathians (Robu et al. 2013, 2018) that are clearly different from all others and could indicate some local effect or a local specialisation in some unusual food. Krajcarz et al. (2016) introduce a correction factor for altitude based on Männel et al. (2007), whereby, the differences in  $\delta^{15}\text{N}$  between high-Alpine and lower elevations are diluted sufficiently to finally show no relevant trend (Krajcarz et al. 2016: Figure 4).

#### *Stable isotopes and global climate*

The isotopic data from the cave bear and herbivorous ungulates that we analyse in this work show calibrated dates that coincide with the end of OIS 3, when climatic fluctuations were extreme (Fletcher et al. 2010).



**Figure 4** (A) Plot of cave altitude above sea level in relation to the chronology. A certain descending trend could suggest a habitat shift of the cave bears towards lower altitudes as the climate cooled down. (B) Variation of the  $\delta^{15}\text{N}$  values adjusted for the altitude (according to Krajcarz et al. 2016) along time. A rising trend is still evident.

After smoothing the climatic curve (Greenland Ice Core Chronology 2005 GICC05 NGRIP, Svensson et al. 2008), the trend over the OIS 3 is a continuous cooling from 52,000 years BP except during two slightly temperate episodes, matching DO events 12 and 8 (Figure 3(A)).

Figure 3(B) shows a clear rise of  $\delta^{15}\text{N}$  in cave bears as the temperature drops. In ungulates, on the other hand, there is no clear trend. The same lack of tendency is observed in the isotopic carbon signature for both cave bears and ungulates (Fig. 3(C)). Another interesting observation is that, except in the case of some Carpathian deposits (Robu et al. 2018), at no time does the mean  $\delta^{15}\text{N}$  of cave bears exceed the mean  $\delta^{15}\text{N}$  of coeval herbivore ungulates, which confirms the preferably herbivorous diet of cave bears and highlights the particular case of some bears in the Oase and Ursilor caves (Romania), whose combination of isotopic values does not yet have a clear explanation, and may represent a case of dietary specialisation that is very particular and of an unknown type.

Regarding the results of  $\delta^{13}\text{C}$ , the possible physiological effect caused by hibernation does not appear to be reflected in the isotopic values of cave bears, which remain approximately within the same range throughout the OIS 3 despite the climatic cooling (Figure 2(C)). During hibernation, bears rely on stored fat, depleted in the heavy isotope (Tieszen and Boutton 1989), a circumstance that has been used to explain the more negative  $\delta^{13}\text{C}$  values in cave bears than in other herbivorous mammals, since at least some proportion of bone collagen would have been synthesised from the fat obtained during the hyperphagia period prior to hibernation (Bocherens et al. 1997). Here, cave bear  $\delta^{13}\text{C}$  values do not appear to vary along the OIS 3 in a similar way to the  $\delta^{15}\text{N}$ .

We could assume that the tendency to increase in  $\delta^{15}\text{N}$  values along OIS 3 could be due to differences in altitude, hence as the climate cooled down, cave bears could move at ever lower altitudes. In fact, if we represent the altitudes of the caves with respect to the chronology of the remains under study (Figure 4), it seems that this effect is evident. Discarding a possible shift at altitude, with  $\delta^{15}\text{N}$  values adjusted to eliminate this effect, as proposed by Krajcarz et al. (2016), we continue to observe the trend of increasing  $\delta^{15}\text{N}$  as OIS 3 progresses.

#### *Regional differences in the evolution of Nitrogen isotope signatures*

It should be noted that temperatures and other parameters such as precipitation or insolation did not vary to the same extent or synchronously throughout Europe. For example, Bocherens et al. (2014a) describe a positive excursion of  $\delta^{15}\text{N}$  values during the Early Aurignacian (roughly around 35 ky) in southwestern France that manifests in all trophic categories studied, which they attribute to increasing aridity due to climate cooling. But in the westernmost regions, the proximity to the ocean and the absence of major orographic barriers seem to have prevented a marked dryness. Significant development of the xerophytic steppe occurs only southwards of  $40^\circ\text{N}$ , in the Mediterranean areas and during the warm pulses of OIS 3 (Fletcher et al. 2010). Marine and continental pollen records from the Atlantic regions further north indicate that xerophytic vegetation showed no significant increases throughout the OIS 3, and only increased during the OIS 2 in areas that were distant from the oceanic influence (Naughton et al. 2007, 2009; De Beaulieu and Reille 2008).

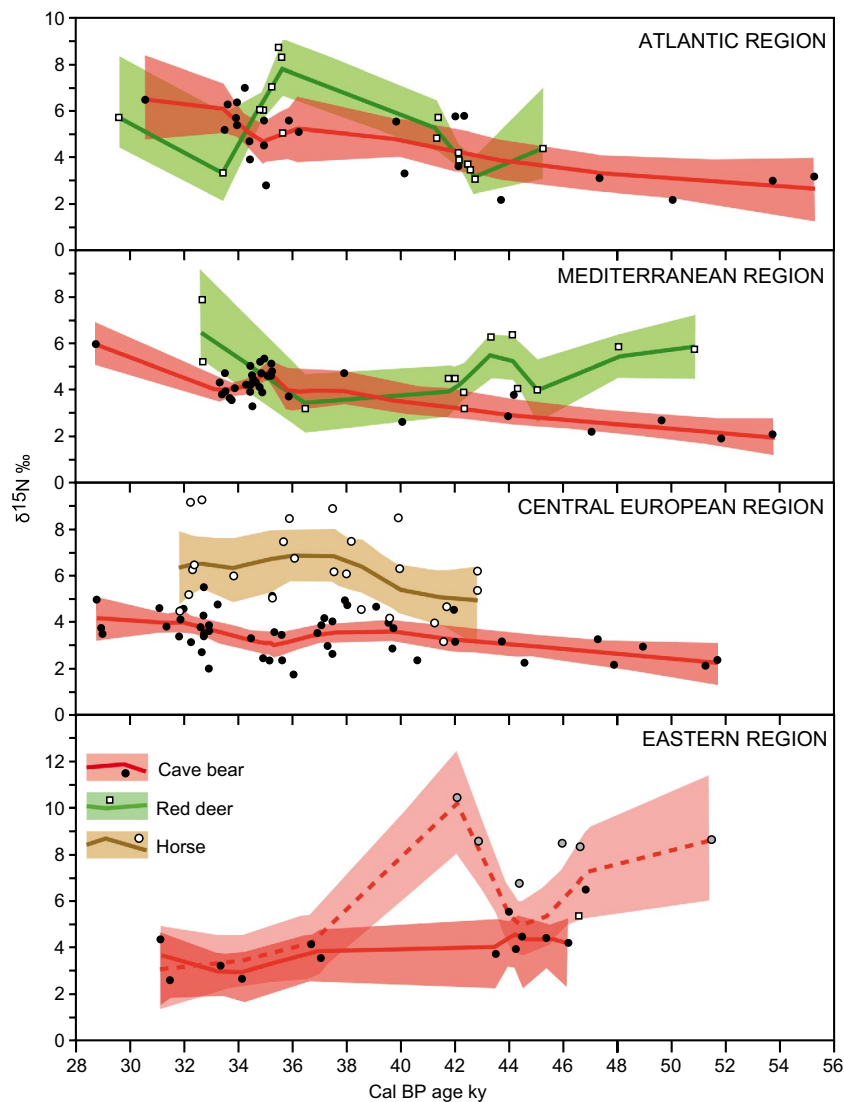
Specifically, in the NW of the Iberian Peninsula the climatic record in stalagmites of O Courel caves, in the same area where the cave bear deposits are located, suggests episodes of high humidity prior to maximum cooling, as does happen towards the end of OIS 3 (Railsback et al. 2017). The microvertebrate assemblages in Cova Eirós also indicate lower temperatures and higher precipitation than they do at present in the region (Rey Rodríguez et al., 2016), hence, at least for this area, we can-not attribute the increase in  $\delta^{15}\text{N}$  of cave bears to an increment in aridity.

Therefore, we look separately at the data from deposits located in different regions, establishing large geographical areas. We follow models of OIS 3 vegetation and climate from Van Andel and Tzedakis (2006) and Van Meerbeeck et al. (2011), as shown in Figure 1. The allocation of deposits to different regions is listed in the supplementary material (Table S1). In some of these regions, data on direct dated ungulates are almost lacking.

Figure 4 shows that the tendency to increase the values of  $\delta^{15}\text{N}$  (adjusted for altitude) of cave bears is maintained in the Atlantic, the Mediterranean and the Central European regions, while the ungulates selected in each case (red deer for the Atlantic and Mediterranean regions, and horse for the Central European region) seem to follow a complex pattern, sometimes with either a reverse trend or no reverse trend.

#### Stable isotopes in perinatal and juvenile individuals

There is another possible approach to the study of the physiological effects of hibernation on the record of stable isotopes of bone collagen in cave bears. In bears, the development of the embryo takes place during the period of hibernation of the mother. Therefore, the embryonic or individual neonate tissues are formed from metabolic products that are derived from the fat storages of the mother.



**Figure 5.** Plots of variation in cave bear  $\delta^{15}\text{N}$  adjusted for altitude along time by regions. For comparison, red deer from the Atlantic and Mediterranean regions and horse from the Central region are included. There are insufficient dated samples from the Eastern region, for which the dashed smoothed line represents the cave bear trend, taking into account the most anomalous values from some Carpathian sites (grey circles). Here, a white square shows the only ungulate (red deer) sample.

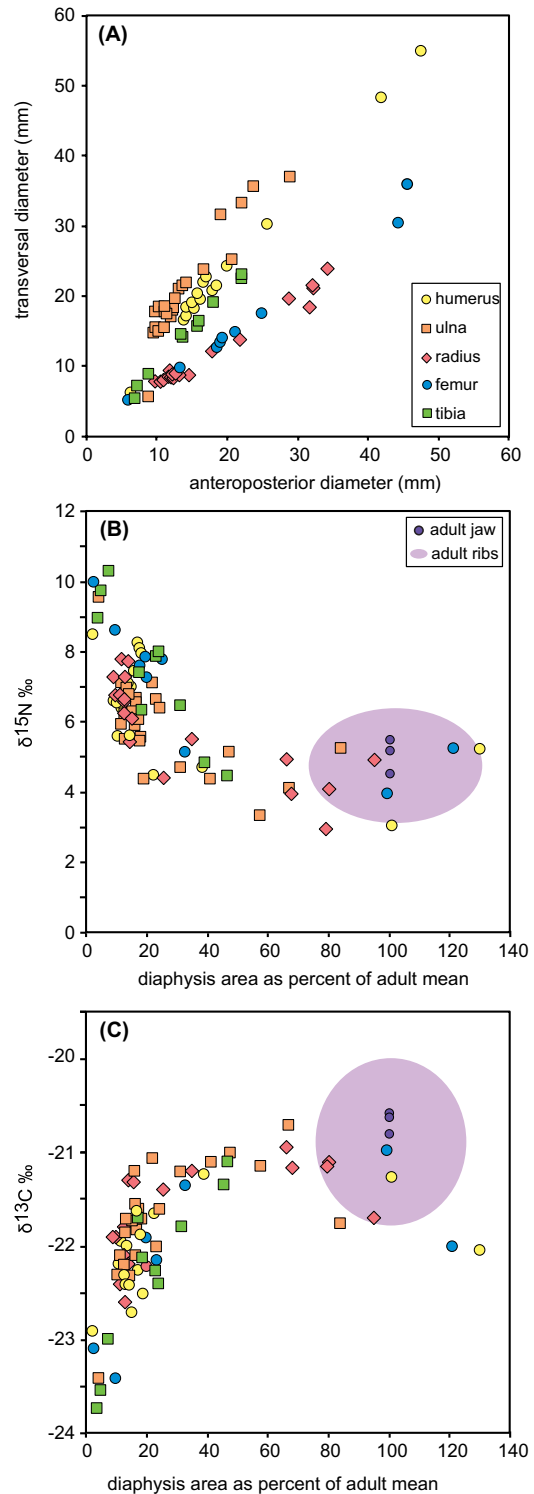
This fact dictates the duration of the embryonic period, which must be short because of the foetal requirement to use glucose for its development, and not just products of the fat catabolism its mother (Power and Schulkin 2016).

Isotopic data of newborn cave bears, whose bone tissue is formed *de novo* during the hibernation period of their mother, show  $\delta^{15}\text{N}$  values that are higher than in adult individuals, and higher than corresponding values that occur with the effect of breastfeeding (Nelson et al. 1998; Bocherens 2004; Pérez-Rama et al. 2011a, 2011b). Nelson et al. (1998) found a difference that is approximately equivalent to two trophic levels in  $\delta^{15}\text{N}$  between infants and adults of cave bears in bone collagen. In the Spanish caves (Eirós, A Ceza, Liñares) the perinatal individuals show  $\delta^{15}\text{N}$  values that are around 5‰ higher than those of adults (Perez-Rama et al., 2011b). This increment is attributable to the sum of two causes: the effect of lactation, that in other species is equivalent to one trophic level (Fogel et al. 1989; Jenkins et al. 2001), and the fact that the foetus is formed during the mother hibernation. Since the bones of adults have a slow turnover, their isotope values reflect a prolonged period during the life of the individual, including the active phase during the warm season in which tissue regeneration is more intense. Therefore, the  $\delta^{15}\text{N}$  value in bone collagen of adults that died during hibernation does not correspond only to that particular period. This does not rule out that the metabolic changes of hibernation that leave an isotopic fingerprint in bone collagen, but its effect may be attenuated or even deleted by the renewal of bone tissue during active periods, at least if the active season was long enough.

To illustrate this effect, we include data from perinatal, infantile and adult individuals from Cova Eirós. It is assumed that the death of bears found in the caves occurred only during hibernation, and thus juveniles will be distributed in age classes (first winter, second winter, etc.), so that it would be possible to classify them by age according to their size (Kurtén 1976; Lidén and Angerbjörn 1999). However, the metric study of a large number of juvenile individuals from Cova Eirós showed that there is no clear critical size in the bones of younger animals (Figure 5(A)). It is possible that the variety of size observed in these juvenile stages is due to an early manifestation of sexual dimorphism, which can be detected in the skull from early stages (Grandal-d'Anglade and López-González 2005). It is also possible that, as with present day bears, differences in offspring size in the initial stages are determined by the number of siblings that make up the litter, the age and experience of the mother, etc. (Pérez-Rama et al. 2011a; Robbins et al. 2012). For this reason, it is not possible to make a clear separation in age classes except, perhaps, in the first age class (perinatal). To avoid a subjective classification by size, Pérez-Rama et al. (2011a) calculated the area of diaphysis of each type of long bone (modelled as an ellipse) in proportion to the area of the average adult bone (Figure 6(A)).

Figure 6(B) shows  $\delta^{15}\text{N}$  values of Cova Eirós individuals of different ages, classified by size as percentages of the average adult size, as described in the above. Perinatal individuals show a much higher isotopic signal than nursing offspring (with a particular signature caused by the lactation effect) and adults.

Thus, the isotopic values of the collagen of the perinatal individual do not resemble those of maternal bone collagen, but largely reflect the physiological effect of maternal hibernation. The recycling of nitrogen by the mother during the gestation of the foetus would result in higher  $\delta^{15}\text{N}$  values in foetal tissues, including bone collagen. This effect in neonates has been observed on several occasions in cave bears (Nelson et al. 1998; Bocherens 2004; Pérez-Rama et al. 2011b; Bocherens 2015; Pérez-Rama et al. 2011a, 2011) and also in brown bears (Bocherens et al. 2004).



**Figure 6.** (A) Area of diaphysis of each type of long bone (modelled as an ellipse from the anteroposterior and transversal diameters) in proportion to the area of the average adult same bone. (B) Nitrogen isotope signatures of perinatal, juvenile and adult bones from Cova Eirós expressed as percentage of the adult size, showing a noticeable offset between perinatal and adult individuals, and a progressive decline for juveniles. (C) Carbon isotope signatures of perinatal, juvenile and adult bones from Cova Eirós, showing an opposite trend to the nitrogen isotopes. Shaded ellipses represent the span in  $\delta^{15}\text{N}$  values and the probable extent of sizes in adult ribs, since it is not possible to separate males and females using this type of bone.

Broadly speaking, the decrease in  $\delta^{15}\text{N}$  of subadult bear values reflects a gradual decline in lactation. Subadults of Eirós show similar values to adults when reaching approximately 35% of the average adult size. A variety of isotopic signatures can be observed in juvenile individuals, which is interpreted as differences in maternal investment for each group of mother-offspring (Pérez-Rama et al. 2011a; Robbins et al. 2012).

Figure 6(C) shows the variation of  $\delta^{13}\text{C}$  with size, i.e. related to age. Because the carbon in new synthesised proteins comes from fat storages, the isotopic signature of perinatal carbon is more negative than that of adults. To this fact is added the consumption of breast milk, that in ursids is very rich in fat (Oftedal et al. 1993). Something similar is observed in other species with high fat content in breast milk, such as otariid seals (Newsome et al. 2006). As the cubs move away from breast milk as the primary feed and incorporate solid food,  $\delta^{13}\text{C}$  values fall to adult levels. In our example from Cova Eirós, this happens when the juvenile individuals reach around 35% of the adult size, as was the case for  $\delta^{15}\text{N}$ .

### **The study of mitochondrial DNA lineages and their relationship to bear behaviour during hibernation**

In describing the caves containing skeletal remains of cave bears, several authors agree in noting the existence of beds or bauges that are hollowed into the sediment of the cave floor, which would be the winter resting place of each individual. The curious element in this regards is that in the larger caves the bauges are found in large numbers, such as in Rouffignac, Chauvet, or Pester cu Oase (Koby 1953; Philippe and Fosse 2003; Quilès et al. 2006) as if several individuals had hibernated at once, using the caves as 'collective dormitories.' This behaviour is not observed in brown bears, either in present day samples current or in fossils, which are usually solitary. Although the remains of the cave bear are accumulated in the caves, it is not possible to know whether they lived simultaneously in the cave, and even the dating of the remains has a sufficient margin of error to prevent knowing the exact date of the death of an individual.

Unexpectedly, a mitochondrial DNA study aimed at identifying the population variability of cave bears from various sites in the Iberian Peninsula (Fortes et al. 2016) brought new light to this question: although cave bears occupied closely situated caves simultaneously, each cave almost exclusively contains a unique lineage of closely related haplotypes. The same is not true for Holocene brown bears of the same region; nor is it true for the red deer of Pleistocene age in the cave of Liñares, interpreted as a group that was trapped in the cave in a single catastrophic episode (López-González et al. 2006), whereby both groups show a marked mitochondrial variability (Rey-Iglesia et al. 2017), thus, indicating the existence of diverse maternal lineages in a group of animals living together.

This remarkable pattern suggests extreme fidelity to their birth site in cave bears, which is best described as homing behaviour, and that cave bears formed stable maternal social groups, at least for hibernation. If this happened, an immediate consequence is that the caves were occupied each winter by groups of bears, i.e. practicing a collective hibernation: at least, the sow with her offspring of different ages and both sexes, and eventually the offspring of the daughter.

The hibernation in family groups of maternal origin offers a new way of imagining the cave bears. We can visualise the groups of individuals, sometimes being very numerous, occupying their nests in large cave halls. The numerous marks of young bear claws on the walls of some caves (Grandal-d'Anglade et al. 2006) bring us a vivid image of the young bears walking through the passages of the cave, climbing and slipping in their games, while their mothers rested in their nests in their winter torpor.

Interestingly, in the case of the caves of the Iberian Peninsula, the only individuals found in caves other than those of their maternal lineage are juveniles. This shows that, despite the homing behaviour described in the above, cave bears could still undergo expansion and occupy new caves. This expansion, apparently mediated by young individuals, could reach a considerable distance: the most similar lineages are geographically very distant, such as Eirós in the northwest of the Iberian Peninsula and Chauvet in the south of France, differentiated by only two base pairs throughout their mitochondrial genome (Fortes et al. 2016).

### **Discussion**

#### ***Could the observed trend be related to a change in cave bear diet?***

When collecting isotope data from a collection of Pleistocene mammals, which at first sight is abundant in the literature, we found a certain lack of absolute dates to enable framing isotopic values and chronology for most species included in this review. It would be desirable to have a larger volume of data in some of the regions concerned. Despite this, it is possible to see some trends in the  $\delta^{15}\text{N}$  isotopic values of the cave bear, which we relate to climate and physiology of this species, rather than to a shift to a more omnivorous diet. This is because the increasing  $\delta^{15}\text{N}$  values are not accompanied by a similar increase in  $\delta^{13}\text{C}$  values, as would be expected if more animal protein existed in the bear diet as OIS 3 progresses (Bocherens et al., 2014c). The isotopic signatures of contemporary carnivores are distinctly different, with higher values in both isotopes (García-Vázquez et al., forthcoming).

The variation in the isotopic values of the bears may have been caused by changes in diet that were not necessarily related to animal protein intake. We could assume that as the climate cooled, the cave bears changed their diet towards more grasses, with higher values of  $\delta^{15}\text{N}$  (Bocherens 2015). However, it can be assumed that the increasingly cold climate would also cause a decrease in the isotopic values of herbaceous vegetation. This may be the reason why ungulates do not show a clear pattern in their  $\delta^{15}\text{N}$  values because, on the one hand, shrub vegetation became scarcer, and also herbaceous vegetation had to suffer a decrease in  $\delta^{15}\text{N}$  values caused by the colder climate.

Drucker et al. (2015) describe changes towards the end of OIS 3 in the feeding of a herbivore, such as the horse, due to inter-specific competition rather than environmental changes. While there may have been interspecific competition among herbivores, including the cave bear, in particular areas it is difficult to imagine that it could have affected cave bears throughout western Europe in the same way and at the same time. The fact that the increase in  $\delta^{15}\text{N}$  is simultaneous in bears in three of the regions considered, seems to point more to a physiological effect than to a synchronous change in their diet.



### *Metabolic mechanism of hibernation and stable isotopes*

Fat is the primary substrate for energy production of hibernating bears (Nelson et al. 1973; Nelson, 1980). One might expect that the  $\delta^{13}\text{C}$  values of the bear decline as its fats are consumed, since fats are depleted in  $^{13}\text{C}$  (DeNiro and Epstein 1977; Tieszen et al. 1983; Tieszen and Bouton 1989). Fats are stored as triglycerids that break down into fatty acids and glycerol. Fatty acids are degraded by beta-oxidation to integrate into the tricarboxylic acid (TCA) cycle and produce energy and  $^{13}\text{C}$ -depleted  $\text{CO}_2$  (Ahlquist et al. 1984) that is expelled with the breath. Glycerol, in turn, can follow several metabolic pathways. One of these pathways is incorporation into glycolysis, so that its carbons eventually enter the TCA cycle. In addition, as demonstrated by Ahquist et al. (1984), by injection of  $^{14}\text{C}$ -labeled glycerol it is possible that the carbonate chain of glycerol may incorporate nitrogen by transamination to produce some non-essential amino acids. From TCA cycle intermediates, some amino acids can be obtained via cataplerotic reactions (Owen et al. 2002). However, according to our results, glycerol only has a minor contribution to the biosynthesis of amino acids originating from the TCA cycle, as was also proposed by Keeling and Nelson (2001). In the hibernating bear, glycerol preferentially follows the gluconeogenesis route (Ahlquist et al. 1984; Sommer et al. 2016) to produce glucose, which is deficient in the hibernating bear and necessary as fuel for important organs such as the brain or red blood cells. Apparently, fats can even enter the gluconeogenesis pathway through the glyoxylate cycle, where metazoans are distinctly in the minority but occurs in bear brown fat (Davis et al. 1990). In this way, the contribution to the bone collagen of carbon from fat, which is isotopically depleted, is small. Differences in  $\delta^{13}\text{C}$  between cave bear populations are more easily explained by the effects of local conditions, which also affect other species, than by the particular metabolism of ursids.

This situation contrasts with what happens in suckling off-spring, where virtually all bone collagen, like all other body proteins, comes from breast milk, which is rich in fat and is  $\delta^{13}\text{C}$  depleted (Keeling and Nelson 2001). Perinatal individuals, thus, show a marked depletion of the isotopic carbon values.

Nitrogen follows totally different pathways in the hibernating bear. The bear does not lose muscle mass during hibernation despite being immobilised for months. However, muscle protein does degrade, as shown by the high creatinine content in blood serum. Creatinine is a marker of protein degradation, together with the urea, which is toxic and under normal conditions would have to be excreted with urine. Hibernating bears have a low urea/creatinine ratio (Nelson et al. 1984; Ramsay et al. 1991; Stenvinkel et al. 2013a), indicating that protein is degraded, but urea does not accumulate. In fact, the bear is capable of preserving its lean tissue by hydrolysing urea and using the nitrogen to synthesise new amino acids (Lundberg et al. 1976; Barboza et al. 1997; Tinker et al. 1998). Vertebrates lack the enzyme necessary for the hydrolysis of urea, that is provided by gut bacteria. Thus, urea is hydrolysed by urease-expressing gut bacteria into ammonia and  $\text{CO}_2$ .

Ammonia is then used by enterocytes to synthesise glutamine, which may be incorporated into proteins (Stenvinkel et al. 2013a). Interestingly, populations of brown bear gut bacteria have a different composition during hibernation than in the active period (Sommer et al. 2016).

### *The effect of climate fluctuations*

Throughout the OIS 3, ungulates show fluctuations in  $\delta^{15}\text{N}$  values that can be related to ecological differences caused by the variety of climates and their evolution in each region. Climatic variations not only alter the phenology of the plants first (growth season, flowering, etc.) and their geographical distribution afterwards (shift of ranges, extirpation, substitution of species, etc.) but also the microbial communities of the soils that are associated with the plants (Booth et al. 2005). How these variations affected the  $\delta^{15}\text{N}$  of soils and vegetables is not fully known. It is true that the altitude produces a decrease of  $\delta^{15}\text{N}$  both in plants and herbivores and, in fact, Krajcarz et al. (2016) found a negative relationship between the values of  $\delta^{15}\text{N}$  of cave bears and the altitude of the deposits. But, it is difficult to imagine that the cave bears fed during the active season in a geographic area that was restricted to the immediate vicinity of their cave. The altitude of the site is probably not the best parameter for determining the geographic area in which bears lived and fed. The homing behaviour that emerges from mitochondrial DNA studies, at least in the Iberian bears, indicates the possibility that, being faithful to their cave of origin, bears returned to the same place every winter, generation after generation, even if environmental conditions had changed substantially and the area around the cave was not suitable for feeding during the active season. If this happened, there will be no direct relationship between the  $\delta^{15}\text{N}$  of the bears and the altitude of their family cave.

However, if we look at the variation in the isotopic signal of bears over time, we find a trend towards increasingly positive values as the climate cooled down. Certainly, the isotopic values do not faithfully follow climatic fluctuations, but we can blame this absence of immediate parallelism to a resilience of soils (Tiunov 2007) and other factors, not only bacterial activity, that influence  $\delta^{15}\text{N}$ . For example, Craine et al. (2015) found a relationship between the proportion of clays and the  $\delta^{15}\text{N}$  of soils. Climate fluctuations also affected soil carbon dynamics, to the extent that small changes in temperature could result in a different release of soil carbon back into the atmosphere (Classen et al. 2015). There is even a relationship between atmospheric  $\text{CO}_2$  concentration and bacterial activity in soils (Booth et al. 2005), so that, from certain atmospheric  $\text{CO}_2$  levels, a co-variation in the isotopic values of C and N could be observed. The whole of this source of variability in the isotopic values of soils and plants can be the origin of the absence of tendencies, at least those that are apparent, in the isotopic values of ungulate herbivores over time. However,  $\delta^{15}\text{N}$  values of cave bears do follow an increasing trend in three of the regions under study.

The length of hibernation in ursids depends on several factors, such as latitude and climate, intensity of rainfall, food availability, or sex (which is related to pregnancy or the presence or absence of offspring). Currently, there are populations of brown bears with hibernations reaching up to six months, while in temperate climates with mild winter, brown bears and other species can even dispense with hibernation (Nores et al., 2010; Schoen et al. 1987).

During the OIS 3, as conditions became more severe, the cave bears would have spent more time in hibernation. We hypothesise that the metabolism during hibernation causes an increase in the isotopic signature of nitrogen, also in bone collagen, and that during the coldest pulses this special signature fails to be cleared during the active season and accumulates at least in bone collagen, so that the general cooling over OIS 3 is reflected in progressively higher  $\delta^{15}\text{N}$  values in the cave bears. But this increase, as with the duration of bear hibernation, has a limit. During particularly cold pulses, a time limit would be reached beyond which the bears would have to leave their shelter. If climate rigor did not allow a long enough plant growth cycle, the permanence of the bears in the area would be unworkable. Consistent with this, the most recent dating (younger than 30 kycalBP) places the last individuals at the end of OIS 3 or even at the onset of OIS 2 in the most western or southern regions: northwest of the Iberian Peninsula (Grandal-Anglade and Vidal-Romani 1997), valleys of the Jura Massif (Bocherens et al., 2014b), north-east Italy (Terlato et al., forthcoming), central Italy (Martini et al. 2014), Croatia (Hofreiter et al. 2004), although recent data from Wojtal et al. (2015) and Baca et al. (2016) show that further north there must have been areas that acted as refuges.

## Conclusions

Certainly, the  $\delta^{13}\text{C}$  signal in cave bear bone collagen is generally more negative than in ungulate herbivores, but there is no observed variation in the values of  $\delta^{13}\text{C}$  along OIS 3 parallel to that of  $\delta^{15}\text{N}$ . Although some of the collagen carbon can come from the degradation of fats, the process of synthesis of amino acids to renew bone collagen must be based mainly on the reuse of carbonate skeletons from the previous degradation of collagen amino acids, without there being an appreciable isotopic fractionation. Nitrogen, however, is actively recycled and reused, so that the isotopic signal is increased. As noted in the above, this hibernation-related elevation of  $\delta^{15}\text{N}$  has been described in blood serum and muscle of present day bears.

Progressively lower temperatures, decreased insolation, increased aridity and subsequent changes in vegetation along OIS3 would lead to increasingly longer hibernations in cave bears. This would cause the effect on bone collagen isotope signature (that could be produced by urea reuse during hibernation) to be recorded with more intensity, leading to higher values of  $\delta^{15}\text{N}$ . Contemporary non-hibernating ungulates lack a similar physiological mechanism, so that trends in  $\delta^{15}\text{N}$  values of cave bear and other herbivores are different. Specifically, in the Atlantic region, where the climate did not become particularly arid, the trend is noticeably reversed. Conversely, in regions with more extreme climate such as the eastern region, the effect of cooling in soils and plants would outweigh the effect caused by hibernation, so that the possible trend that is proposed would be masked.

It is difficult to determine whether this increase in  $\delta^{15}\text{N}$  in cave bears towards the end of OIS 3 is due to a physiological effect of hibernation that is recorded in their collagen only when the active period is shorter. Climate variation, both globally and locally, and its influence on the values of isotopic baseline, can interact with the values recorded in collagen and prevent a clear

interpretation. With current data, it is difficult to know whether this trend is due to an effect of increasingly longer hibernations, an effect that we know exists from the study of perinatal individuals.

The dilemma is whether adult bone collagen can record an increase in  $\delta^{15}\text{N}$  due to hibernation, as seen in current bear blood serum or neonatal bones, and whether this variation may have been maintained in the bones of bears living in colder periods. An experimental study is not possible at this stage, since it is practically impossible to reproduce in present day bears the conditions in which the cave bears lived at the end of the OIS 3, at least with respect to the time period necessary to check whether there is an effect on the adult bone collagen or not. More data are needed, not only from cave bears but also from other herbivores, covering a longer time interval, in order to see if the suggested trend is real or is only the result of data scarcity in certain cases. At the same time, other approaches, such as the study of the isotopic signal of isolated amino acids (Naito et al. 2016), comparing individuals from colder to warmer regions or periods, could shed new light on this challenge.

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